Caractérisation du contenu protéomique et des propriétés immunomodulatrices des vésicules extracellulaires dérivées de la bactérie probiotique Propionibacterium freudenreichii CIRM-BIA129

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Par

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Characterization of the proteomic content and immunomodulatory properties of the extracellular vesicles derived from the probiotic *Propionibacterium freudenreichii* CIRM-BIA129

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"I want to learn more and more to see as beautiful what is necessary in things;
then I shall be one of those who makes things beautiful."

Friedrich Nietzsche

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I learned from a song that we are the borders that we crossed. I had to cross several borders during these years and, fortunately, I was not alone. This is the opportunity to say **thank you**, ***obrigado*** and ***merci*** to the people that were with me and helped me to become who I am today.

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“Caminhando com minha guitarra,
Somos as fronteiras que cruzei.
A cada passo andado,
Sou o caminho que me é dado.
Para sempre cantarei!”
Francisco, El Hombre

“Walking with my guitar,
we are the borders that I crossed.
With each step taken,
I am the path given to me.
I will always sing!”
Francisco, El Hombre

Abstract

Extracellular vesicles (EVs) are ubiquitous mediators of the intercellular communication and consist in an export system involved in essential processes of life, such as nutrition, adaptation, defense and host-microbe interaction. EVs are spherical non-replicating nanoparticles composed of a lipid bilayer and an internal aqueous lumen that shelters a diverse set of molecules, including proteins, nucleic acids, lipids, glycans and other metabolites. The research on bacteria-derived EVs focused initially in Gram-negative and pathogenic bacteria, but the scenario changed in the last years, including EVs derived from other groups, such as Gram-positive and non-pathogenic bacteria. A relevant Gram-positive and probiotic bacterium is *Propionibacterium freudenreichii*, which has been long and safely used in the production of Swiss-type cheese, organic acids and vitamin B12. *P. freudenreichii* has emerged in the probiotics landscape due to notable properties, such as anti-pathogenic and anti-cancer activities, modulation of the microbiota composition and immunomodulation. Some of these properties were attributed to surface-exposed or secreted factors. Therefore, we aimed to investigate whether *P. freudenreichii* produced EVs that could mediate its beneficial properties, particularly immunomodulation. Accordingly, the bacterium was cultured in milk ultrafiltrate (UF) or yeast extract-lactate (YEL) medium until the beginning of the stationary phase. Protocols for EV purification based on size-exclusion chromatography (SEC) or density gradient ultracentrifugation (UC) were applied. The resulting samples presented nano-sized particles with typical morphology, confirming EV production by *P. freudenreichii*. EV protein content was investigated with shotgun proteomics approaches, unveiling a diverse set of proteins that included immunomodulatory and cytoplasmic proteins. The protein cargo and interactomics analysis indicated potential immunomodulatory roles for *P. freudenreichii*-derived EVs. That was confirmed with the EV-mediated reduction of IL-8 release and NF-κB activity in human intestinal epithelial cells treated with bacterial LPS, indicating an anti-inflammatory role. Moreover, EVs properties were dependent on the culture medium used to grow bacteria, since they varied for EVs derived from UF or YEL, including EV size, abundance, protein content and anti-inflammatory activity. Furthermore, the method used for EV purification also impacted EVs properties, particularly protein content. Although there were exclusive proteins for some conditions, hundreds of proteins were identified in all the studied conditions, indicating the existence of a conserved core proteome. Altogether, these findings contribute to the understanding of the mechanisms underlying the probiotic effect in *P. freudenreichii* and reveal novel possibilities for the developing of optimizable and safe nanotechnological delivery systems, potentially holding a great impact in human health.

Keywords: extracellular vesicles, probiotics, propionobacteria, proteomics, immunomodulation, anti-inflammatory, NF-κB.

Résumé

Les vésicules extracellulaires (VEs) sont des médiateurs ubiquitaires de la communication intercellulaire et consistent en un système d'exportation impliqué dans des processus essentiels de la vie, tels que la nutrition, l'adaptation, la défense et l'interaction hôte-microbe. Les VEs sont des nanoparticules sphériques non réplicatives composées d'une bicouche lipidique et d'une lumière aqueuse interne qui abrite un ensemble diversifié de molécules, notamment des protéines, des acides nucléiques, des lipides, des glycanes et d'autres métabolites. La recherche sur les VEs dérivés de bactéries s'est concentrée initialement sur les bactéries Gram-négatives et pathogènes, mais le scénario a changé au cours des dernières années, intégrant les VEs dérivés d'autres groupes, comme les bactéries Gram-positives et non pathogènes. Une bactérie Gram-positive et probiotique pertinente est *Propionibacterium freudenreichii*, qui est utilisée depuis longtemps et en toute sécurité dans la production de fromage de type suisse, d'acides organiques et de vitamine B12. *P. freudenreichii* a émergé dans le panorama des probiotiques en raison de propriétés notables, telles que les activités anti-pathogènes et anticancéreuses, la modulation de la composition du microbiote et l'immunomodulation. Certaines de ces propriétés ont été attribuées à des facteurs exposés à la surface ou sécrétés. Par conséquent, nous avons cherché à déterminer si *P. freudenreichii* produisait des VEs qui pourraient médier ses propriétés bénéfiques, en particulier l'immunomodulation. En conséquence, la bactérie a été cultivée en milieu ultrafiltrat de lait (UF) ou extrait de levure-lactate (YEL) jusqu'au début de la phase stationnaire. Des protocoles de purification de VEs basés sur la chromatographie d'exclusion de taille (SEC) ou l'ultracentrifugation (UC) à gradient de densité ont été appliqués. Les échantillons résultants présentaient des particules de taille nanométrique avec une morphologie typique, confirmant la production de VEs par *P. freudenreichii*. Le contenu en protéines de VEs a été étudiée avec des approches de protéomiques shotgun, dévoilant un ensemble diversifié comprenant des protéines immunomodulatrices et cytoplasmiques. L'analyse du contenu protéique et d'interactomique ont indiqué des rôles immunomodulateurs potentiels pour les VEs dérivés de *P. freudenreichii*. Cela a été confirmé par la réduction médiée par les VEs de la libération d'IL-8 et de l'activité de NF-kB dans les cellules épithéliales intestinales humaines traitées avec du LPS bactérien, indiquant un rôle anti-inflammatoire. De plus, les propriétés des VEs dépendaient du milieu de culture utilisé pour cultiver les bactéries, car elles variaient pour les VEs dérivés de l'UF ou du YEL, y compris la taille, l'abondance, le contenu en protéines et l'activité anti-inflammatoire des VEs. En outre, la méthode utilisée pour la purification des VEs a également eu un impact sur les propriétés des VEs, en particulier le contenu en protéines. Bien qu'il y ait eu des protéines exclusives pour certaines conditions, des centaines de protéines ont été identifiées dans toutes les conditions étudiées, indiquant l'existence d'un protéome

central conservé. Dans l'ensemble, ces résultats contribuent à la compréhension des mécanismes sous-jacents à l'effet probiotique chez *P. freudenreichii* et révèlent de nouvelles possibilités pour le développement de systèmes nanotechnologiques de livraison optimisables et sûrs, susceptibles d'avoir un grand impact sur la santé humaine.

Mots clés : vésicules extracellulaires, probiotiques, propionobactéries, protéomique, immunomodulation, anti-inflammatoire, NF-κB.

Resumo

As vesículas extracelulares (VEs) são mediadores ubíquos da comunicação intercelular e consistem em um sistema de exportação envolvido em processos essenciais da vida, como a nutrição, a adaptação, a defesa e a interação entre hospedeiro e micro-organismos. VEs são nanopartículas esféricas não replicantes compostas por uma bicamada lipídica e um lúmen aquoso interno que abriga um conjunto diversificado de moléculas, incluindo proteínas, ácidos nucléicos, lipídios, carboidratos e outros metabólitos. A pesquisa em VEs derivadas de bactérias focou inicialmente em bactérias Gram-negativas e patogênicas, mas o cenário mudou nos últimos anos, incluindo VEs derivadas de outros grupos, como bactérias Gram-positivas e não patogênicas. Uma bactéria Gram-positiva e probiótica relevante é *Propionibacterium freudenreichii*, que tem sido usada há muito tempo e com segurança na produção de queijo do tipo suíço, ácidos orgânicos e vitamina B12. *P. freudenreichii* emergiu no panorama dos probióticos devido a propriedades notáveis, como atividades antipatogênica e anticâncer, modulação da composição da microbiota e imunomodulação. Algumas dessas propriedades foram atribuídas a fatores secretados ou expostos superficialmente. Portanto, nosso objetivo foi investigar se *P. freudenreichii* produz VEs que poderiam mediar suas propriedades benéficas, particularmente a imunomodulação. Dessa forma, a bactéria foi cultivada em meio ultrafiltrado do leite (UF) ou extrato de levedura-lactato (YEL) até o início da fase estacionária. Protocolos para purificação de VEs com base em cromatografia de exclusão molecular (SEC) ou ultracentrifugação (UC) com gradiente de densidade foram aplicados. As amostras resultantes apresentaram nanopartículas com morfologia típica, confirmando a produção de VEs por *P. freudenreichii*. O conteúdo proteico das VEs foi investigado com abordagens de proteômica *shotgun*, revelando um conjunto proteômico diversificado, incluindo proteínas citoplasmáticas e imunomoduladoras. O conteúdo proteômico e a análise interatômica indicaram potenciais funções imunomoduladoras para as VEs derivadas de *P. freudenreichii*. Isso foi confirmado com a redução mediada por VEs da liberação de IL-8 e da atividade de NF-κB em células epiteliais intestinais humanas tratadas com LPS bacteriano, indicando uma atividade anti-inflamatória. Além disso, as propriedades das VEs foram dependentes do meio de cultura usado para cultivar bactérias, uma vez que variaram para VEs derivadas de UF ou YEL, incluindo o tamanho, a abundância, o conteúdo proteômico e atividade anti-inflamatória das VEs. Além disso, o método usado para purificação de VEs também impactou essas propriedades, particularmente o conteúdo proteômico. Embora existissem proteínas exclusivas para algumas condições, centenas de proteínas foram identificadas em todas as condições estudadas, indicando a existência de um proteoma central conservado. Em conjunto, esses achados contribuem para a compreensão dos mecanismos subjacentes ao efeito probiótico em *P. freudenreichii* e revelam novas possibilidades para o desenvolvimento

de nano-sistemas de entrega otimizáveis e seguros, tendo potencialmente um grande impacto na saúde humana.

Palavras-chaves: vesículas extracelulares, probióticos, propionibactérias, proteômica, imunomodulação, anti-inflamatório, NF-κB.

List of symbols and abbreviations

5-FU	5-Flourouracil
ABC transporters	ATP-binding cassette transporters
ACNQ	2-amino-3-carboxy-1,4-naphthoquinone
AFM	Atomic force microscopy
AI	Artificial intelligence
AMPK	5' adenosine monophosphate-activated protein kinase
AP-MS	Affinity purification coupled to mass spectrometry
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BFT	Bacteroides fragilis toxin
CFU	Colony-forming unit
CM	cytoplasmic membrane
CMOS	complementary metal-oxide semiconductor
CMV(s)	cytoplasmic membrane vesicle(s)
CO_2	Carbon dioxide
COG(s)	Cluster(s) of Orthologous Groups
DHNA	1,4-dihydroxy-2-naphthoic acid
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulphate
EOMV(s)	explosive outer-membrane vesicle(s)
EV(s)	extracellular vesicle(s)
FDR	False discovery rate
FT-ICR	Fourier-transform ion cyclotron resonance
GC-content	Guanine-cytosine content
GI	GenInfo Identifier
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GRAS	Generally recognized as safe
HGT	Horizontal gene transfer
HIV-1	Human immunodeficiency virus 1
HPRD	Human Protein Reference Database
IFNγ	Interferon gamma
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17a	Interleukin 17a
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6

IL-8	Interleukin 8
IM	Inner membrane
ISEV	International Society for Extracellular Vesicles
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-ESI MS/MS	Liquid chromatography coupled to electrospray ionization tandem mass spectrometry
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MINT	Molecular Interaction Database
ML	Machine learning
MS	Mass spectrometry
MUC2	Mucin 2
MV(s)	Membrane vesicle(s)
NADH	Nicotinamide adenine dinucleotide (reduced)
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NTA	Nanoparticle tracking analysis
OIMV(s)	Outer-inner membrane vesicle(s)
OM	Outer membrane
OMV(s)	Outer-membrane vesicle(s)
PBMCs	Peripheral blood mononuclear cells
PCA	Principal components analysis
PG	Peptidoglycan
PPAR-γ	Peroxisome proliferator-activated receptor γ
PPI(s)	Protein-protein interaction(s)
PQS	Pseudomonas quinolone signal
PSA	Polysaccharide A
PSMα	Phenol-soluble modulin α
QPS	Qualified presumption of safety
RNA(s)	Ribonucleic acid(s)
RNA-seq	RNA sequencing
RT-PCR	Reverse transcription polymerase chain reaction
SCFA(s)	Short-chain fatty acid(s)
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
SIGNR1	Murine homolog of human dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)
SLH domain	S-layer homology domain
sRNA(s)	Small ribonucleic acid(s)
STRING	Search Tool for Recurring Instances of Neighbouring Genes
TBS	Tris-Buffered Saline
TEM	Transmission electron microscopy
TICAM-1	TIR domain-containing adapter molecule 1
TIR	Toll/interleukin-1 receptor
TLR2	Toll-like receptor 2

TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
TM	Transmembrane
TNBS	Trinitrobenzenesulphonic acid
TNFα	Tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
TRPS	Tunable resistive pulse sensing
TSMS(s)	Tube-shaped membranous structure(s)
UC	Ultracentrifugation
UF	Milk ultrafiltrate
YEL	Yeast extract-lactate
ZO-1	Zonula occludens-1

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Presentation

Communication is a key aspect of life, as living beings continually interact with each other and with the environment. At the cellular level, communication is achieved by sharing several types of molecules, including proteins, nucleic acids and other metabolites. Some decades ago, some of these communicating molecules were found safely wrapped by extracellular vesicles (EVs), which are lipidic nanosized packages that transport biological information from cell to cell. Currently, this is thought to be a ubiquitous phenomenon, in all kingdoms of life, although the confirmation of EV production is lacking in several species. That was the case of Gram-positive probiotic *Propionibacterium freudenreichii*, although some evidences suggested that its beneficial properties could be mediated by an export system like EVs. Therefore, this thesis project was conceived to study if *P. freudenreichii* produced EVs that could be implicated in its beneficial properties, particularly immunomodulation.

In this section, the international collaboration and the funding that supported the development of this work are presented. Furthermore, the organization of the thesis is explained.

1. International collaboration

This thesis is the result of an international collaboration between Brazilian and French institutions, as part of an International Joint Laboratory that formalized an ongoing collaboration since 2000. Accordingly, this thesis was carried out under the joint supervision of:

- Dr. Vasco Ariston de Carvalho Azevedo, from the Laboratory of Cellular and Molecular Genetics (Laboratório de Genética Celular e Molecular, LGCM), in Belo Horizonte, Brazil.
- Dr. Eric Guédon, from Science and Technology of Milk and Eggs (Science et Technologie de Lait et de l'Œuf, STLO), in Rennes, France.

LGCM is associated to the Institute of Biological Sciences (Instituto de Ciências Biológicas, ICB) and the Interunit Postgraduation Program in Bioinformatics (Programa Interunidades de Pós-graduação em Bioinformática, PGBioinfo), both from the Federal University of Minas Gerais (Universidade Federal de Minas Gerais, UFMG).

STLO is associated to the French National Research Institute for Agriculture, Food and Environment (Institut National de Recherche pour l'Agriculture, l'alimentation et l'Environnement, INRAE) and the French School of Agriculture, Food, Horticultural and

Landscape Sciences (AGROCAMPUS OUEST), an internal school of the National Institute of Higher Education for Agriculture, Food and the Environment (Insitut Agro).

2. Funding

This work has received financial support from INRAE (Rennes, France), Institut Agro (Rennes, France) and the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES) of the Brazilian Ministry of Education (numbers 99999.000058/2017-03 and 88882.349289/2019-01).

3. Organization

This thesis is divided in sections as follows:

- **Chapter 1** contains a bibliographic review providing the theoretical background of this work, addressing topics like bacterial EVs, their roles as mediators of the host-microbe interaction, experimental and *in silico* approaches in EV research, as well as a comprehensive review of *P. freudenreichii* probiotic traits.
- **Chapter 2** addresses succinctly the research context, the hypothesis and the objectives.
- **Chapter 3** introduces an original research article, which demonstrates that *P. freudenreichii* produces EVs with anti-inflammatory activity *in vitro*.
- **Chapter 4** introduces a second original research article, which demonstrates that the properties of *P. freudenreichii*-derived EVs are dependent on the bacterial growth conditions.
- **Chapter 5** introduces additional studies about *P. freudenreichii*-derived EVs purified by another method and a comparative analysis of the protein content in all studied conditions.
- **Chapter 6** presents a general discussion and the perspectives of the work developed during this thesis.
- **Thesis outputs** presents a list of the products derived from this thesis, including research articles, scientific communication and patent.
- **Résumé étendu** presents an extended abstract written in French.
- **References** contains the list of scientific studies cited in the thesis.
- **Annexes** presents supplemental information.

Chapter 1 – Introduction

Abstract

The production of extracellular vesicles (EVs) is now recognized as a ubiquitous phenomenon, occurring in all kingdoms of life and comprising a secretory pathway involved in intercellular communication. EVs are natural spherical nanoparticles, composed of a lipid bilayer and an internal lumen carrying a biological cargo. EVs are agents of intercellular exchange and many bacteria were reported to produce them, including pathogenic, probiotic and commensal bacteria. Although bacterial EVs were mainly characterized in Gram-negative and pathogenic bacteria, in the last years, there was an increase in reports of EVs production by several beneficial bacteria, including Gram-negative *Akkermansia muciniphila*, *Bacteroides* spp., *Escherichia coli* Nissle 1917, *Lysobacter* spp., and Gram-positive *Lactobacillus* spp. and *Bifidobacterium* spp. The EVs produced by these bacteria exerted diverse beneficial roles, including anti-pathogenic, immunomodulatory, anti-obesity and antidepressant activities, as well as the enhancement of epithelial barrier function and gut mobility. These reports and the next to come will help to decode the crosstalk between micro-organisms and their hosts, particularly the interaction of the intestinal microbiota and human cells. Therefore, the characterization of the properties of EVs derived from beneficial bacteria hold a great potential for diverse clinical and technological applications. One of such beneficial bacteria whose importance is increasingly recognized is *Propionibacterium freudenreichii*, a Gram-positive dairy probiotic species that has been used as starter in the production of Swiss-type cheese for a long time. It was first identified more than one century ago and it has been increasingly studied since then for its interesting properties and versatility. In a technological context, this bacterium was studied for the optimization of cheese production, such as ripening and aroma compounds, but also for vitamin B12 and organic acids production. Furthermore, it has emerged in the probiotics landscape due to several beneficial traits, including tolerance to stress in the gastrointestinal tract, ability of adhesion to host cells, anti-pathogenic activity, anticancer and immunomodulatory properties. These beneficial properties have been confirmed with *in vitro* and *in vivo* investigations. Several omics approaches were employed to study the molecular mechanisms involved in these processes, resulting in the identification of important molecular actors, such as surface proteins, short-chain fatty acids and bifidogenic factors. The diversity in the species was showed to be an important aspect to take into consideration, since many of these properties are strain-dependent. Several studies have performed multi-strain screening to identify outstanding strains and optimizations have been proposed to understand or enhance technological and probiotic properties in specific contexts. New studies should dive further into the molecular mechanisms related to the beneficial properties of this species and its products, while considering the complexities of strain diversity and the interactions with the host and the microbiota. In this chapter, the aforementioned aspects will be reviewed in detail.

1. Bacterial extracellular vesicles: transportable packages of biological information

Cells from all sorts of living beings communicate with each other and with the environment in order to modulate processes that are essential for life, such as nutrition, defense and overall normal physiology (Tricarico et al., 2017). The fulfillment of this function was first considered restricted to soluble factors, such as cytokines, growth factors, peptides, and other signaling molecules (Antunes and Ferreira, 2009; Maacha et al., 2019). However, life has provided natural packages to safely wrap biological information and guarantee its delivery. It was arranged to generically designate these packages as extracellular vesicles (EVs) (Théry et al., 2018).

EVs are spherical nanostructured particles, composed of a marginal lipid bilayer and an internal aqueous lumen that harbors a potentially diverse biological cargo (Figure 1) (Théry et al., 2018). This cargo may include DNA, RNA, proteins, lipids, carbohydrates and small molecules (Kim et al., 2015b; Bitto and Kaparakis-Liaskos, 2017; Skotland et al., 2017; Jeppesen et al., 2019; Williams et al., 2019a). EVs were reported to additionally contain proteins, lipoproteins, glycoproteins, polysaccharides, lipoteichoic acid (LTA) and other elements on their surface or imbedded in their lipid bilayers (Salverda et al., 2016; Bitto and Kaparakis-Liaskos, 2017; Charoenviriyakul et al., 2018; Shiraishi et al., 2018; Valguarnera et al., 2018; Williams et al., 2019b; Cerezo-Magaña et al., 2020). Depending on specific features, such as size and origin, EVs have also been referred to as exosomes, microvesicles, microparticles, ectosomes, oncosomes, apoptotic bodies, outer membrane vesicles, membrane vesicles and others (Théry et al., 2018).

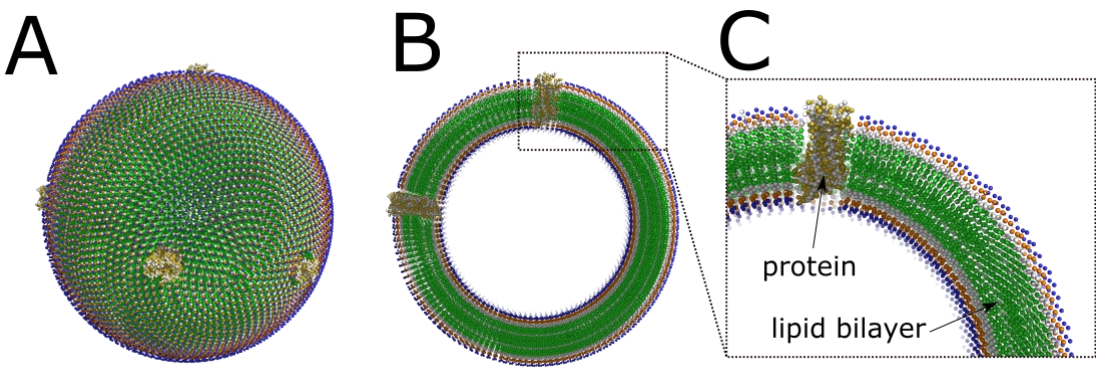

Figure 1. Schematic representation of an EV. A) Overview. B) Sectional view. C) Detail of lipid bilayer and embedded protein. Based on Van Niel et al. (2018).

Mounting evidence confirmed EV release by cells in all kingdoms of life, including bacteria, archaea, fungi, plants and human cells (Deatheragea and Cooksona, 2012; Brown et al., 2015; Woith et al., 2019). Initially, there was the assumption that their major role was the disposal of unnecessary material (Johnstone et al., 1987). However, by the end of the 20th century, a paradigm shift took place with the postulation that EVs could perform alternative roles, such as antigen presenting and immunomodulation

(Raposo et al., 1996). Thereafter, a multitude of biological roles have been associated to EVs, both in normal physiology and in pathology (Van Niel et al., 2018; Woith et al., 2019). For example, in a human perspective, EVs were associated to implications as diverse as neurological pathologies (Park et al., 2017; Choi et al., 2019), cancer (Anfossi and Calin, 2020), and microbiota-mediated improvement of inflammatory diseases (Kang et al., 2013; Chu et al., 2016; Fábrega et al., 2017).

1.1. Bacteria-derived EVs

EVs derived from bacteria have raised particular interest, due to implications in infection (Pathirana and Kaparakis-Liaskos, 2016; Ñahui Palomino et al., 2019), antibiotic resistance (Uddin et al., 2020), microbiota-derived beneficial effects (Kang et al., 2013; Chu et al., 2016; Fábrega et al., 2017), among others. Bacterial EVs were first microscopically documented in Gram-negative bacteria, specifically *Escherichia coli* grew in lysine-limiting condition (Work et al., 1966). Due to differences in cellular envelope, EV production was initially considered restricted to Gram-negative bacteria, which present an exposed outer membrane (Schwechheimer and Kuehn, 2015). Contrastingly, Gram-positive bacteria have a thick peptidoglycan layer enclosing the cell wall, which could function as an impenetrable barrier for EV release (Rohde, 2019). However, EV production by Gram-positive bacteria was reported, after more than two decades since its identification in *E. coli* (Dorward and Garon, 1990). At that point, researchers constated that the Gram-positive bacteria *Bacillus cereus* and *Bacillus subtilis* also produced EVs (Dorward and Garon, 1990). However, the first comprehensive analysis of the properties and content of EVs derived from Gram-positive bacteria was performed only in 2009 with *Staphylococcus aureus* (Lee et al., 2009). Nowadays, it is clear that Gram-negative, Gram-positive and mycolic acid-containing bacteria produce EVs with diverse cargo (Brown et al., 2015; Kim et al., 2015b; Toyofuku et al., 2019).

1.2. Biogenesis of bacterial EVs

Several types of EVs were recognized to be produced by bacteria (Figure 2), with different routes of formation (membrane blebbing or endolysin-triggered cell lysis) and origins (outer membrane or cytoplasmic membrane) (Toyofuku et al., 2019). Membrane blebbing has been extensively demonstrated for Gram-negative bacteria and consists in the protrusion of vesicles from regions of the outer membrane with weakened peptidoglycan crosslinking (Song et al., 2008; Deatherage et al., 2009; Wessel et al., 2013). Contrastingly, endolysin-triggered cell lysis is based on the enzymatic action of endolysins that act on peptidoglycan layer, demonstrated for both Gram-negative (Turnbull et al., 2016) and Gram-positive (Toyofuku et al., 2017) bacteria. Regarding nomenclature, Gram-negative bacteria are associated to the production of outer-membrane vesicles (OMVs), explosive outer-membrane vesicles (EOMVs), outer-inner membrane vesicles (OIMVs), and tube-shaped membranous structures (TSMSs);

whereas Gram-positive bacteria are associated to cytoplasmic membrane vesicles (CMVs or MVs) and TSMSs (Toyofuku et al., 2019; Nagakubo et al., 2020). However, the generic term “extracellular vesicles” or “EVs” encompass all types of vesicles (Théry et al., 2018).

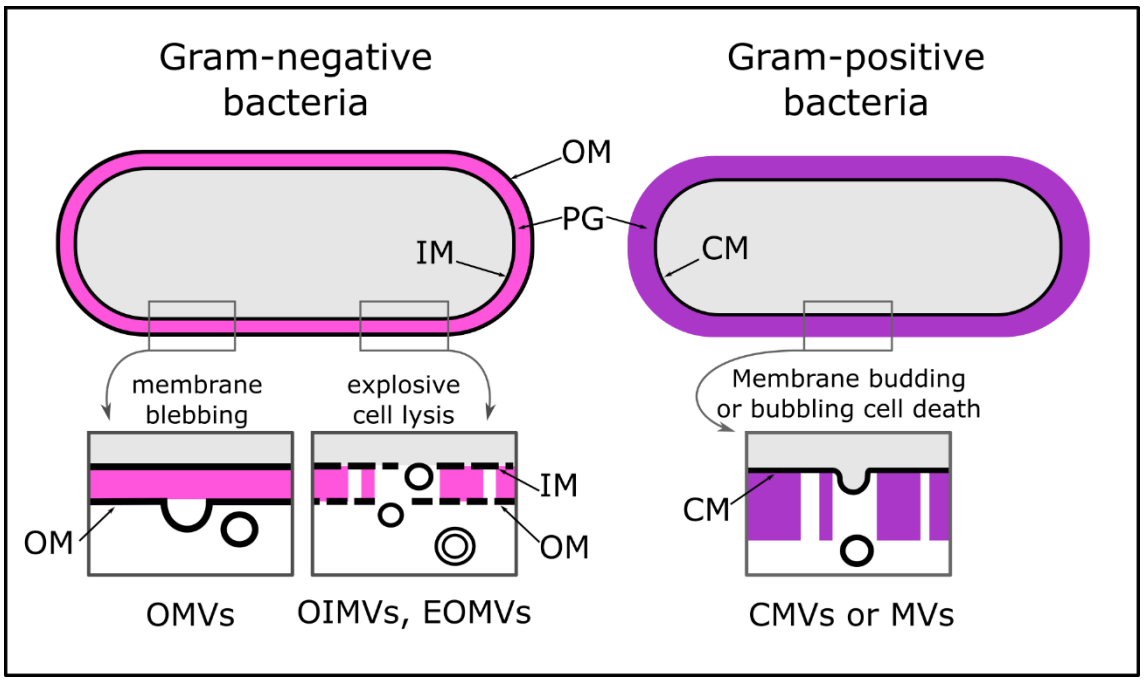

Figure 2. Types of EVs produced by Gram-negative and Gram-positive bacteria. OM: outer membrane, PG: peptidoglycan, IM: inner membrane, CM: cytoplasmic membrane, OMVs: outer-membrane vesicles, EOMVs: explosive outer-membrane vesicles, OIMVs: outer-inner membrane vesicles, CMVs or MVs: cytoplasmic membrane vesicles or membrane vesicles. Based on Toyofuku et al. (2019) and Nagakubo et al. (2020).

Regarding mechanisms of biogenesis, studies with different bacteria species focused on OMVs and MVs, and indicated that, although encompassing complex genetic and environmental determinants, EV formation is mainly related to factors affecting membrane homeostasis and cell wall permeability (Volgers et al., 2018; Briaud and Carroll, 2020).

1.2.1. *Gram-negative bacteria*

The cellular envelope of Gram-negative bacteria is composed of an outer membrane (OM), an inner membrane (IM) and a periplasmic space in between, which contains a thin peptidoglycan (PG) layer covalently and non-covalently linked to the membranes via lipoproteins (Ruiz et al., 2006; Silhavy et al., 2010; Schwechheimer and Kuehn, 2015). Several factors affecting envelope structure were identified as important for EV formation, including the interactions between PG layer and the membranes, the turgor pressor mediated by misfolded or fragmented proteins and OM modifications (Figure 3) (Schwechheimer and Kuehn, 2015).

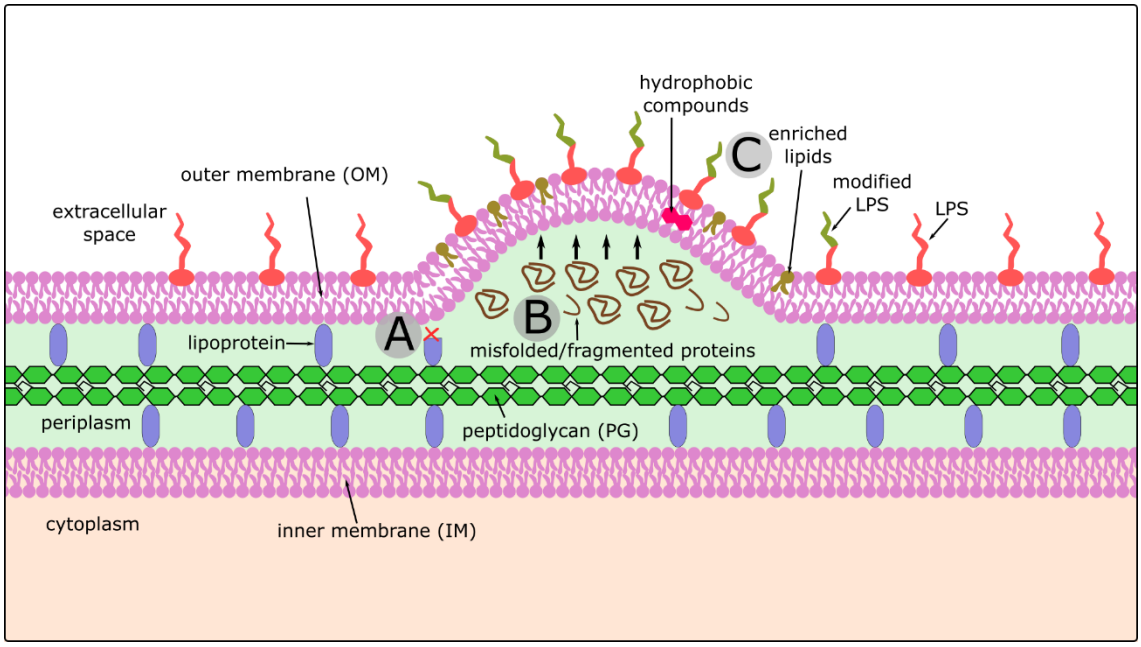

Figure 3. Factors affecting EV biogenesis in Gram-negative bacteria. A) Modulation of PG-membrane crosslinking. B) Turgor pressure mediated by misfolded or fragmented proteins. C) Alterations in membrane homeostasis, including LPS modifications, enrichment of specific lipids and interactions with hydrophobic compounds. Based on Brown et al. (2015), Haurat et al. (2015) and Schwechheimer and Kuehn (2015).

The modulation of the envelope cross-linking involves the depletion of OM proteins and lipoproteins, as demonstrated with mutant studies in *E. coli* (Sonntag et al., 1978; Wensink and Witholt, 1981; Schwechheimer et al., 2015). OM proteins also impacted EV production in *Salmonella enterica* serovar Typhimurium (Deatherage et al., 2009), *Acinetobacter baumannii* (Moon et al., 2012), *Pseudomonas aeruginosa* (Wessel et al., 2013), *Porphyromonas gingivalis* (Iwami et al., 2007) and *Vibrio cholerae* (Song et al., 2008).

Additionally, the accumulation of proteins, PG fragments and other envelope components in the periplasmic space and the consequent increase in turgor pressure was also associated to EV production (Zhou et al., 1998; Schwechheimer et al., 2014). The evidences include EVs derived from *V. cholerae*, *Helicobacter pylori*, *P. aeruginosa*, *Neisseria gonorrhoea*, and *P. gingivalis,* which contained PG-derived fragments (Zhou et al., 1998; Kaparakis et al., 2010; Bielig et al., 2011). Furthermore, the accumulation of lipopolysaccharide (LPS), PG fragments and misfolded proteins was associated to a stress response, involving increased periplasmic pression and EV production in *E. coli* and *S. enterica* serovar Typhimurium (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013; Schwechheimer et al., 2014). In this context, it was suggested that one of the functional aspects of EV production might comprise a regulated stress response for the removal of undesirable molecules (McBroom and Kuehn, 2007).

Membrane homeostasis is also considered determinant for EV biogenesis, since it is related to curvature tendency (Schertzer and Whiteley, 2012). Membrane homeostasis may be affected by lipid composition, as showed in *P. aeruginosa* and *Lysobacter* sp. XL1, whose EVs were enriched in specific types of lipids, such as phosphatidylglycerol, stearic acid, and cardiolipin (Tashiro et al., 2011; Kudryakova et

al., 2017). Furthermore, mutants for genes related to lipid transport in *Haemophilus influenzae*, *V. cholerae* and *N. gonorrhoeae* resulted in increased EV production (Roier et al., 2016; Baarda et al., 2019). Alterations in LPS, another component of the OM, were also associated to EV formation in *P. aeruginosa*, *S. enterica* serovar Typhimurium, and *Citrobacter rodentium* (Sabra et al., 2003; Elhenawy et al., 2016; Sinha et al., 2019).

EV biogenesis may also be mediated by the addition of hydrophobic compounds in the outer leaflet of the OM (Tashiro et al., 2010a; Schertzer and Whiteley, 2012). One of such compounds is 2-heptyl-3-hydroxy-4-quinolone, the hydrophobic *Pseudomonas* quinolone signal (PQS), related to quorum sensing and the transcriptional regulation of virulence genes (Mashburn and Whiteley, 2005; Lin et al., 2018). Mutant studies showed that PQS is required and sufficient for EV formation in *P. aeruginosa*, acting via direct interaction with OM components, including phospholipids and LPS, and inducing curvatures in the lipidic structure (Mashburn and Whiteley, 2005; Mashburn-Warren et al., 2008; Schertzer and Whiteley, 2012). Interestingly, PQS induced EV production in other bacteria species, including Gram-negative *E. coli* K12, *Burkholderia* spp and *Pseudomonas* spp., as well as Gram-positive *Bacillus subtilis* 168 (Tashiro et al., 2010a). Other quorum sensing molecules have been associated to EV production in other bacteria species, such as *Stenotrophomonas maltophilia* and *Chromobacterium violaceum* (Devos et al., 2015; Batista et al., 2020).

Furthermore, EV production in Gram-negative bacteria increased in response to antibiotics stress, as verified for pathogenic strains of *E. coli* exposed to gentamicin, ciprofloxacin, meropenem, fosfomycin and polymyxin B (Bielaszewska et al., 2017; Chan et al., 2017). In multidrug-resistant strain *A. baumannii* DU202, imipenem treatment increased EV production, modulated the EV protein cargo and cytotoxic activity (Yun et al., 2018). In *Burkholderia cepacian*, subinhibitory concentrations of ceftazidime increased EV production, with the enhancing of pro-inflammatory activity (Kim et al., 2020d). These increases in EV production due to antibiotic exposure are related to specific interferences in bacterial envelope homeostasis (Bielaszewska et al., 2017; Yun et al., 2018).

1.2.2. *Gram-positive and mycolic acid-containing bacteria*

Relative to research on Gram-negative bacteria, there are fewer data on the biogenesis of EVs in Gram-positive and mycolic acid-containing bacteria (Briaud and Carroll, 2020). However, there are evidences that the enzymatic remodeling of the thick peptidoglycan wall, interaction with membrane-targeting molecules and turgor pression might be important factors for EV production (Figure 4) (Toyofuku et al., 2017; Schlatterer et al., 2018).

The first proteomic study in EVs derived from Gram-positive bacteria, with *S. aureus*, identified penicillin-binding proteins, lipoteichoic acid synthase and peptidoglycan hydrolase, which are related to cell wall remodeling and could be associated to EV biogenesis (Lee et al., 2009). Proteomics studies in other species,

including *Listeria monocytogenes*, *Bacillus anthracis* and *Mycobacterium tuberculosis* also identified EV proteins with cell wall remodeling roles (Rivera et al., 2010; Prados-Rosales et al., 2011; Lee et al., 2013a; Resch et al., 2016). In *B. subtilis*, EV formation was associated to the expression of endolysin, a PG-hydrolyzing enzyme encoded by a defective prophage, which resulted in the creation of holes in the cell wall and the release of EVs into the extracellular space (Toyofuku et al., 2017). Although the cell wall did not completely disintegrate, there was loss of membrane integrity and cell death (Toyofuku et al., 2017).

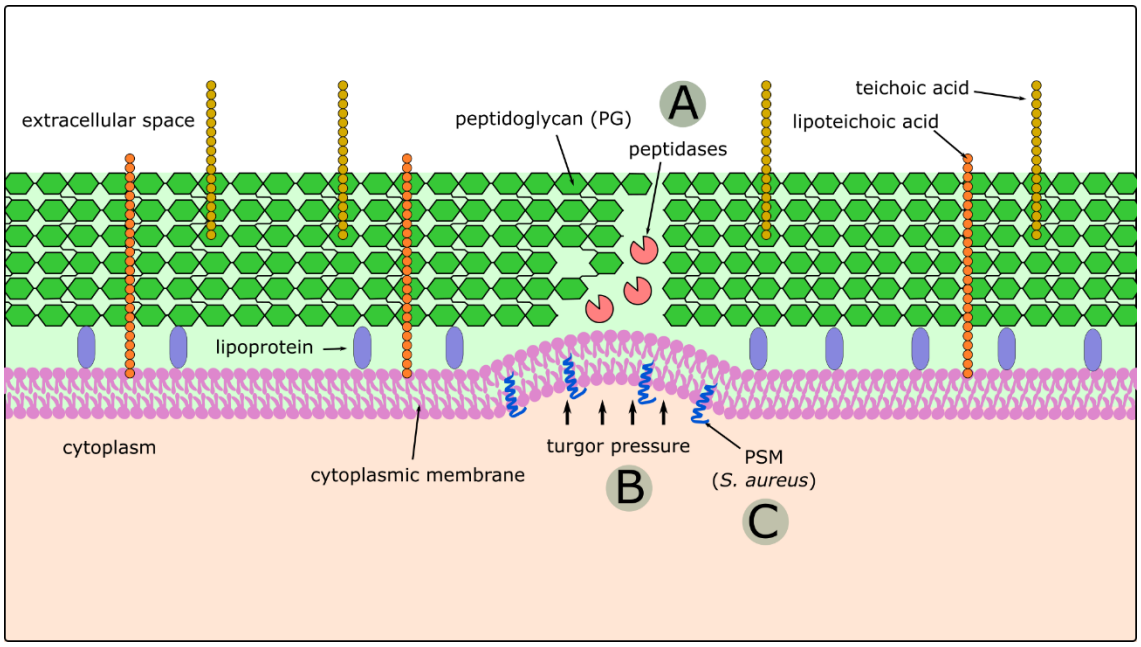

Figure 4. Factors affecting EV biogenesis in Gram-positive bacteria. A) PG layer remodeling through enzymatic action. B) Turgor pressure over the cytoplasmic membrane. C) Interactions with membrane-targeting molecules, such as phenol-soluble modulins produced by *S. aureus*. Based on Brown et al. (2015) and Wang et al. (2018).

In *S. aureus*, the surfactant-like phenol-soluble modulin α (PSMα) was associated to EV production (Schlatterer et al., 2018; Wang et al., 2018). These are pro-inflammatory peptides required for the mobilization of lipoproteins and the export of cytoplasmic proteins (Ebner et al., 2017). The deletion of *psmα* genes resulted in reduced EV yield, indicating that PSMα is important for EV production via membrane targeting (Wang et al., 2018). Moreover, the same study showed that the mutant for PG hydrolase Sle1 presented a reduction in EV yield (Wang et al., 2018). Finally, a deletion of penicillin-binding protein 4 (pbp4) gene showed reductions in PG cross-linking and increased EV production and size (Wang et al., 2018). Therefore, this study showed the multifactorial control of EV release in *S. aureus*, including PSMα-mediated membrane disrupting and PG remodeling (Wang et al., 2018). Furthermore, another mutant study with *S. aureus* showed that PSMα increased bacterial membrane fluidity and promoted EV release dependent on the existence of a turgor pressure in bacterial cells (Schlatterer et al., 2018). The importance of membrane fluidity in EV biogenesis was also demonstrated in a mutant strain of *S. aureus* which was deficient in the lipidation and

maturation of lipoproteins, resulting in increased EV yield (Wang et al., 2020c). The absence of functional lipoproteins was associated to augmented cytoplasmic membrane fluidity, as measured by excimer-forming lipid technique (Wang et al., 2020c). These studies with *S. aureus* reinforced that a combination of factors might be determinant for EV production and release.

Antibiotics were also associated to the control of EV biogenesis in Gram-positive bacteria (Andreoni et al., 2018; Kim et al., 2020c). In *S. aureus*, the treatment with quinolone ciprofloxacin in a phage-bearing strain resulted in increased EV production, via endolysin-triggered cell death (Andreoni et al., 2018). Contrastingly, the treatment with β-lactam flucloxacillin or ceftaroline increased EV production in a phage-independent manner, probably via another mechanism related to cell wall weakening (Andreoni et al., 2018). Interestingly, EVs produced under antibiotic stress also served bacteria as an decoy defense mechanism, even containing antibiotic-hydrolyzing enzymes, which is coherent with increased EV production in these situations (Lee et al., 2013b; Andreoni et al., 2018; Kim et al., 2020c).

1.3. Content of bacterial EVs

Since EVs are derived from cellular membranes, their composition naturally includes lipids (Nagakubo et al., 2020). Additionally, several other types of biomolecules have been identified in bacterial EVs cargo, including proteins of diverse origins, DNA, RNA and metabolites (Toyofuku, 2019; Nagakubo et al., 2020). EVs content has been extensively characterized by multi-omics approaches to shed light over their biogenesis, cargo sorting and functional aspects (Choi et al., 2015a). Although many aspects of bacterial EV formation and content sorting remain elusive, there is evidence that this is not a random, but rather controlled process (Schwechheimer et al., 2013; Volgers et al., 2018; Caruana and Walper, 2020).

1.3.1. *Proteins*

For the characterization of EV cargo, much attention has been allotted to protein identification, since proteins can be more directly associated to EV functions (Lee et al., 2016). EV protein content may be investigated by several approaches, including the determination of total protein content by conventional colorimetric analyses, such as bicinchoninic acid (BCA) and Bradford assays (Hartjes et al., 2019). More directed assays have also been applied to detect specific proteins in EVs, including immunoblotting and immunosorbent assays (Hartjes et al., 2019). However, over the last years, the high-throughput methodologies based on mass spectrometry (MS) have been crucial for boosting the knowledge about EV protein content (Kim et al., 2015b; Rosa-Fernandes et al., 2017). Particularly, bottom-up MS-based strategies have been widely applied in EV research, which involve the extraction of proteins, their digestion into peptides and separation by gel electrophoresis or liquid chromatography. Then, the resulting fractions of peptides are ionized and fragmented in the gas phase of mass spectrometers, with the measurement of their mass/charge ratio (Rosa-Fernandes et al., 2017). The

interpretation of mass/charge spectra intensities and their comparison to theoretical spectra allow protein identification and quantification (Wysocki et al., 2005). Several approaches are applied in MS-based EV proteomics, including shotgun proteomics and targeted proteomics, in which peptide ions are heuristically or selectively fragmented, respectively (Rosa-Fernandes et al., 2017).

In the case of bacteria, early studies with OMVs applied polyacrylamide gel electrophoresis and immunoblotting for specific protein identification, but MS-based approaches dominated since 2007, with the proteomic characterization of EVs derived from *E. coli*, which were enriched in outer membrane proteins (Kesty and Kuehn, 2004; Lee et al., 2007, 2016). For Gram-positive bacteria, in 2009, the first comprehensive characterization of EV content applied liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) for proteomic characterization of *S. aureus*-derived EVs, identifying mainly cytoplasmic proteins related to metabolism, information storage and processing, and virulence (Lee et al., 2009).

Since these initial observations, the protein cargo of bacterial EVs has been consistently associated with their origin. For instance, for Gram-negative bacteria, certain lipoproteins present in the outer membrane, such as outer membrane proteins A (OmpA), C (OmpC) and F (OmpF) were also identified in OMVs (Kesty and Kuehn, 2004; Kim et al., 2015b). The same is true for proteins from the periplasmic space, such as alkaline phosphatases and multidrug efflux pump subunits, as well as adhesins, invasins and other proteins that function as virulence factors (Kesty and Kuehn, 2004; Kim et al., 2015b). However, these EVs contained not only outer membrane and periplasmic proteins, as one would expect, but inner membrane proteins and cytoplasmic proteins were also consistently identified (Kadurugamuwa and Beveridge, 1995; Oliver et al., 2017; Nagakubo et al., 2020). The occurrence of such proteins from other cellular localizations in EVs derived from Gram-negative bacteria was justified by the enzymatic degradation of the peptidoglycan layer, which could trigger an explosive cell lysis and result in membrane fragments that round up and wrap intracellular components (Toyofuku et al., 2019; Nagakubo et al., 2020). Therefore, these corresponded to other types of EVs, such as EOMVs and OIMVs, resulting from cell lysis and rearrangement of fragments derived from Gram-negative bacteria membranes (Toyofuku et al., 2019).

In the case of Gram-positive bacteria, proteins related to peptidoglycan hydrolysis or cross-linking were also reported in EVs (Lee et al., 2009; Wang et al., 2018; Briaud and Carroll, 2020). Moreover, since EV formation involves budding of the cytoplasmic membrane, several cytoplasmic and membrane proteins were also identified in EVs derived from Gram-positive bacteria (Lee et al., 2009; Kim et al., 2015b). Furthermore, in pathogenic *S. aureus*, EVs were demonstrated to carry several virulence factors (Lee et al., 2009, 2013b; Kim et al., 2015b) and in probiotic *Lactobacillus* spp., EVs carried proteins related to probiotic effect (Rubio et al., 2017; Ñahui Palomino et al., 2019).

Overall, it is currently considered that, depending on origin and environmental conditions, bacteria-derived EVs transport diverse groups of proteins, including outer membrane proteins, hydrolyzing enzymes and multidrug efflux pumps related to antibiotic resistance, virulence factors, ABC transporters, motility-related proteins, proteases, chaperones, proteins implicated in adhesion, invasion, killing of competing bacteria, coagulation and host cell modulation, as well as several cytoplasmic proteins, such as ribosomal proteins, DNA-processing enzymes and metabolic enzymes (Lee et al., 2009; Nagakubo et al., 2020; Uddin et al., 2020).

Some evidences suggest that the packaging of such proteins inside EVs is not a random process, although the precise mechanisms for cargo sorting remain unknow (Bonnington and Kuehn, 2014; Briaud and Carroll, 2020; Nagakubo et al., 2020). These evidences include the enrichment of certain types of proteins in EVs, such as lipoproteins in EVs derived from *S. aureus* (Lee et al., 2009; Tartaglia et al., 2020) and cytoplasmic proteins in EVs derived from Gram-negative bacteria (Kadurugamuwa and Beveridge, 1995; Oliver et al., 2017; Clarke, 2018). Moreover, a multi-strain study indicated that the EV proteome of *S. aureus* is conserved in the analyzed strains (Tartaglia et al., 2020). In Gram-negative bacteria, cargo selection was associated to cell envelope stress responses, since the accumulation of misfolded proteins in the periplasmic space triggered OMVs formation (McBroom and Kuehn, 2007; Schwechheimer et al., 2013). Moreover, specific proteins, such as chaperones and some enzymes, are implicated in the selection of other proteins to be packaged inside EVs (Altindis et al., 2014; Hussain and Bernstein, 2018). Furthermore, there are also evidences of lipid-dependent (Roier et al., 2016; Yokoyama et al., 2017), size-dependent (Turner et al., 2018) and isoelectric point-dependent (Elhenawy et al., 2014) cargo selections.

1.3.2. *DNA*

Early reports of bacterial EVs containing DNA date from the decade of 1980, when its protection against enzymatic degradation during *H. influenzae* transformation was associated to membranous structures so-called “transformasomes” (Kahn et al., 1983; Gill et al., 2019). Then, subsequent studies showed that the transfer of virulence genes was also mediated by vesicles in *E. coli* O157:H7 (Kolling and Matthews, 1999; Yaron et al., 2000). Currently, several reports show that DNA of different origins is packaged inside bacterial EVs, including chromosome fragments, natural plasmids, artificial vectors and even viral DNA (Pérez-Cruz et al., 2013; Fulsundar et al., 2014; Domingues and Nielsen, 2017). Sizes may achieve up to hundreds of kilobases, but smaller fragments are more common (Domingues and Nielsen, 2017). Regarding mechanisms of content selection, little is known, although it is assumed that DNA may end up in EVs via cytoplasmic or periplasmic routes, as well as cell death or even EV lyses and rebounding (Domingues and Nielsen, 2017; Uddin et al., 2020). Furthermore, it is unclear if sequences are randomly packaged inside EVs, or if there is an active transport into EVs (Domingues and Nielsen, 2017).

As other sorts of characterization, the investigation of DNA content and functional implications in EVs derived from Gram-negative bacteria is relatively more advanced than in other bacteria (Uddin et al., 2020). Vesicle-mediated horizontal gene transfer (HGT) has been demonstrated for several Gram-negative donor species, such as *E. coli* (Yaron et al., 2000), *N. gonorrhoeae* (Dorward et al., 1989), *A. baumannii* (Rumbo et al., 2011), *Acinetobacter baylyi* (Fulsundar et al., 2014), *P. gingivalis* (Ho et al., 2015), *Thermus thermophilus* and *Thermus scotoductus* (Blesa and Berenguer, 2015). In some of these studies, DNA was tracked with techniques such as immunogold labelling (Fulsundar et al., 2014) and transformation rate was higher for EV-associated DNA than naked DNA (Rumbo et al., 2011). Fewer data are available for Gram-positive bacteria, but a pioneer study with *Ruminococcus* spp. of ruminal origin showed these bacteria were capable of transferring DNA via EVs and that the transformation restored the ability of mutants to degrade crystalline cellulose (Klieve et al., 2005). The EV-mediated

transfer of DNA was also reported in other Gram-positive bacteria, such as *Streptococcus mutans* (Liao et al., 2014), *Clostridium perfringens* (Jiang et al., 2014) and *S. aureus* (Rodriguez and Kuehn, 2020). Evidently, the horizontal gene transfer mediated by EVs has functional implications in antibiotic resistance, virulence and bacterial evolution (Gill et al., 2019).

1.3.3. *RNA*

RNA was also detected in association with bacterial EVs, in experiments with *N. gonorrhoeae* in the decade of 1980 (Dorward et al., 1989). After that, a long time passed before the emergence of reports characterizing comprehensively the RNA content of bacteria-derived EVs (Biller et al., 2014; Ghosal et al., 2015). A pioneer study with marine cyanobacterium *Prochlorococcus marinus* employed RNA sequencing on Illumina platform to investigate EVs cargo, resulting in the detection of ribosomal RNA (rRNA) in more than 94% of total sample and reaching a coverage of 95% of the open reading frames in the genome (Biller et al., 2014). Since them, researchers found RNA in EVs derived from several bacteria, such as Gram-negative *E. coli* (Ghosal et al., 2015; Blenkiron et al., 2016; Dauros Singorenko et al., 2017), *P. gingivalis* (Ho et al., 2015; Choi et al., 2017), *Aggregatibacter actinomycetesmcomitans* (Choi et al., 2017), *P. aeruginosa* (Koeppen et al., 2016), *V. cholerae* (Sjöström et al., 2015), and *Treponema denticola* (Choi et al., 2017); as well as Gram-positive *Streptococcus pyogenes* (Resch et al., 2016), *Streptococcus sanguinis* (Choi et al., 2018), *L. monocytogenes* (Frantz et al., 2019) and *S. aureus* (Rodriguez and Kuehn, 2020).

Cutting-edge technologies in transcriptomics allowed the description of a great diversity of RNA types associated to bacterial EVs, although the lack of standardization of protocols remains a great challenge to be addressed (Dauros-Singorenko et al., 2018). The referred diversity includes ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA) and several types of small RNA (sRNA) (Gill et al., 2019). The detection of enriched mRNAs in bacterial EVs suggested that they could trigger protein synthesis in recipient cells (Resch et al., 2016; Gill et al., 2019). In turn, sRNAs are known to regulate gene expression post-transcriptionally and those sRNAs detected in EVs derived from *P. aeruginosa* and *L. monocytogenes* had targets in human host cells (Koeppen et al., 2016; Frantz et al., 2019).

Whether RNA content in EVs is merely representative of the physiological state of parental cells in the absence of a selection process, or if there is an active loading into EVs consistent with the enrichment of specific transcripts, remains an open question (Lécrivain and Beckmann, 2020). Chances are this sort of enquiries will be elucidated soon, with the rising application of high-throughput and single-vesicle RNA sequencing techniques (Lécrivain and Beckmann, 2020; Wang et al., 2020b).

1.3.4. *Other molecules*

Although the majority of the studies on the content of bacteria-derived EVs focused in proteins and nucleic acids, the importance of other types of cargo is increasingly recognized, including lipids, glycans and small molecules (Kuipers et al., 2018). For example, two recent studies highlighted the importance of lipidomics

approaches to comprehend the modulation and biogenesis of EVs (Jasim et al., 2018; Kim et al., 2020b). In polymyxin-susceptible strains of *Klebsiella pneumoniae*, the lipid content of the derived EVs was modulated by the treatment with polymyxin B, with a reduction in glycerophospholipids, fatty acids, lysoglycerophosphates and sphingolipids (Jasim et al., 2018). In another study, a comparative analysis of the lipidomic profile was conducted with *Lactobacillus plantarum* APsulloc 331261 isolated from green tea leaves and its EVs, showing that several lipid classes were enriched in EVs relative to parental cells, including diacylglycerol, triacylglycerol, phosphatidylcholine, phosphatidylserine and lysophosphatidylserine (Kim et al., 2020b).

Regarding more general metabolomics approaches, recent studies showed that EVs may deliver several types of metabolites with impact on their roles (Bryant et al., 2017; Feitosa-Junior et al., 2019; Maccelli et al., 2020). The EVs derived from *Bacteroides thetaiotaomicron* obtained in different conditions presented a highly conserved metabolomic profile, enriched with colonization-related and mouse-digestible metabolites. *In silico* analysis allowed the unveiling of previously unknown metabolic interactions between the two organisms (Bryant et al., 2017). The EVs derived from strains of *Xylella fastidiosa* were analyzed by MS, resulting in a metabolomic profile of 275 compounds, including fatty acids, amino acids, carbohydrates and phenolic compounds. Among those, the unsaturated fatty acids of diffusible signaling factor family are of special interest, since they participate in the regulation of the expression of pathogenic determinants (Feitosa-Junior et al., 2019). Finally, an untargeted metabolomic approach based on fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry allowed the identification of several metabolites associated to the EVs from *Lactobacillus reuteri* DSM 17938, including amino acids, lipids, fatty acids, organic acids, polyalcohols, nucleotides, and vitamins, some of which are potentially associated to antimicrobial activity (Maccelli et al., 2020).

1.4. Functional aspects of bacterial EVs

The diversity of composition and biogenesis mechanisms of bacterial EVs has been linked to a series of functional consequences (Toyofuku et al., 2019). Although the majority of bacterial EV research has focused in pathogenesis (Kim et al., 2015b), the roles of EVs are also associated to interactions with the environment or microbial communities, as well as beneficial effects in the interactions with the host (Figure 5) (Caruana and Walper, 2020).

1.4.1. *Bacteria-bacteria communication*

In the context of the interactions in microbial communities, the propagation of quorum sensing molecules are of crucial importance, since they are implicated in processes such as pathogenesis and nutrition (Caruana and Walper, 2020). Some of these molecules are hydrophobic and their diffusion is facilitated by bacterial EVs, such as the quinolone signal of *P. aeruginosa* (Mashburn and Whiteley, 2005), the N-acyl

homoserine lactone signal of *Paracoccus denitrificans* (Morinaga et al., 2018) and the long-chain amino ketone molecule of *V. harveyi* (Brameyer et al., 2018). Additionally, EVs are reported as components of biofilm matrices, with functions of structural support, cooperation, nutrition and survival (Flemming et al., 2016; Caruana and Walper, 2020). As an example, in *P. aeruginosa*, the EVs implicated in biofilm formation also packaged virulence factors and bound antibiotics (Schooling and Beveridge, 2006). Furthermore, *S. aureus* EVs were able to increase bacterial adhesion and cell aggregation, contributing to biofilm formation (He et al., 2017).

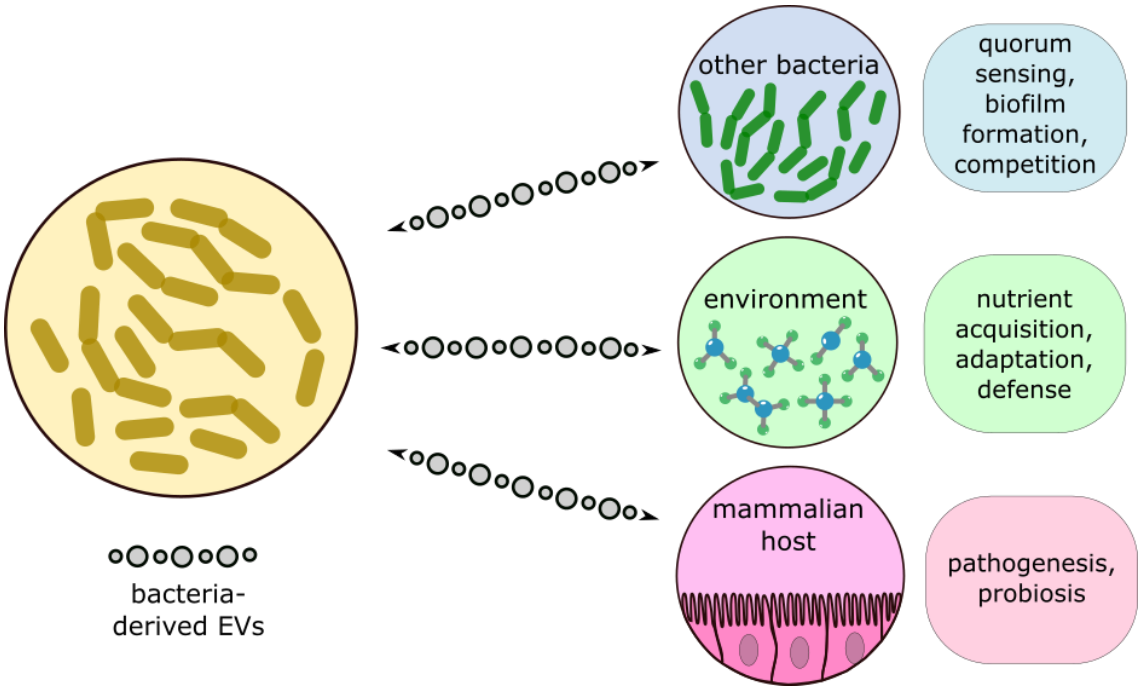

Figure 5. Overview of bacterial EVs roles as mediators of interactions with other bacteria, the environment or mammalian hosts. Based on Caruana and Walper (2020).

Besides interactions that may benefit other bacteria, EVs also mediate antagonistic interactions and competition (Li et al., 1998). Some of them export antimicrobial factors targeting other bacteria, that is the case of *P. aeruginosa* (Tashiro et al., 2013), *Myxococcus xanthus* (Berleman et al., 2014), and *Cystobacter velatus* (Yun et al., 2018). The probiotic *Lactobacillus acidophilus* was reported to deliver bacteriocin peptides to opportunistic pathogens through EVs (Dean et al., 2020) and the environmental bacterium *C. violaceum* produced EVs containing violacein, a hydrophobic microbicidal purple pigment, with roles in interbacterial competition (Batista et al., 2020).

1.4.2. *Bacteria-environment communication*

EVs functional roles also concern adaptations to a changing environment. That is the case of iron acquisition, especially in environments where this is a limiting condition. In such conditions, EVs derived from *M. tuberculosis* and *P. aeruginosa* were showed to contain iron-chelating proteins (Prados-Rosales et al., 2014; Lin et al., 2017). EV-mediated adaptation also occurs in the human gastrointestinal tract, since *Bacteroides fragilis* and *B. thetaiotaomicron* produce EVs implicated in polysaccharide breakdown, which is also beneficial for other species in the intestine, since they could consume the resulting products, even if they could not metabolize those polysaccharides themselves (Elhenawy et al., 2014). In marine ecosystems, EVs produced by the photosynthetic cyanobacterium *Prochlorococcus* had implications in marine carbon flux, since they supported the growth of heterotrophic marine bacteria and interacted with virus particles (Biller et al., 2014, 2017).

Microbial defense is also achieved by EVs, particularly regarding resistance to antibiotics. This is carried out by enzymatic degradation, such as with the EV-mediated export of beta-lactamases in *S. aureus* (Lee et al., 2013b), *Moraxella catarrhalis* (Schaar et al., 2013), *H. influenzae* (Schaar et al., 2014) and *P. aeruginosa* (Ciofu et al., 2000). As an example, *S. aureus*-derived EVs containing beta-lactamase were able to confer a transient resistance against ampicillin to susceptible surrounding Gram-negative and Gram-positive bacteria, including different strains of *E. coli*, *Salmonella enterica* serovar Enteritidis, *Staphylococcus epidermidis*, and *S. aureus* (Lee et al., 2013b). EVs may also act as decoys in the depletion of lipophilic antimicrobials that are titrated out by them, as demonstrated in strains of *E. coli* (Manning and Kuehn, 2011) and in *M. catarrhalis* (Roszkowiak et al., 2019).

1.4.3. *Bacteria-host communication*

Some bacteria communicate not only with neighboring bacteria, but also with mammalian host cells. Indeed, due to the immediate implications in human health and disease, these interactions mediated by bacterial EVs have been extensively researched, especially regarding pathogenesis (Pathirana and Kaparakis-Liaskos, 2016; Cecil et al., 2019). EVs derived from pathogenic bacteria were investigated in niches as diverse as human lungs, oral cavity, and gastrointestinal tract (Caruana and Walper, 2020). One of the key features of EV-mediated pathogenesis is the delivery of virulence factors and toxins, which results in cytotoxicity and has been verified in species such as *E. coli* (Bielaszewska et al., 2013), *Shigella dysenteriae* (Dutta et al., 2004), *Campylobacter jejuni* (Lindmark et al., 2009), *V. cholerae* (Chatterjee and Chaudhuri, 2011) and *S. aureus* (Thay et al., 2013). In the human oral cavity, bacterial EVs are associated with periodontal plaque formation, particularly *P. gingivalis*-derived EVs, that carry cysteine proteases linked to bacterial spread throughout the periodontal tissue (Nakao et al., 2014). In the gastrointestinal tract, an example of EV-mediated pathogenesis is triggered by *H. pylori*, whose EVs carry the vacuolating cytotoxin VacA, resulting in the dysregulation of inflammatory processes, such as by the induction of T cell apoptosis (Fiocca et al., 1999; Winter et al., 2014). Moreover, bacteria-derived EVs exerted immunomodulatory roles mediated by toll-like receptor 2 (TLR2) or 4 (TLR4), as

demonstrated for *B. fragilis* (Shen et al., 2012), *Brucella abortus* (Pollak et al., 2012) and mycobacteria species (Prados-Rosales et al., 2011).

Contrastingly, bacterial-derived EVs may also be beneficial to the host. That is the case of commensal or probiotic bacteria whose EVs produce anti-inflammatory effects, including *Akkermansia muciniphila* (Ashrafian et al., 2019), *B. fragilis* (Shen et al., 2012), *E. coli* Nissle 1917 (Fábrega et al., 2017), *Bifidobacterium bifidum* (López et al., 2012), and several *Lactobacillus* species (Seo et al., 2018; Vargoorani et al., 2020). These beneficial effects mediated by bacterial-derived EVs will be reviewed in detail in section 4.

1.5. Clinical and technological applications of bacterial EVs

Bacteria-derived EVs present a broad range of potential applications, since they promote content exchange, resist to enzymatic degradation and low pH, as well as spread to distant tissues without self-replicating (Stentz et al., 2018). For example, EVs have been regarded as promising vaccine candidates (Gerritzen et al., 2017), since they are implicated in the interaction with mammal hosts and in the induction of a broad range of immune responses (Bitto and Kaparakis-Liaskos, 2017). Moreover, EVs are stable in different temperatures and chemical environments (Arigita et al., 2004). Another remarkable feature is self-adjuvanting properties of EVs, which simplify vaccine design, eliminating the need for additional adjuvants, and may result in more efficient products (Bitto and Kaparakis-Liaskos, 2017; Gerritzen et al., 2017). Several bacterial EV-based vaccines showed positive results *in vivo*, including those derived from *Bordetella pertussis* (Raeven et al., 2016), *A. baumannii* (McConnell et al., 2011) and *S. aureus* (Choi et al., 2015b).

EVs also find applications in drug delivery, similarly to their synthetic counterparts, liposomes, widely used for drug encapsulation due to favorable pharmacokinetic properties (Nguyen et al., 2016; Jain and Pillai, 2017; Wang et al., 2019). Furthermore, bacteria-derived EVs show potential advantages over conventional drug delivery systems, such as protection against enzymatic degradation and naturally occurring surface proteins for targeting (Wang et al., 2019). As an example, OMVs derived from *B. thetaiotaomicron* were employed for the delivery of proteins to the gastrointestinal and respiratory tract in mice (Carvalho et al., 2019a) and *E. coli*-derived EVs were bioengineered to reduce toxic outer membrane components and deliver chemotherapeutic drugs to tumor cells and tissues (Kim et al., 2017).

EVs derived from non-pathogenic bacteria also find promising applications in biotechnological and food industries (Liu et al., 2018b). For example, EVs naturally harboring nucleic acids could be employed as systems for the delivery of DNA or RNA to recipient cells, conferring them resistance to stress or enhancing productivity (Liu et al., 2018b). Moreover, EVs derived from probiotic bacteria show potential applications as anti-inflammatory and anti-pathogenic tools (Bitto and Kaparakis-Liaskos, 2017). For example, EVs derived from several probiotic bacteria of the *Lactobacillus* genus showed anti-inflammatory properties *in vitro* and *in vivo* (Kim et al., 2018b; Molina-Tijeras et al.,

2019; Choi et al., 2020b; Vargoorani et al., 2020). Moreover, the anti-pathogenic activity associated to some bacterial EVs suggest that they could be used in infection therapy, especially those carrying antimicrobial compounds and bacteriolytic enzymes (Liu et al., 2018b; Yun et al., 2018; Ñahui Palomino et al., 2019; Dean et al., 2020). These applications are already diverse and promising, but there is potential for further improvement with the modulation of EV properties.

1.6. Modulation of bacterial EVs properties

Since bacterial EVs biogenesis is triggered by genetic and environmental factors, and EVs content is related to their functions, there has been some efforts to understand and modulate EVs properties, in order to improve their applicability (Orench-Rivera and Kuehn, 2016). As an example, some studies identified genetic factors related to increased production of EVs, such as a genetic mutation in probiotic *E. coli* Nissle 1917 (Pérez-Cruz et al., 2016) and DNA inversions at distantly localized promoters in *B. fragilis* (Nakayama-Imaohji et al., 2016). Besides genetic elements, culture medium supplementation with glycine also resulted in increased production of EVs and reduced endotoxin concentration in *E. coli* Nissle 1917 (Hirayama and Nakao, 2020). Therefore, these genetic and environmental triggers could be manipulated to increase EV production in technological contexts.

Besides EV concentration, some studies showed that it is also possible to modulate their content and functions. Environmental conditions, such as antibiotic treatment (Chan et al., 2017; Yun et al., 2018), salt stress (Lee et al., 2018; Jun et al., 2019), pH (Lynch et al., 2019), bacterial growth phase (Tashiro et al., 2010b; Zavan et al., 2019) and culture media composition (Keenan and Allardyce, 2000; Bager et al., 2013; Adriani et al., 2018; Hong et al., 2019; Taboada et al., 2019) were capable of modulating bacterial EV proteomic content and hold potential for designing content optimization.

Lastly, nanotechnology and biotechnology have provided additional tools for modulating EV properties. For example, *L. plantarum*-derived EVs were coupled with microparticles to allow controlled administration (Kuhn et al., 2020). Additionally, EVs derived from pathogenic *E. coli* were coated with gold nanoparticles in order to enhance stability and elicit an immunological response with promising applications in antibacterial vaccines development (Gao et al., 2015). In a biotechnological perspective, EVs derived from *E. coli* Nissle 1917 were engineered to carry recombinant subunit vaccines (Rosenthal et al., 2014) and other OMVs may be used for enzyme encapsulation, with potential applications in bioremediation, enzyme catalysis and vaccine development (Turner et al., 2019).

2. Experimental approaches in bacterial EVs research

2.1. Preparation of bacterial EVs samples

In laboratory settings, EVs are generally released by bacteria grew in complex culture media and coexist with bacterial cells, spent medium components, and secreted proteins, lipids and metabolites. In addition, EV concentration varies largely, depending on growth conditions and bacterial strains (Klimentová and Stulík, 2015; Orench-Rivera and Kuehn, 2016). Therefore, the obtention of high quality EV samples require an initial step to separate the supernatants from bacterial cells, a concentration step to reach valuable yields and a final separation step to recover EVs and deplete medium contaminants (Figure 6) (Brown et al., 2015; Klimentová and Stulík, 2015).

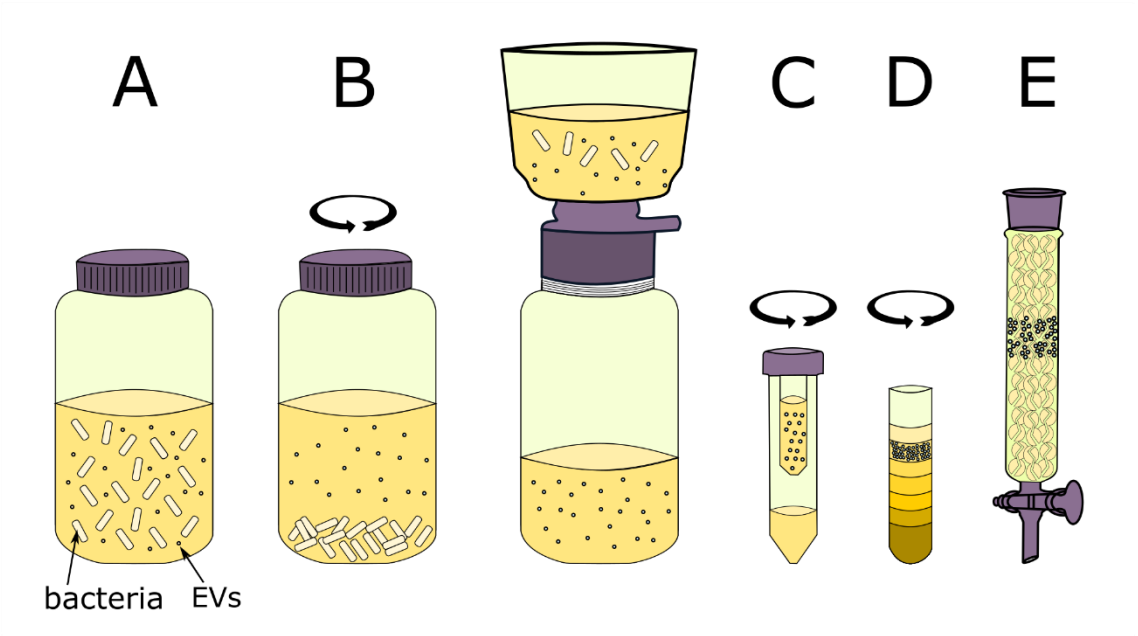

Figure 6. Example of EV purification protocol. A) Starting culture. B) Initial step, comprising centrifugation and filtration. C) Concentration of the supernatants with centrifugal ultrafiltration units. D-E) Final separation by D) ultracentrifugation with density gradient or E) size-exclusion chromatography. Adapted from Brown et al. (2015).

2.1.1. *Initial step*

In the first step of EV obtention, the supernatants of fresh bacterial cultures are isolated from bacterial cells, which is normally performed with low-speed centrifugation and filtration (Klimentová and Stulík, 2015). Centrifugation at low speeds is generally enough to precipitate bacterial cells, but further steps of differential centrifugation might be included to eliminate cell debris and large aggregates (Brown et al., 2015; Klimentová and Stulík, 2015). To ensure the elimination of residual bacterial cells, the

resultant supernatants are then filtered through pores of 0.22 µm, although EVs may be lost at some extent in this step, particularly large aggregates (Klimentová and Stulík, 2015).

2.1.2. *Concentration step*

After the obtention of clear cell-free supernatants, their volumes may be reduced in order to concentrate EVs. One of the common approaches is ultrafiltration through membranes of molecular weight cut-off varying from 10 to 100 kDa, which results in EV concentration in the retentate and depletion of contaminant molecules (Brown et al., 2015; Klimentová and Stulík, 2015; Vergauwen et al., 2017). Among the available settings for ultrafiltration, there are stirred cells, tangential flow devices and centrifugal units. In stirred cells, a gentle pressure direct large volumes of supernatant through the membrane while stirring avoids excessive membrane clogging. Tangential flow devices direct the flow of the solution parallelly to the membrane, also protecting it from clogging. Centrifugal units make use of the centrifugal force to direct the solution through membranes, although they are generally available in tubes of smaller volumes (Klimentová and Stulík, 2015). Another concentration approach is precipitation, which is based on the addition of salts, such as ammonium sulphate, to the solution containing EVs. The salt changes the way EVs interact with the solvent, i.e., the patterns of hydrogen bonds and surface charges, thus reducing their solubility and facilitating precipitation (Klimentová and Stulík, 2015).

2.1.3. *Separation step*

Concentrated samples containing EVs are then submitted to a final separation step, with the aim of further depleting contaminants, such as proteins, lipids and other residual molecules from culture media. One of the oldest and most used approaches for EV separation is ultracentrifugation (UC), which makes use of high-speed centrifugation to differentially sediment EVs and contaminants (McNamara and Dittmer, 2020). The protocols vary regarding number of UC steps, centrifugal g-forces, inclusion of density gradients and washing steps. An UC round of 100,000×g for 1-2 h is usually employed to obtain a pellet of vesicles, while contaminant macromolecules are discarded with the supernatants. The EV pellet can be resuspended and washed with an extra UC round to improve purity. Additionally, an UC round with a density gradient or cushion may also be included to improve EV purification based on density. In this case, iodixanol, sucrose or commercial solutions with varying density in the vertical axis are employed for the recovery of EVs in specific density fractions (Klimentová and Stulík, 2015; Monguió-

Tortajada et al., 2019a). In spite of its popularity, straightforward implementation and reliability, UC-based methods present some drawbacks, such as being time consuming and operator-dependent, EV aggregation after pelleting and EV damaging due to shearing forces (Mol et al., 2017; Monguió-Tortajada et al., 2019a).

Alternatively, the separation step may also be performed by size-based methods, such as size exclusion chromatography (SEC). In this case, the chromatographic column contains a buffer as mobile phase and a porous polymeric matrix, such as Sepharose CL-2B, as stationary phase. The pores in the polymeric matrix have a specific molecular weight cut-off that allows a differential elution, since smaller molecules (e.g., proteins) enter the pores and slow down, whereas bigger particles (e.g., EVs) will flow outside the pores and elute first (Monguió-Tortajada et al., 2019a). SEC may result in EV preparations with higher purity, intact structure and activity, besides being user-friendly, scalable and less time-consuming (Nordin et al., 2015; Benedikter et al., 2017; Mol et al., 2017; Monguió-Tortajada et al., 2019a). Some of the drawbacks of SEC are the limitation in the input volume, possible sample dilution and possible co-purification of EVs with viruses, since they have similar sizes (McNamara and Dittmer, 2020).

Besides size-exclusion chromatography and ultracentrifugation, other methods have been used for EV separation, such as ultrafiltration, precipitation, immunoaffinity capture and microfluidics (Théry et al., 2018; Sidhom et al., 2020). Although these alternative methods have not been extensively and individually applied in bacterial EV separation, each one has its own set of advantages and disadvantages, holding promise for future applications (Klimentová and Stulík, 2015; Sidhom et al., 2020). Some methods may be used in combinations and the best methodological approach for EV separation depends on the scientific question and the desired downstream applications (Théry et al., 2018).

2.2. Biophysical characterization of bacterial EVs

EV obtention must be validated with the characterization by multiple and complementary techniques, to assure the quality of separation and to assess the likelihood that certain biomarkers or activities are associated with EVs, and not co-isolated contaminants (Théry et al., 2018). The physical characterization of EVs focus mainly in the determination of morphology and size distribution, and may be achieved by direct high-resolution imaging or indirect optical or electrical detection (Szatanek et al., 2017; Hartjes et al., 2019).

2.2.1. *Microscopy techniques*

Electron microscopy is one of the most conventional methodologies for EV imaging, employing an electron beam for the obtention of high-resolution images, including inner structure analysis with transmission electron microscopy (TEM) (Figure 7) and topographic analysis with scanning electron microscopy (SEM) (Hartjes et al., 2019). These techniques have been successfully applied for the morphology and size characterization of isolated EVs and EVs being released by bacteria (Lee et al., 2009; Elhenawy et al., 2016; Valguarnera et al., 2018). Nevertheless, sample preparation includes fixation and drying steps that may change native EV structure, and it may be difficult to obtain representative size measures, due to the low number of analyzed EVs (Hartjes et al., 2019).

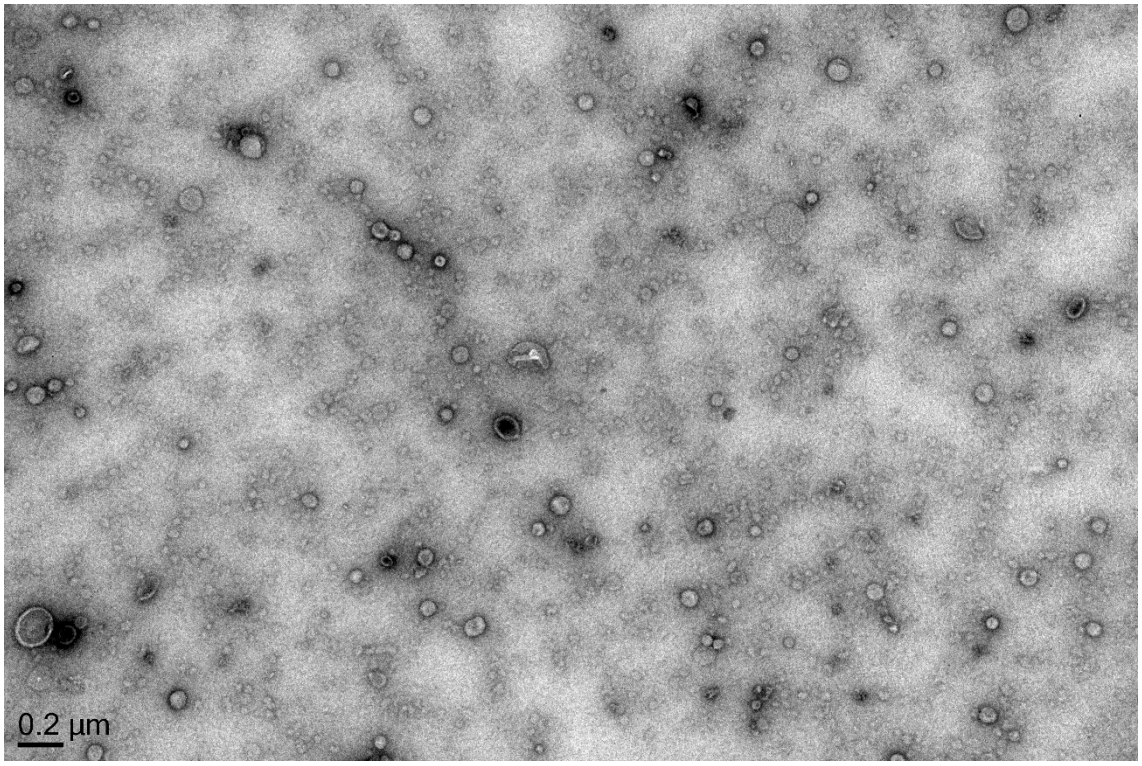

Figure 7. TEM image showing spherical nanosized EVs derived from *S. aureus*. Image acquired under a Jeol 1400 transmission electron microscope after uranyl acetate negative coloration by Rodovalho (2018).

Another commonly used image technique is atomic force microscopy (AFM), which records the interactions between a thin probing tip at the end of a microcantilever and the sample surface (Kikuchi et al., 2020). As the system scans the surface, the tip changes its vertical position in response to the topography of the sample, and the voltage used to move the tip up or down corresponds to the height data that is used in the construction of a topographic image with nanometer resolution (Allison et al., 2010). As advantages of this approach, there are minimum deformation during sample

preparation, as it does not require labeling; and the measurement of unique properties, such as EV stiffness and elasticity (Hartjes et al., 2019). However, it is a technique with low throughput and that requires specific operation skills, which has limited its wide application in EV research (Hartjes et al., 2019).

Other techniques applied to bacterial EV research include electron cryo-tomography, that allowed three-dimensional characterizations and reconstructions, providing insights into mechanisms of biogenesis (Toyofuku et al., 2017; Tartaglia et al., 2018); confocal laser scanning microscopy and confocal fluorescence microscopy, that allowed the monitoring of EV uptake by receptor cells (Ellis and Kuehn, 2010; Ling et al., 2019).

2.2.2. *Light scattering techniques*

Some techniques rely on the Brownian motion of EVs in suspension in order to measure their size distribution. That is the case of dynamic light scattering (DLS), which is based on the incidence of a monochromatic laser beam in the suspension, and the scattering of the light that finds freely defusing particles, such as EVs. The temporal fluctuations of scattered light intensity allow the calculation of particles sizes (Szatanek et al., 2017; Hartjes et al., 2019). The advantages of this method are the simplicity, speed and detection of a wide range of sizes, whereas the disadvantages include the limitation to monodisperse samples and the low resolution for subpopulations detection (Szatanek et al., 2017; Hartjes et al., 2019).

The light scattered by particles undergoing Brownian motion is also the basis of nanoparticle tracking analysis (NTA), extensively used in EV research. In this case, scattered light is used to track and record individual particles movement over time. Thus, it is possible to reconstruct individual particles trajectories and calculate their sizes through the application of Stokes-Einstein equation (Szatanek et al., 2017; Hartjes et al., 2019). The advantages include the possibility to analyze polydisperse samples, high resolution, possibility of integrating fluorescence detection, speed and simplicity, whereas the limitations include limited concentration ranges for reliable measures and the impact of physical parameters, such as temperature and viscosity (Szatanek et al., 2017; Hartjes et al., 2019).

2.2.3. *Other techniques*

Although initially applied to cells, flow cytometry is another technique that finds applications in EV research. As cells (or EVs) pass individually through a flow chamber,

they are illuminated by laser beams, and the scattered light allows the determination of size, granularity and composition of the particles. Although this is a high-throughput methodology, extensively applied in cellular biology, most of the conventional flow cytometers are unreliable for the detection of smaller EVs (Szatanek et al., 2017; Hartjes et al., 2019). Fluorescent labeling by monoclonal antibodies or in the interior of EVs have been proposed to overcome these limitations (Szatanek et al., 2017).

Finally, tunable resistive pulse sensing (TRPS) has been increasingly applied for measurements of EV size, concentration and surface charge. The methodology is based on the recording of changes in electrical current as particles pass through a nanopore, which can be related to particle volume (Hartjes et al., 2019). The technique does not require EV labeling and it is accurate, although the application to heterogeneous samples is still limited (Hartjes et al., 2019).

3. Bioinformatics approaches in EV research

Several fields in Cellular and Molecular Biology, including EV research, have meaningfully benefited from the development and cost reduction in high-throughput methodologies, which established the so-called “omics” sciences (Aizat et al., 2018; Chitoiu et al., 2020). These advances included the next generations of DNA and RNA sequencing, as well as improvements of mass spectrometry for analysis of proteins and metabolites (Aizat et al., 2018). This breakthrough allowed a whole new approach: rather than focusing in individual genes, transcripts, proteins or metabolites, research transitioned to a holistic perspective of biology, considering entire and heterogeneous sets of biological entities and their interactions (Aizat et al., 2018). At the same time, heterogeneity among organisms, cells and EVs subtypes is now being addressed with emergent approaches that account for local variability in large-scale contexts (Gho and Lee, 2017; Chitoiu et al., 2020). The immense volume of produced data, as well as their intrinsic meanings and relationships, relies on Bioinformatics analysis to dissect properties, find patterns, compare groups, organize and interpret biological information (Aizat et al., 2018).

3.1. Exploratory analysis

The omics high-throughput data represent an enormous challenge for analysis, since the results are generally expressed as long lists of biological entities (e.g., genes or proteins) and possibly their quantitative measures (e.g., expression), which turns individual literature search and interpretation into an impractical approach (Reimand et al., 2019). Bioinformatics offers diverse tools to deal with this complexity, attempting to find intrinsic patterns and relationships to make sense of omics-derived big data (Bessarabova et al., 2012; Carnielli et al., 2015; Keerthikumar et al., 2017).

One of such tools is functional enrichment analysis, which implements the reduction of long lists of biological entities into smaller functional categories, such as biochemical pathways (Reimand et al., 2019). That is possible because several individual genes, transcripts or proteins are involved in broader, yet less numerous, biological processes (Bessarabova et al., 2012; Reimand et al., 2019). In pathway enrichment analysis, the overrepresentation of pathways in an experimental-derived list of genes, transcripts or proteins is statistically tested relative to what is expected by chance (Reimand et al., 2019). For each pathway, the analysis considers both the number of elements detected in the experiment and the total of annotated elements, as well as quantitative measures, such as relative ranking (Reimand et al., 2019). The visualization of the results allow the identification of the main enriched biological themes and their relationships (Reimand et al., 2019). Several tools are freely available to conduct and visualize enrichment analysis, including g-Profiler (Raudvere et al., 2019), GSEA (Subramanian et al., 2005), FunRich (Pathan et al., 2015) and Enrichment map (Merico et al., 2010).

The lists of biological entities derived from high-throughput approaches may also be described according to their classification into categories, such as the Clusters of Orthologous Groups (COGs) of proteins (Galperin et al., 2018). This clusterization approach was first proposed in 1997, with the delineation of 720 functional clusters, each comprising orthologous proteins or proteins sets from at least three lineages (Tatusov, 1997). As orthologs typically share the same function, that allowed functional transfer and it was largely adopted for genome-wide functional annotation, particularly in poorly characterized genomes (Galperin et al., 2015). Therefore, a gene or protein set can be analyzed regarding the frequencies of COG categories that occur within that set.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) has also been traditionally used for the interpretation of high-throughput molecular datasets (Kanehisa and Goto, 2000; Kanehisa et al., 2012). KEGG is a resource that integrates multiple databases of systems information (e.g., pathways, functional modules), genomic information (e.g., genes, genomes and orthologs), and chemical information (e.g., enzymes, reactions, compounds) (Kanehisa et al., 2012). It offers manually curated resources and it is widely used for ortholog-based high-throughput annotation of genes and proteins and dissection of functions in a determined dataset (Kanehisa et al., 2016). Similarly, other relevant tools are used to interpret high-throughput molecular data, including Gene Ontology (Gene Ontology Consortium, 2019) and Reactome (Jassal et al., 2019).

Finally, exploratory analysis of proteomic data may be complemented with categorical classification of subcellular localizations. The predictors of subcellular localization are generally based on the analysis of signal peptides, transmembrane motifs, evolutionary relationships, amino acid compositions or integrative approaches (Nielsen et al., 2019). Commonly used tools include CELLO (Yu et al., 2004), PSORTb (Yu et al., 2010), SignalP (Almagro Armenteros et al., 2019), LipoP (Juncker et al., 2003), among others. These predictions are useful because subcellular localization is generally related to protein function (Hu et al., 2012). In the case of EV research, this is particularly important, because it might improve the understanding of biogenesis and content sorting in the different types of EVs (Toyofuku et al., 2019).

3.2. Interactions networks

Another way to make sense of high-throughput data is to analyze how individual biological entities connect with each other, allowing the construction of interactomes, i.e., a representation of the molecular interactions in certain contexts that provides an holistic view on cellular processes (Koh et al., 2012; Hawe et al., 2019). Interactomes may be represented as biological interactions networks, comprising a set of nodes (or points), which represent proteins, DNA, RNA or small molecules, and a set of edges (or lines), which represent the interactions between these molecules (Chisanga et al., 2017). Some examples of biological interactions networks include protein-protein interactions (PPIs) networks (Figure 8), transcriptional regulatory networks, metabolic networks and even heterogeneous networks based on the integration of multi-omics data (Chisanga et al., 2017; Hawe et al., 2019). Since proteins constitute the backbones of cellular processes, including signaling and metabolic pathways, the majority of experimentally

and computationally determined interactions are PPIs (Koh et al., 2012; Chisanga et al., 2017).

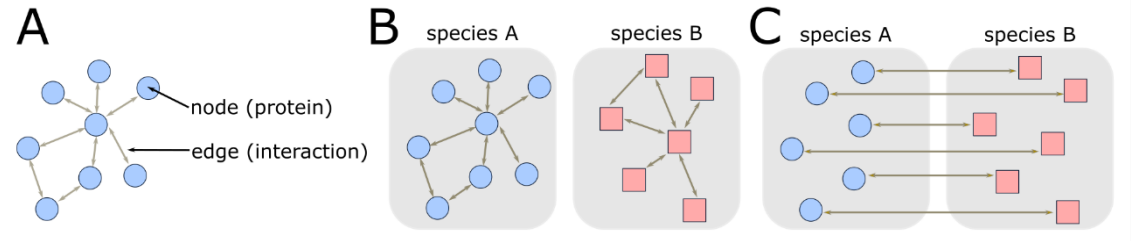

Figure 8. Graphical representation of PPIs networks. A) Representation of proteins as nodes and interactions as edges. Examples of B) intra-species and C) inter-species networks. Based on Keskin et al. (2016).

PPIs correspond to physical contacts between proteins, that are specific (non-accidental), non-generic (not corresponding to generic functions, such as protein synthesis and degradation), and context-dependent (depending on cell type, environmental conditions, etc.) (de Las Rivas and Fontanillo, 2010). PPIs may be determined experimentally by pairwise methodologies, such as yeast two-hybrid (Y2H), in which two moieties of a transcription factor are individually linked to the proteins whose interaction will be tested; if those proteins interact, the moieties bind, activating the transcription machinery and a reporter system (e.g., β-galactosidase activity) (Koh et al., 2012). Other methodologies are based on the detection of whole complexes, such as affinity purification coupled to mass spectrometry (AP-MS), in which a single protein is used as bait in a matrix and the passage of a sample through the matrix results in the retention of interacting partners, its preys, later identified by mass spectrometry (de Las Rivas and Fontanillo, 2010; Koh et al., 2012). These methodologies are also liable of generating high-throughput data, that have been stored in curated interactions databases, such as the Human Protein Reference Database (HPRD) (Goel et al., 2012), Molecular Interaction Database (MINT) (Zanzoni et al., 2002), the Search Tool for Recurring Instances of Neighbouring Genes (STRING) (Snel, 2000) and the IntAct molecular interaction database (IntAct) (Hermjakob, 2004).

However, experimental procedures for PPIs identification have a series of downsides: they are time consuming, labor intensive, and their applicability and scalability are limited, since high-throughput methodologies are still prone to many false positives and false negatives (Ding and Kihara, 2018). A number of computational approaches were developed to circumvent these limitations and complement interactomes via *in silico* predictions (Figure 9), which consider, for each potentially interacting protein pair, features based on proteins sequences, structures, functions, networks, gene expression measures and comparative genomics (Ding and Kihara, 2018). Among structure-based methods, there are molecular docking and dynamics, which model proteins interactions in atomistic scale by computing interactions forces, yet at a high computational cost and depending on structure availability (Keskin et al., 2016). Other methods, based on statistics and machine learning, use features of known interacting protein pairs to build prediction models for novel pairs, being applicable at

large scale (Keskin et al., 2016; Lian et al., 2019). Evolution-based methods are also available, such as interolog methodology, that accounts for the conservation of PPIs among species, transferring known interactions of orthologous genes to the tested proteins (Folador et al., 2014; Keskin et al., 2016). Other prediction methods are based on literature text mining, residues coupling, co-occurrence, co-expression and gene/domain fusion information (Keskin et al., 2016).

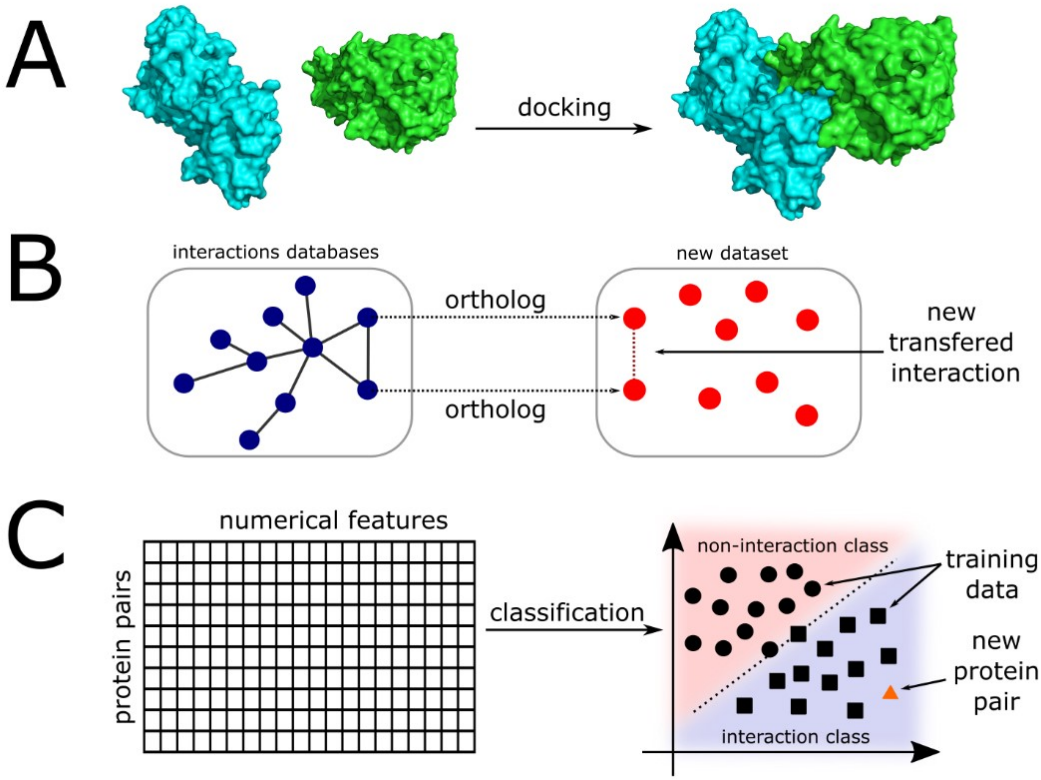

Figure 9. Examples of computational approaches for PPI prediction. A) Molecular docking, based on three-dimensional structures of potentially interacting pairs. B) Interolog methodology, based on interaction transfer for evolutionarily-related proteins. C) Machine learning approaches, which convert potentially interacting pairs in numerical representations for classification. Adapted from Keskin et al. (2016).

The prediction of PPIs find applications in several fields in microbiology, including virus-host interactions (Zhou et al., 2018), bacteria-host interactions (Lian et al., 2019), and microbiome-host interactions (Coelho et al., 2014). In EV research, the application of such techniques is still restricted, with few examples in eukaryote-derived EVs, such as the evaluation of EV purification quality (Choi et al., 2020a) and the identification of functional modules for cancer prognosis (Cao et al., 2019; Xu et al., 2020). However, the application of such methodologies that account for the complexity of EVs cargo is recommended to obtain a complete perspective of their functional roles in diverse contexts (Gho and Lee, 2017).

3.3. Machine learning

Another approach that has helped to unveil the complexity of molecular biology in the big data era is machine learning (ML). ML is a subfield of artificial intelligence (AI), comprising statistical and mathematical models that are able to improve their outcomes through experience (Kelchtermans et al., 2014; Hamet and Tremblay, 2017). In that sense, the models improve their performances due to some sort of information received, through the exploitation of regularities and pattern detection (Kelchtermans et al., 2014). The general ML workflow involves the processing of input data, the training of the model and its use to make predictions on new data (Camacho et al., 2018).

ML algorithms are often classified into categories, such as unsupervised and supervised learning. Unsupervised learning is used when input data is not labeled, meaning that their categories are unknown. In such cases, the goal might be to create groups (or clusters) from subsets of the data, based on similarity patterns. Principal components analysis (PCA) and hierarchical clustering are common examples (Camacho et al., 2018). Contrastingly, in supervised learning, labels are available and they are used to train the model, in such a way, that it will be able to predict the categories or values of new, unlabeled data. (Camacho et al., 2018). Supervised learning encompasses regression analysis, when the outputs are continuous variables, and classification analysis, when the outputs are discrete variables (Baştanlar and Özuysal, 2014).

ML finds applications on several fields in bioinformatics, including PPIs predictions (Ahmed et al., 2018; Lian et al., 2019), protein function prediction (Bonetta and Valentino, 2020) and several aspects of proteomics based on mass spectrometry (Kelchtermans et al., 2014). Microbiology meets ML in applications as diverse as bacterial species identification (Roux-Dalvai et al., 2019), metagenome-based disease prediction (LaPierre et al., 2019), bacterial infection diagnosis (Rawson et al., 2019), fighting microbial resistance (Macesic et al., 2017) and even bioterrorism (Reardon, 2019). EV research has also benefited from ML methods, with applications in motif detection in miRNAs from exosomes (Gaur and Chaturvedi, 2019), diagnosis of traumatic brain injury based on EV miRNA signatures (Ko et al., 2018), proteomic profiling of EVs from Alzheimer's disease (Muraoka et al., 2020) and identification of EV proteins as biomarkers for cancer detection (Hoshino et al., 2020).

3.4. Dedicated data repositories

In order to store and organize omics data derived from EV research, some freely available repositories were created, including ExoCarta, Vesiclepedia and EVpedia (Keerthikumar et al., 2017). ExoCarta is a web-based compendium of exosome cargo, including data mainly of mammal origin, purification protocols and biomarkers identification (Mathivanan and Simpson, 2009). It was the first initiative of this kind, launched in 2009 and initially focusing in exosome protein and RNA content (Mathivanan and Simpson, 2009). Subsequently, updates in the repository included lipids as a new feature and implemented community involvement in data uploading

(Mathivanan et al., 2012). Although the volume of data increased in the repository, the active participation of the community was still limited, and the heterogeneity in purification protocols required caution for the applicability of the datasets (Simpson et al., 2012). The last improvements in the repository included the adaptation to annotation standards proposed by the International Society for Extracellular Vesicles (ISEV), and the addition of tools for analysis of PPI networks, Gene Ontology and biological pathways (Keerthikumar et al., 2016).

As ExoCarta was limited to exosomes, the introduction of Vesiclepedia in 2012 extended data availability, including omics data from other types of EVs and organisms (Kalra et al., 2012). Subsequent updates in Vesiclepedia repository included quality metrics calculated from experimental parameters related to EV-TRACK initiative (Van Deun et al., 2017), as well as the functionality of FunRich, an enrichment analysis tool (Pathan et al., 2015, 2019). Another repository was launched in 2013, named EVpedia, which contained datasets from both prokaryotic and eukaryotic EVs (Kim et al., 2013). EVpedia also included tools for enrichment, network and ortholog analysis (Kim et al., 2013). A subsequent update included the category of metabolomics and a personalization function for account creation and storage of private datasets (Kim et al., 2015a).

In summary, the availability of such repositories allowed the centralization and organization of high-throughput datasets from EV cargo of diverse origins, facilitating data acquisition for comparative studies, biomarker discovery and potential insights about cargo sorting and EV biogenesis (Kim et al., 2015a; Keerthikumar et al., 2017). Nevertheless, there is still room for improvement, particularly in what refers to community engagement and protocol standardization (Kim et al., 2015a; Keerthikumar et al., 2017).

3.5. Perspectives

As an emerging field, the research on EVs, particularly on those of bacterial origin, has been associated to more traditional bioinformatics analysis, including biological entities identification and quantification on high-throughput datasets and descriptive exploratory analysis (Keerthikumar et al., 2017). However, the tendency of holistic and integrative approaches that started to be applied mainly in eukaryotic-derived EVs, such as interactions networks analysis and machine-learning, are also promising for EVs of other origins (Carnielli et al., 2015; Gho and Lee, 2017; Gézsi et al., 2019).

Furthermore, recent developments hold promises for data generation and bioinformatics analysis in EV research. That includes single-EV sequencing, which will allow the investigation of individual EVs and the identification of cargo and function heterogeneities in EVs subpopulations, currently hidden in bulk approaches (Pick et al., 2018). Novel nanotechnological microfluidics-based systems will also contribute to EV isolation, detection and analysis, enriching characterization and improving the quality of downstream analysis, while offering advantages, such as low cost, high throughput and precision (Guo et al., 2018).

Furthermore, some promising computational methods have extended their application to EV research, such as deep learning, a subfield of machine learning based on several layers of neural networks that can automatically extract features from high-throughput omics data and achieve accurate predictions (Zhang et al., 2019c). Some recent deep learning applications in EV research include the identification of EV transport proteins from sequence features, such as amino acid composition (Le et al., 2019), the segmentation of small EVs using transmission electron microscopy images (Gómez-de-Mariscal et al., 2019), imaging analyses of EVs *in vivo* (Kranich et al., 2020) and early-stage EV-mediated diagnosis of lung cancer (Shin et al., 2020).

Taken together, these and other experimental and computational improvements hold a great potential to bring novel insights into biogenesis, cargo sorting and functional roles of bacterial EVs (Gho and Lee, 2017).

4. EVs as mediators of the interaction between beneficial bacteria and the host

As previously discussed, there are more reports of EV occurrence in pathogenic bacteria than in beneficial bacteria (Kim et al., 2015b; Molina-Tijeras et al., 2019). EVs derived from pathogenic bacteria deliver virulence factors and other molecules to host cells, that promote immunomodulation and contribute to pathogenesis (Ellis and Kuehn, 2010; Macia et al., 2019). Contrastingly, beneficial bacteria produce EVs that trigger immune tolerance and anti-inflammatory effects, which highlights their therapeutic potential (Macia et al., 2019; Molina-Tijeras et al., 2019). Overall, beneficial bacteria-derived EVs present the typical spheric membranous shape, size range of 20 to 400 nm in diameter, and several molecules in their interior or at their surface, depending on the producing bacterium (Figure 10). In the next subsections, the reports of EV production in several Gram-negative and Gram-positive beneficial bacteria are reviewed in detail, with focus on their content and activity.

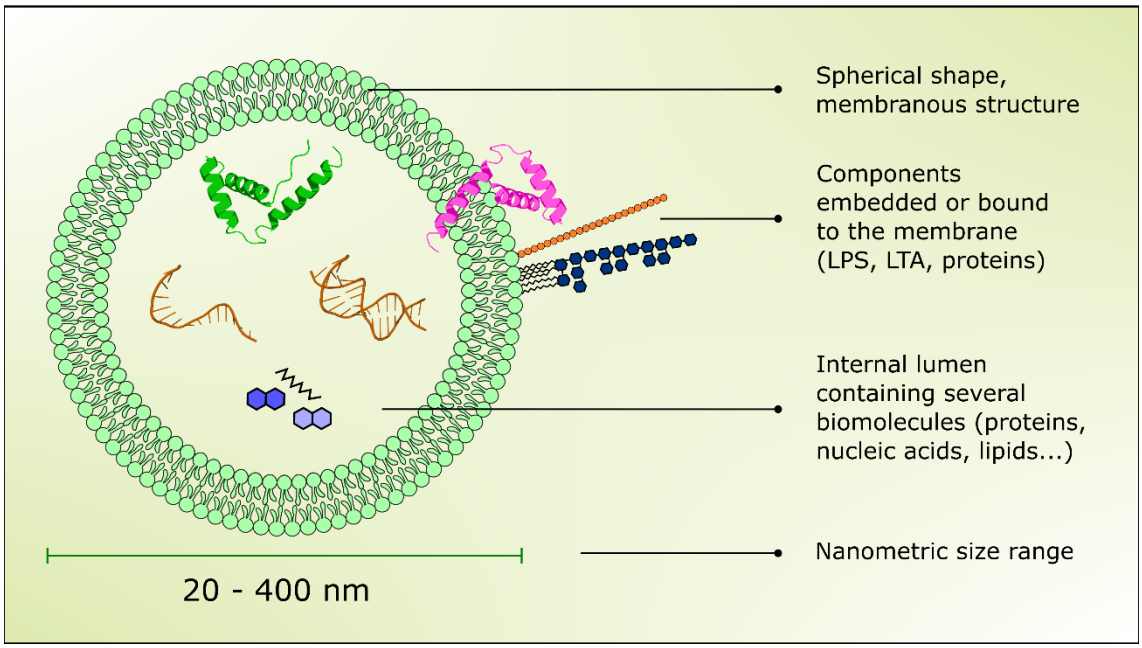

Figure 10. Key aspects of beneficial bacteria-derived EVs. Based on Liu et al. (2018).

4.1. EVs produced by Gram-negative beneficial bacteria

Studies of EVs derived from Gram-negative beneficial bacteria have focused mainly in the classical probiotic *Escherichia coli* Nissle 1917. However, the next-generation probiotics, such as *Akkermansia muciniphila* and *Bacteroides fragilis*, are increasingly investigated for EV production (Molina-Tijeras et al., 2019).

4.1.1. *Escherichia coli* Nissle 1917-derived EVs

The strain *E. coli* Nissle 1917 is the most commonly studied Gram-negative probiotic bacterium (Sonnenborn, 2016; Behrouzi et al., 2020). It was firstly isolated by Alfred Nissle, in 1917, from the feces of a healthy soldier (Nissle, 1918). Since them, scientific interest has arisen for its role as gut colonizer and its beneficial properties, including the treatment of ulcerative colitis (Scaldaferri et al., 2016), genetic engineering for vaccine development (Ou et al., 2016) and anticancer therapy (Yu et al., 2020).

The first study reporting the occurrence of EVs in this probiotic strain performed a comprehensive characterization of their proteome, finding proteins inside EVs that were associated to adhesion to host tissues and bacterial survival, which was suggested to contribute to the ability of this strain to colonize the human gut (Aguilera et al., 2014). Another study performed a comprehensive proteomic characterization of *E. coli* Nissle 1917 and an uropathogenic strain (*E. coli* 536), comparing the method of purification of EVs (ultracentrifugation, density gradient centrifugation or size exclusion chromatography), and growth conditions (iron-restricted and iron-supplemented). The EVs derived from *E. coli* 536 were enriched in functional terms related to ribosome, while the EVs derived of *E. coli* Nissle 1917 were enriched in proteins related to glycolysis and ligase activity. Furthermore, some proteins consistently enriched in specific conditions were identified as potential markers of purity and culture conditions of EV preparations (Hong et al., 2019). The proteome of *E. coli* Nissle 1917-derived EVs and their role in the modulation of mouse macrophage RAW264.7 cells immune response was also investigated. Proteomics allowed the identification of proteins related to probiotic traits, such as intestinal adhesion and colonization (flagellins), bacterial survival (transporters), antimicrobial activities (murein hydrolase), and interaction with the host (outer membrane proteins). Regarding functional roles, EVs were internalized by macrophages, promoted their proliferation, phagocytic and antibacterial functions. EVs also induced anti-inflammatory cytokines (IL-10) and modulated the production of Th1 and Th2-polarizing cytokines (Hu et al., 2020).

Other studies further clarified the immunomodulatory roles of *E. coli* Nissle 1917-derived EVs. For instance, EVs could activate cytokine production by peripheral blood mononuclear cells (PBMCs) in co-culture with Caco-2 Cells, which was used as a model of intact intestinal mucosa. Particularly, the increase in the expression of the anti-inflammatory cytokine IL-10 was significantly higher for the group stimulated by *E. coli* Nissle 1917-derived EVs. Colonic mucosa explants were also used as an *ex vivo* model, confirming that EVs triggered the modulation of the expression of immunomodulatory mediators, evidencing the ability of microbiota-derived EVs to promote signaling through the intestinal epithelial barrier (Fábrega et al., 2016). A follow-up study confirmed that *in vivo*, showing that the oral administration *E. coli* Nissle 1917-derived

EVs has intestinal anti-inflammatory effects *in vivo*, in mice with DSS-induced colitis. These results show that EVs reproduce the effects of viable probiotic administration, with the improvement of clinical signs, colonic histology and cytokine profile (Fábrega et al., 2017).

Epithelial barrier enhancement was another important role associated to probiotic-derived EVs. The EVs and soluble fractions from *E. coli* Nissle 1917 and ECOR63 regulated the expression of tight junction proteins in intestinal epithelial cells Caco-2 and T-84. That included the upregulation of ZO-1 and claudin-14, and the down-regulation of claudin-2, thus reinforcing the epithelial barrier (Alvarez et al., 2016). A similar role was verified when polarized T-84 and Caco-2 cells were infected with enteropathogenic *E. coli*, simulating tight junctions' disruption and gut permeability increase. EVs derived from *E. coli* Nissle 1917 and ECOR63 counteracted the intestinal epithelial barrier dysfunction by modulating occludin and claudin-14 expression, retaining ZO-1 and occludin at tight junctions and ameliorating F-actin disorganization (Alvarez et al., 2019).

Finally, the internalization pathway of EVs was also investigated. EVs derived from strains *E. coli* Nissle 1917 and ECOR12 were internalized by the human intestinal epithelial cell lines Caco-2 and HT-29, as well as the mucus-secreting HT29-MTX. The internalization occurred by clathrin-mediated endocytosis and that EVs were then sorted to lysosomal compartments. EVs inhibited HT-29 cell growth, but did not affect cell viability, while also inducing DNA damage and repair (Cañas et al., 2016). A follow-up study showed that the EVs derived from the same strains were also able to activate immune responses mediated by intracellular receptor NOD1 in intestinal epithelial cells, possibly through the intracellular delivery of peptidoglycan fragments (Cañas et al., 2018).

4.1.2. *Akkermansia muciniphila*-derived EVs

Recent studies have unraveled beneficial bacteria other than traditional probiotics, namely next generation probiotics, with improved effectiveness and disease-specificity (Chang et al., 2019; Zhang et al., 2019b). One example is *A. muciniphila*, that is reported to reverse obesity and insulin resistance (Chang et al., 2019). *A. muciniphila* is a Gram-negative bacterium of oval shape, that can use mucin as its single source of carbon, nitrogen, and energy, being widely distributed in human and animal intestine (Zhai et al., 2019; Zhang et al., 2019b).

A pioneer study evaluated EVs derived from mice stool by conducting a comprehensive metagenomics characterization. These results were associated to progression of colitis, in order to identify significant changes in EV composition in the

disease state, finding that *A. muciniphila*-derived EVs had an inverse relationship with colitis progression. Therefore, *A. muciniphila*-derived EVs were further analyzed *in vivo*, displaying protective effects that ameliorated the severity of colitis induced by dextran sodium sulfate in C57BL/6 mice (Kang et al., 2013). Another study showed that fecal samples of healthy individuals are enriched in *A. muciniphila*-derived EVs in comparison to patients with type 2 diabetes. Moreover, the administration of theses EVs to mice with diabetes induced by high-fat diet resulted in enhancement of tight junction function and improved glucose tolerance. Finally, the barrier integrity of human epithelial Caco-2 cells was improved with the administration of EVs, through 5' adenosine monophosphate-activated protein kinase (AMPK) activation (Chelakkot et al., 2018).

Another study indicated that *A. muciniphila*-derived EVs could be employed in the therapy of obesity, as shown for C57BL/6 mice with obesity induced by a high-fat diet. The EVs derived from *A. muciniphila* strain MucT reduced the food intake and weight gain in mice, improving intestinal barrier integrity and reducing inflammation. Lipid metabolism and expression of inflammatory markers in adipose tissues were also affected by the treatment with EVs (Ashrafian et al., 2019).

4.1.3. *Bacteroides fragilis*-derived EVs

B. fragilis are Gram-negative bacteria commonly found in the human gastrointestinal, oral, upper respiratory and genital tract (Sun et al., 2019). The strains in this species are divided in an enterotoxigenic group, which produces *B. fragilis* toxin (BFT) and triggers energy metabolism and intestinal dysfunction; and a nontoxigenic group, comprising beneficial commensal bacteria that compete with pathogenic bacteria and deliver beneficial molecules, such as polysaccharide A (PSA) (Sun et al., 2019; Valguarnera and Wardenburg, 2020). Since the beneficial strains of *B. fragilis* display health-promoting traits, such as anti-inflammatory and anticancer effects, they have also been regarded as next-generation probiotics (Chang et al., 2019).

The delivery of beneficial effector molecules derived from *B. fragilis* to host cells is reported to occur via EVs (Sun et al., 2019). For instance, EVs derived from *B. fragilis* strain NCTC9343 protected BALB/c mice from 2,4,6-trinitrobenzenesulfonic acid-induced colitis and intestinal inflammation, with the suppression of the production of tumor necrosis factor (TNFα) and the development of Th17 cells. These EVs were internalized by dendritic cells, which induced the differentiation of IL-10-producing Treg cells. One of the main factors in the promoted immunomodulation was capsular polysaccharide (PSA), which was packed inside EVs for its deliverance to the mammalian host (Shen et al., 2012). Another study used Caco-2 cell line as a human intestinal

epithelial cell model to evaluate the immunomodulatory roles of *B. fragilis* ATCC 23745-derived EVs. Higher concentrations of EVs increased TLR2 and TLR4 gene expressions, as well as modulated cytokines concentrations, decreasing interferon gamma (IFNγ) and increasing IL-4 and IL-10 concentrations (Badi et al., 2019).

A comparison of the EVs derived from enteropathogenic *B. fragilis* BOB25 and non-toxigenic *B. fragilis* 323-J-86 was also performed, with regard to their proteomic and metabolomic content. *B. fragilis* 323-J-86-derived EVs had enzymes related to polysaccharide metabolism, suggesting a nutrition role in a community context; whereas *B. fragilis* BOB25-derived EVs had virulence factors and enzymes comprising whole pathways, related to their stability and persistence. This study suggested that biochemical processes were still active in *B. fragilis* BOB25-derived EVs after secretion, turning them into an active and self-sufficient biological system (Zakharzhevskaya et al., 2017).

4.1.4. EVs derived from other Gram-negative probiotic bacteria

Other examples of Gram-negative bacteria that were reported to produce EVs with beneficial properties include *Lysobacter* and *Pseudoalteromonas* genera. *Lysobacter* sp. XL1 produced EVs containing components of bacterial outer membrane and bacteriolytic endopeptidases. These EVs showed bacteriolytic activity against Gram-positive *Staphylococcus aureus* 209-P and Gram-negative *Erwinia marcescens* EC1 (Vasilyeva et al., 2008). The EVs derived from *Lysobacter enzymogenes* strain C3 showed anti-fungal activity, inhibiting the growth of the yeast *Saccharomyces cerevisiae* and of the filamentous fungus *Fusarium subglutinans* (Meers et al., 2018). Finally, isolates of *P. piscicida* produced EVs with digestive activity against *Vibrio parahaemolyticus,* suggesting that EVs may be part of a mechanism of bacterial competition (Richards et al., 2017).

Some *Bacteroides* species other than *B. fragilis* also produces EVs. It is the case of *B. thetaiotaomicron*, reported to secrete EVs containing large quantities of glycosidases and proteases, which is coherent with its role as a component of human microbiota, helping in the digestion of dietary polysaccharides, and making this digestion products available to other members of the microbiota (Valguarnera et al., 2018). Additionally, the symbiotic commensal *Bacteroides vulgatus* mpk was reported to produce EVs that are essential for the induction of dendritic cells semi-maturation, resulting in a tolerant dendritic cells phenotype (Maerz et al., 2018).

4.2. EVs produced by Gram-positive beneficial bacteria

Studies concerning EV production in Gram-positive bacteria have emerged only since 2009, with the first comprehensive characterization of *Staphylococcus aureus*-derived EVs (Lee et al., 2009). In the case of Gram-positive beneficial bacteria, there are even fewer reports of EV production, mainly for *Lactobacillus* and *Bifidobacterium* species (Molina-Tijeras et al., 2019).

4.2.1. *Lactobacillus*-derived EVs

Lactobacillus is the main genus of Gram-positive probiotic bacteria that has been studied for EVs production. This is a complex and diverse genus, comprising hundreds of species of pronounced technological and scientific interest, including members of the normal commensal human flora, particularly in the gastrointestinal tract and vagina (Goldstein et al., 2015; Hatti-Kaul et al., 2018). Some species in this genus have been considered occasional opportunistic pathogens, but reports of serious infections are rare and often associated to comorbidities (Goldstein et al., 2015). These bacteria also have a long history of use in food production, including cheese, yogurt, and fermented mustard, cabbage, and olives (Goldstein et al., 2015; Şanlier et al., 2019). Although a recent reclassification was proposed in the genus (Zheng et al., 2020), we kept the traditional nomenclature, as used in the published reports.

Regarding EV production, one of the most studied species in the genus is *Lactobacillus plantarum*. The strain *L. plantarum* WCFS1 was showed to produce EVs within the typical shape and sizes (31-200 nm). Their content was investigated, allowing the identification of 31 proteins, half of which were associated to the membrane. When the nematode *Caenorhabditis elegans* was used as a model of infection with vancomycin-resistant *Enterococcus faecalis* C68, the pre-treatment with *L. plantarum*-derived EVs resulted in significantly longer survival of *C. elegans* in comparison with the control group. Furthermore, in addition to this protective effect against infections, EVs were showed to enhance the expression of host defense genes, reproducing the effects of parental bacterial cells (Li et al., 2017). The EVs derived from another strain, *L. plantarum* KCTC 11401BP, showed antidepressant-like effects *in vitro* and *in vivo*. The study investigated the expression of the brain-derived neurotrophic factor (BDNF), an important regulator of synaptic transmission and plasticity. *L. plantarum*-derived EVs increased the expression of BDNF in hippocampal HT22 cells, reversing the effects of stress hormone glucocorticoid. Furthermore, *L. plantarum*-derived EVs promoted an increased BDNF expression in the hippocampus of mice with stress-induced depression (Choi et al., 2019).

The EVs derived from the strain *L. plantarum* KCTC 11401BP also presented immunomodulatory properties and a protective effect in the context of skin inflammation induced by *S. aureus*. When keratinocytes and macrophages were treated with *S. aureus*-derived EVs, the pre-treatment with *L. plantarum*-derived EVs was able to decrease IL-6 levels and restore cell viability. *In vivo*, the administration of *L. plantarum*-derived EVs to mouse with atopic dermatitis induced by *S. aureus* resulted in the reduction of epidermal thickening and IL-4 levels (Kim et al., 2018a). Finally, the strain *L. plantarum* APsulloc 33126 produced EVs that induced M2 macrophage polarization *in vitro* and an anti-inflammatory phenomenon in the human skin cells, via modulation of the secretion of IL-10, IL-1β and GM-CSF (Kim et al., 2020e).

Other studies focused on the species *L. rhamnosus*, which also produces EVs. In the case of strain *L. rhamnosus* JB-1, EVs were spherical as typical, ranging in size from 50 to 150 nm. Their protein cargo consisted of 13 EV-exclusive proteins and 12 proteins shared with bacterial cells, which were mainly related to stress response and glycolysis. Regarding biological activity, the EVs derived from *L. rhamnosus* JB-1 showed immune and neuronal effects that were similar to those of the parent bacteria, including an increase in functional regulatory T cell population in a murine model (Al-Nedawi et al., 2015). The EVs derived from strain *L. rhamnosus* GG, by the other side, showed a concentration-dependent cytotoxic effect against HepG2 hepatic cancer cells, via the intrinsic pathway of apoptosis (Behzadi et al., 2017). The cell-free supernatants and EVs of strains *L. rhamnosus* GG and *L. reuteri* DSM 17938 were also evaluated regarding their anti-inflammatory properties, showing a reduction in IFNγ responses from T-cells and NK cells in a monocyte dependent manner (Mata Forsberg et al., 2019).

The species *L. casei* and *L. paracasei* have also been studied for its EVs. *L. casei* BL23 produced EVs ranging in diameter from 26 to 70 nm, containing DNA, RNA and proteins (Rubio et al., 2017). Regarding proteomic composition, 103 proteins were identified, 13 of which were exclusive from EVs. The protein cargo included stress, metabolic, ribosomal and cell wall-associated proteins, as well as a adhesion protein that was exclusively identified in EVs (Rubio et al., 2017). Another strain, *L. casei* ATCC 393, produced EVs with anti-inflammatory activity towards colon carcinoma Caco-2 cells, decreasing TLR9 gene expression and the level of IFNγ, while increasing IL-4 and IL-10 levels (Vargoorani et al., 2020). *L. paracasei* EVs also displayed anti-inflammatory activity, both *in vitro* and *in vivo* (Choi et al., 2020b). *In vitro* experiments showed that EVs reduced the expression of pro-inflammatory cytokines and increased the expression of anti-inflammatory cytokines in HT-29 human colorectal cancer cells, whereas *in vivo* experiments showed EVs promoted in mice a protection against DSS-induced colitis (Choi et al., 2020b).

The strain *L. reuteri* DSM 17938 has also been reported to produce extracellular vesicles. A study investigated both biofilm and planktonic phenotypes of this strain,

finding that extracellular DNA was associated to EVs, especially for the biofilm phenotype (Grande et al., 2017; Puca et al., 2019). Another study with the same strain showed that *L. reuteri*-derived EVs could reproduce the effects of the parental bacteria on gastrointestinal motility in mice (West et al., 2020). By the other hand, the strain *L. sakei* NBRC15893, isolated from a traditional seed mash used for brewing sake, produced EVs capable of enhancing IgA production by murine Peyer's patch cells and activating the mucosal immune system via TLR2 signaling *in vitro* (Yamasaki-Yashiki et al., 2019). The strain *L. gasseri* JCM 1131T produced EVs with surface-exposed LTA, which could have dramatic effects in the interactions with the host (Shiraishi et al., 2018).

Other studies of *Lactobacillus*-derived EVs compared their properties, content and activity according to strain of origin. A comprehensive study compared the EVs produced by *L. acidophilus* ATCC 53544, *L. casei* ATCC 393, and *L. reuteri* ATCC 23272. They shared a similar bimodal size distribution ranging from 20 to 50 nm and from 100 to 150 nm in diameter. Moreover, a proteomic analysis showed there was little consistency of EV protein content among the strains, and that *L. acidophilus* ATCC 53544 produced EVs enriched in components of the bacteriocin pathway (Dean et al., 2019). A follow-up study further investigated the EVs of this last strain, which were reported to transport bacteriocin peptides to *L. delbrueckii* ATCC 15808, an opportunistic pathogen, inhibiting its growth and compromising its membrane integrity. This finding suggested EVs may have additional roles in competition within the complex communities of the gut microbiome (Dean et al., 2020).

A comparison was also performed with kefir-derived strains *Lactobacillus kefir* KCTC 3611, *Lactobacillus kefiranofaciens* KCTC 5075, and *Lactobacillus kefirgranum* KCTC 5086, that produced EVs with size distribution ranging from 80 and 400 nm. These EVs presented anti-inflammatory effect both on colon carcinoma Caco-2 cells and on a mouse model of inflammatory bowel disease induced by 2,4,6-trinitrobenzene sulfonic acid. Their anti-inflammatory effect was enhanced when EVs from 3 strains were combined in the treatment (Seo et al., 2018). The strains of human vaginal origin *L. crispatus* BC3, *L. crispatus* BC5, *L. gasseri* BC12, and *L. gasseri* BC13, were reported to produce EVs with antiviral activity (anti-HIV-1) in human T cells *in vitro* and/or human tissues *ex vivo*. EVs showed a differential inhibition of HIV-1 infection, which was attributed to their composition of proteins and metabolites. More active EVs had lactate and high amounts of amino acids, as well as several specific proteins, including enolase, chaperonin and elongation factor Tu (Ñahui Palomino et al., 2019).

4.2.2. *Bifidobacterium*-derived EVs

Bifidobacterium is another important probiotic genus for which EVs occurrence has been reported. Currently, this taxon contains more than 70 species, which have been isolated from different ecological niches, including milk, sewage, human blood, the oral cavity, and the gastrointestinal tract (GIT) of mammals, birds, and insects (Alessandri et al., 2019). Particularly, bifidobacteria are among the dominant bacterial populations and the first colonizers of the human gut, with some species being involved in a vertical route of colonization, from mother to offspring (Milani et al., 2016; Chander Roland et al., 2018; Alessandri et al., 2019).

Regarding EVs described in this genus, there is much less reports than for *Lactobacillus*. For instance, the strain *Bifidobacterium bifidum* LMG13195 produced EVs with immunomodulatory activity *in vitro*. Dendritic cells isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors and exposed to these EVs induced relatively high IL-10 production and Treg cell differentiation (López et al., 2012). Moreover, the EVs derived from strain *B. longum* KACC 91563 induced apoptosis of mast cells and alleviated food allergy symptoms in BALB/c mice with food allergy induced by ovalbumin and alum (Kim et al., 2016a). Finally, another strain, *B. longum* NCC2705, was reported to produce EVs when grown in supernatants of human fecal fermentation broth. These EVs contained double-stranded DNA and mucin-binding proteins, such as GroEL, elongation factor Tu (EF-Tu) and transaldolase (Tal), suggesting that EVs may promote colonization of bifidobacteria (Nishiyama et al., 2020).

4.3. Applications of beneficial bacteria-derived EVs

As detailed, the EVs derived from Gram-negative and Gram-positive beneficial bacteria presented several beneficial properties towards the host, both *in vitro* and *in vivo* (Figure 11), even reproducing properties of the producing bacteria (Al-Nedawi et al., 2015; West et al., 2020). Besides the characterization of these beneficial properties, some studies proposed interesting applications for the EVs derived from these bacteria, including vaccines, drug delivery and biosensing (Gilmore et al., 2021).

EV-based vaccines are increasingly considered due to interesting features, such as the high immunogenicity and self-adjuvant potential, the simple and cost-effective production, and safety as non-replicative nanoparticles (Balhuizen et al., 2021; Gilmore et al., 2021). As an example, the safety and effectiveness of vaccines based on bacteria-derived EVs were attested with human trials in Cuba, Norway and New Zealand to prevent meningococcal serogroup B disease (Nøkleby et al., 2007; Oster et al., 2007; Holst et al., 2013). Regarding non-pathogenic bacteria, bioengineered *E. coli* Nissle 1917 produced EVs expressing exogenous antigens on their surface (referred as pathogen-like particles), capable of inducing self-adjuvanted humoral and T_H1 cellular responses in

BALB/c mice (Rosenthal et al., 2014). Moreover, EVs derived from *B. bifidum* LMG 13195 were suggested to serve as adjuvants for immunotherapy, since they induced T regulatory cells differentiation *in vitro* (López et al., 2012). Likewise, EVs derived from *E. coli* DH5α expressing the outer membrane protein Omp22 of *A. baumannii* protected mice against challenge with a clinical isolate and confirmed EVs feasibility as vaccine platform (Huang et al., 2016b). Recently, the strain *E. coli* BL21(DE3)Δ60 was designed with bioinformatics tools to produce a generalized vaccine platform, with minimal endogenous proteome, increased loading capacity for recombinant proteins and enhanced EVs productivity (Zanella et al., 2021).

Strains of *B. thetaiotaomicron* were also bioengineered to produce EVs that incorporated bacterial, viral and human proteins at their interior or surface, serving as both vaccine and drug delivery platforms (Carvalho et al., 2019a). The antigens Fraction 1 (F1) and LcrV (V antigen) from *Yersinia pestis* were expressed in the membrane or lumen of the EVs and delivered to the gastrointestinal and respiratory tracts of cynomolgus macaques (Carvalho et al., 2019b). The approach resulted in the induction of both humoral and cellular responses, self-adjuvanticity and absence of adverse effects (Carvalho et al., 2019b). In another study, antigens from *S. enterica* ser. Typhimurium and influenza A virus were also expressed, packaged and delivered by EVs, triggering mucosal and systemic immune responses in C57BL/6 mice (Carvalho et al., 2019a). The human protein keratinocyte growth factor-2 was also delivered by this approach, showing a therapeutic effect against DSS-induced colitis in mice (Carvalho et al., 2019a).

EVs also find applications in drug delivery, bioimaging and diagnosis. For example, the *msbB* mutant of *E. coli* W3110 was engineered to produce EVs with an affibody for the human epidermal growth factor receptor 2 (HER2) displayed on their surface and the small interfering RNA (siRNA) targeting kinesin spindle protein in their interior (Gujrati et al., 2014a). These EVs could specifically trigger tumor regression *in vivo*, with reduced side effects (Gujrati et al., 2014a). In another study, the msbB mutant of *E. coli* W3110-K12 expressing a tyrosinase transgene was used to produce EVs encapsulating melanin, which was efficient as a platform for both optoacoustic imaging and photothermal therapy targeting tumor cells in mice, thus comprising a promising theranostics application (Gujrati et al., 2019). Likewise, bacterial EVs were purified from human stool and analyzed for their metagenomic and metabolomic profile, allowing the establishment of associations of bacterial taxa and specific metabolites with colorectal cancer, and the development of a diagnostic model (Kim et al., 2020a).

Although many applications were initially proposed for EVs derived from pathogenic bacteria, the tendency is an increase in the applications for EVs derived from beneficial bacteria, since they display several advantages, such as the absence or

reduced content of toxins, simple purification and possibility of bioengineering for content and yield modulation (Bitto and Kaparakis-Liaskos, 2017; Liu et al., 2018a).

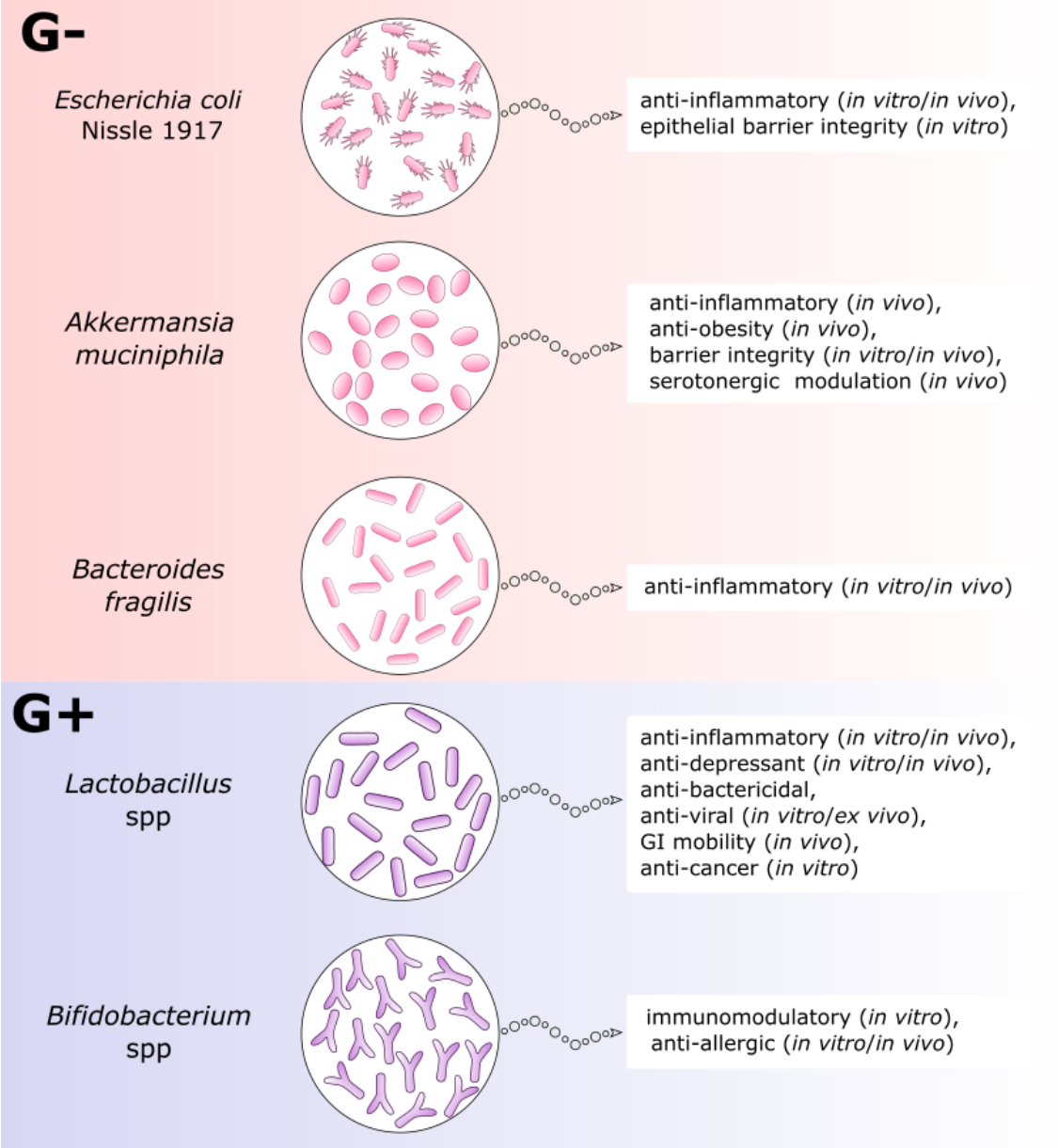

Figure 11. Summary of potential activities reported for the EVs derived from Gram-negative (G-) and Gram-positive (G+) beneficial bacteria. Based on Molina-Tijeras et al. (2019).

5. *Propionibacterium freudenreichii*: general characteristics and probiotic traits

The denomination “probiotics” comprises living organisms, including bacteria and yeasts, with health-promoting properties and suitable for safe consumption, as confirmed by their dietary uses for thousands of years of human history (Ozen and Dinleyici, 2015; Gasbarrini et al., 2016; de Melo Pereira et al., 2018). Lactic acid bacteria and bifidobacteria comprise traditional probiotic bacteria species, widely researched and commercialized (de Melo Pereira et al., 2018; Douillard and de Vos, 2019). However, different species have emerged in probiotics landscape, such as the dairy species *Propionibacterium freudenreichii* (Rabah et al., 2017; Douillard and de Vos, 2019), which are phylogenetically close to bifidobacteria (Figure 12) (Douillard and de Vos, 2019).

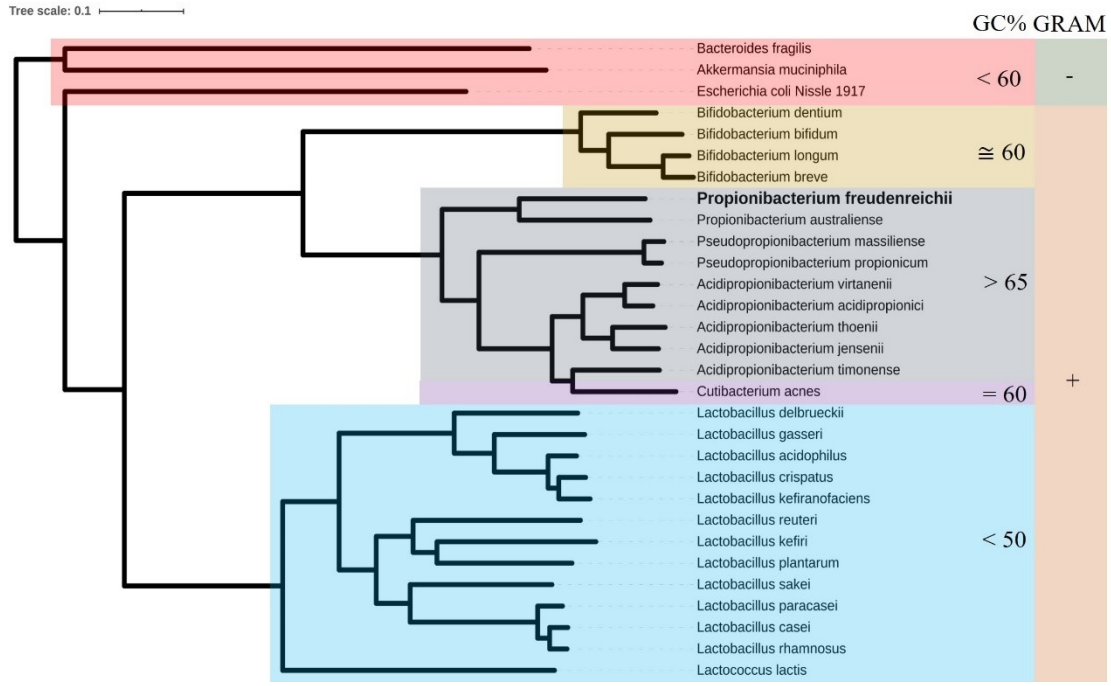

Figure 12. Phylogenetic tree showing evolutionary relatedness between health-promoting *P. freudenreichii* species, other probiotic or closely-related species. Cortesy of Diego L. N. Rodrigues (2021), constructed with Neighbor-Joining method, using reference sequences of common proteins retrieved from the NCBI.

The former *Propionibacterium* genus encompassed a group of microorganisms with importance in industry and health, due to the production of valuable metabolites, food, cosmetic and pharmacological products (Piwowarek et al., 2018b). Previously, this genus included classic dairy propionibacteria species and skin-associated pathogenic propionibacteria (Scholz and Kilian, 2016). However, a genome-based taxonomy reevaluation proposed the reclassification of cutaneous propionibacteria into the *Cutibacterium* genus, together with the inclusion of two other new genera for classic propionibacteria, *Acidipropionibacterium* and *Pseudopropionibacterium* (Scholz and Kilian, 2016). *P. freudenreichii*, which is one of the most notable dairy propionibacteria species, kept its former taxonomic classification (Scholz and Kilian, 2016).

5.1. General characteristics

P. freudenreichii is a Gram-positive, high GC-content, mesophilic, aerotolerant, non-motile, non-spore forming bacterium, that shows low nutritional requirements and survives in several environments (Falentin et al., 2010; Thierry et al., 2011; Rabah et al., 2017). Regarding morphology, it is a pleomorphic rod microorganism, with aggregation tendency, forming clusters that resemble Chinese characters (Figure 13) (Rabah et al., 2017). This bacterium was first described by Orla Jensen and von Freudenreich in 1906, isolated from samples of Emmental cheese (Von Freudenreichii and Orla-Jensen, 1906). Recently, *P. freudenreichii* strains have been identified in fecal samples from a discrete cohort of human preterm breast-fed infants, suggesting that it could be a component of the healthy human gut microbiota (Colliou et al., 2017).

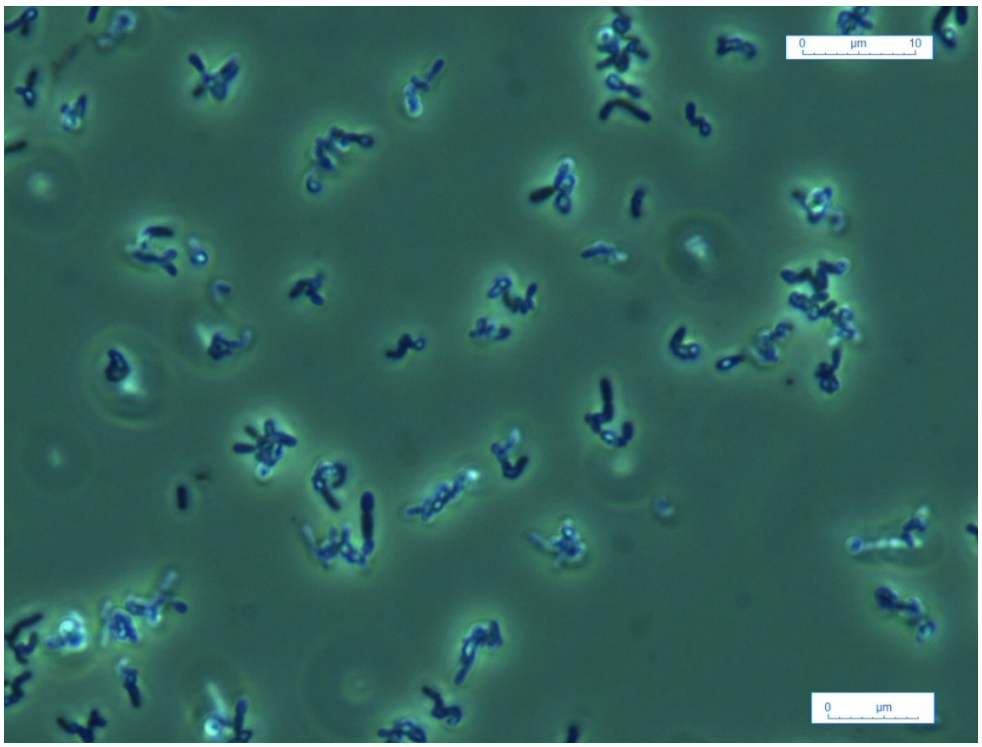

Figure 13. Optical microscopy image showing the morphological aspect of a *P. freudenreichii* CIRM-BIA129 culture, with typical aggregates. Image acquired with an Olympus BX51TF phase contrast microscope by Rodovalho (2018).

P. freudenreichii is able to use several carbon sources (e.g., glycerol, erythriol, L-arabinose, adonitol, galactose, D-glucose, D-fructose, D-manose, inositol, arbutine, esculine, lactose, lactate and gluconate) in the fermentation process to produce propionate, together with acetate, succinate and carbon dioxide (CO_2) (Thierry et al., 2011; Loux et al., 2015; Ojala et al., 2017). Unlike other species, *P. freudenreichii* is able to reduce pyruvate into propionate via the transcarboxylase cycle (also referred to as Wood–Werkman cycle), which is a cyclic process coupled to oxidative phosphorylation,

that allows a higher ATP yield than in other propionate-producing bacteria (Thierry et al., 2011). Pyruvate is a metabolic node molecule, which may be used either for the NADH-generating synthesis of acetate, or for the NADH-consuming synthesis of propionate (Deborde and Boyaval, 2000). In a strain-dependent manner, the bacterium modulates the proportions of pyruvate that are reduced into propionate or oxidized into acetate and CO_2, thus maintaining the redox equilibrium (Thierry et al., 2011).

5.2. Technological importance

The species *P. freudenreichii* is widely used for the production of Swiss-type cheeses, such as Emmental (Figure 14) (Gagnaire et al., 2015; Rabah et al., 2017). In such dairy matrices, the CO_2 gas that is produced during fermentation forms bubbles that diffuse slowly, creating characteristic holes, or “eyes”, in cheese architecture (Thierry et al., 2011; Ojala et al., 2017). Cheese flavor is related to propionate and acetate, as well as the products of amino acids catabolism and fat hydrolysis by propionibacteria (Poonam et al., 2012; Abeijón Mukdsi et al., 2014). Importantly, these dairy products containing *P. freudenreichii* displayed anti-inflammatory properties *in vivo* (Plé et al., 2016; Rabah et al., 2018a, 2020), increasing the recognition of this bacterium and of its products as health-promoting. Therefore, propionibacteria are considered 2-in-1 bacteria, with both fermentative and probiotic properties, which makes them ideal for the development of health-promoting fermented food (Rabah et al., 2017, 2020).

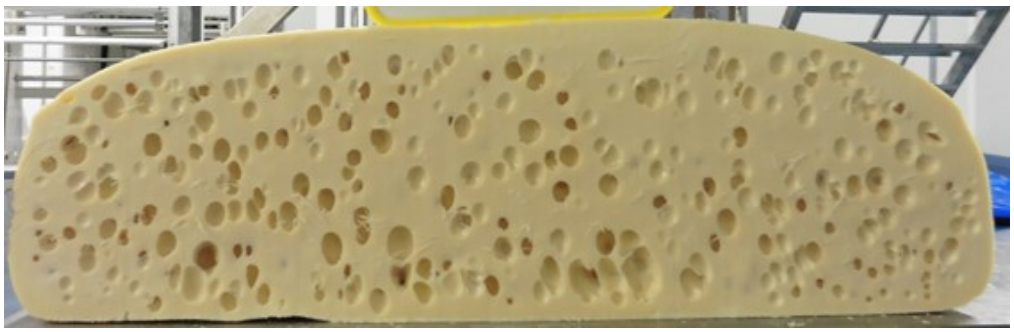

Figure 14. Emmental cheese produced with *P. freudenreichii* CIRM-BIA129. Photography courtesy from the study of Rabah et al. (2018).

This bacterium is also well recognized to encompass a pathway for vitamin B12 (cobalamin) synthesis (Falentin et al., 2010; Thierry et al., 2011). This vitamin is a complex organic molecule and a co-factor of methylmalonyl-CoA mutase, which catalyzes a crucial step in the fermentative route to produce propionate (Takahashi-Iñiguez et al., 2012). Therefore, the growth conditions of *P. freudenreichii* have been optimized for the production of vitamin B12, using substrates such as cereal matrices (Chamlagain et al., 2018; Wang et al., 2020a), waste frying sunflower oil (Hajfarajollah et al., 2015), tofu wastewater (Yu et al., 2015) and soybean agroindustry residue (Assis

et al., 2020). Moreover, *P. freudenreichii* has been genetically engineered to enhance vitamin B12 and propionate production (Wang et al., 2015; Piwowarek et al., 2018b).

The production of vitamin B12, organic acids, trehalose and other metabolites, together with the safe use as cheese ripening starter and probiotic characteristics, make this bacterium attractive for several biotechnological and industrial applications (Rabah et al., 2017; Pillai et al., 2018; Piwowarek et al., 2018b; Gaucher et al., 2019a). A wide range of genetic and environmental optimizations have been conducted to improve these properties (Pillai et al., 2018; Piwowarek et al., 2018b). Moreover, some optimizations of the growth and processing conditions allowed the improvement of resistance towards storage and towards several industrial processes, such as freeze-drying and spray-drying (Gaucher et al., 2019b, 2019c, 2019a, 2020b).

5.3. Strain variability

The interesting properties of this bacterium, such as health-promoting features, and participation in vitamin B12 and cheese production, were showed to be strain-dependent, suggesting the need for analysis that account for that variability (Thierry et al., 2011). As an example, some strains presented differences concerning nitrogen and sugar degradation, which had a genetic origin, probably horizontal transfers, duplications, transpositions and other mutations (Loux et al., 2015). This strain diversity was confirmed at the genomic level by another study and attributed to transposable elements, in such a way that genome plasticity enabled bacterial adaptation to several environments (Deptula et al., 2017).

In view of this strain-related variability, there have been efforts to specify criteria for the selection of probiotic strains. These criteria include tolerance to stresses encountered in the gastrointestinal tract, ability of adhesion to host cells, anti-pathogenic activity, anticancer and immunomodulatory properties, industrial requirements and molecular characterization using omics methodologies (de Melo Pereira et al., 2018). Mounting evidence shows that *P. freudenreichii* fulfills these criteria (Rabah et al., 2017).

5.4. Stress tolerance

Regarding stress tolerance in the gastrointestinal tract (GIT), some *P. freudenrichii* strains presented adaptations, including morphological and proteomic modifications (Jan et al., 2001; Leverrier et al., 2003, 2004). The acid tolerance response in the strain *P. freudenreichii* SI41 was investigated physiologically and morphologically with a kinetic study of stress proteins during acid adaptation. As a result, biotin carboxyl carrier and proteins involved in DNA synthesis and repair were associated to early stress response, whereas chaperonins GroEL and GroES were associated to late stress response (Jan et al., 2001). Analysis with the same strain showed that bile salts (a mixture of cholate and deoxycholate) triggered drastic morphological changes and induced proteins related to

signal sensing and transduction, general stress and an alternative sigma factor (Leverrier et al., 2003). The same strain was used in a follow-up comprehensive study that included heat, acid, and bile salts conditions to study *P. freudenreichii* tolerance. As a result, each form of stress induced specific proteins, but six of them were common to all stresses, including chaperones and proteins involved in energetic metabolism and oxidative stress (Leverrier et al., 2004). An *in vitro* study that involved 13 strains of *P. freudenrichii* showed that most of them had high capacity of tolerance to simulated gastric juices with varying pH and small intestine conditions (Huang and Adams, 2004).

Moreover, this resistance was also evidenced *in vivo*. The mRNA of *P. freudenreichii* methylmalonyl-transcarboxylase was detected in human fecal samples using real time reverse transcriptase polymerase chain reaction (RT-PCR), which indicated that the bacterium survives and remains metabolically active, transcribing genes within the human digestive tract (Hervé et al., 2007). A multi-strain study using human microbiota-associated rats monitored intestinal microbiota composition and short-chain fatty acids production, confirming that *P. freudenreichii* stress tolerance in the GIT is also strain-dependent (Lan et al., 2007a). *P. freudenreichii* CIRM-BIA1 was showed to adapt metabolically and physiologically to the colon environment of pigs, with changes in carbohydrate metabolism, down-regulation of stress genes and up-regulation of cell division genes (Saraoui et al., 2013). Furthermore, the use of food vehicles for *P. freudenreichii* delivery, such as cheese and fermented milk, improved its resistance towards the GIT stressing environment (Cousin et al., 2012b; Gagnaire et al., 2015; Rabah et al., 2018b, 2018a, 2020).

Other aspects of *P. freudenreichii* resistance to stress conditions have also been studied, such as long-term nutritional shortage (Aburjaile et al., 2016b, 2016a). A screening was performed with eight *P. freudenreichii* strains, which were incubated for several days after the beginning of the stationary phase, without further supplementation of nutrients. They displayed high survival rates and no lysis, indicating that these strains adapt to long-term nutritional shortage, using a viable, but nonculturable state (Aburjaile et al., 2016a). The strain *P. freudenreichii* CIRM-BIA138 was further studied in these conditions of incubation, and it was shown that a high population was maintained, even after exhaustion of lactate, the preferred carbon source. RNA-seq analysis showed that several metabolic and information processing pathways were down-regulated (Aburjaile et al., 2016b).

Another important feature of *P. freudenreichii* during stress response is the accumulation of trehalose. A study that investigated this bacterium during adaptation to osmotic, oxidative and acid stress, showed that the trehalose-6-phosphate synthase/phosphatase (OtsA–OtsB) pathway, related to trehalose synthesis, was enhanced in these conditions (Cardoso et al., 2007). Another study focused in stress response in the cold (4 ºC), a condition that mimicked cheese ripening. As a result, seven *P. freudenreichii* strains displayed a slowed-down cell machinery, cold stress response and the accumulation of trehalose and glycogen (Dalmasso et al., 2012). The accumulation of trehalose, together with glycine betaine, was also verified in a technological context, when bacteria's viability was increased during spray drying and storage, through the optimization of the growth medium composition and thermal adaptation (Gaucher et al., 2019b). The ratio of the concentrations of these intracellular

osmoprotectants, trehalose and glycine betaine, was further shown to modulate the stress tolerance during the technological processes of freeze-drying and of spray-drying (Gaucher et al., 2020b).

5.5. Adhesion properties

The ability of adhesion to host cells is another important feature of probiotics, which favors their local beneficial action. In the case of *P. freudenreichii*, the adhesion of some strains to pig ileum cells (IPEC-J2) was between 25 and 35%, and that proportion was higher with the addition of $CaCl_2$ (Campaniello et al., 2015). In the case of human host, several bacterial strains were tested for adhesion to HT-29 colon cells *in vitro*; the most adhesive strain was *P. freudenreichii* CIRM-BIA129 and surface layer protein B (slpB) key role in adhesion was demonstrated by using gene inactivation (do Carmo et al., 2017, 2018). Surface polysaccharides are also components involved in adhesion properties of the species (Guyomarc'h et al., 2020). Another study demonstrated that adhesion to immobilized mucus could be improved by administration of strains combinations, such as *P. freudenreichii* ssp. *shermanii* JS in combination with *Bifidobacterium breve* or *Lactobacillus rhamnosus* strains (Collado et al., 2007).

5.6. Anti-pathogenic activity

There are also several evidences of an anti-pathogenic activity in this species. *P. freudenreichii* JS reduced by 39% the adhesion of *S. aureus* to human intestinal mucus and by 27% its viability, probably due to the production of organic acids (Vesterlund et al., 2006). *P. freudenreichii* PTCC 1674 was reported to secrete a lipopeptide biosurfactant with antimicrobial activity mainly against *Rhodococcus erythropolis*, and anti-adhesive activity mainly against *P. aeruginosa* (Hajfarajollah et al., 2014). Moreover, *P. freudenreichii* DSM 20270 significantly inhibited *E. coli* O157:H7 growth *in vitro* (Campaniello et al., 2015).

P. freudenreichii also showed anti-pathogenic properties in animals. *P. freudenreichii* B-3523 and B-4327 impacted *Salmonella* strains multiplication, motility and adhesion to avian epithelial cells *in vitro* (Nair and Kollanoor-Johny, 2017). The follow up study indicated that the cell-free culture supernatants of the same probiotic strains were bactericidal against multidrug-resistant *Salmonella enterica* serovar Heidelberg. *In vivo* assays further showed that the probiotic strains reduced the pathogen cecal colonization and dissemination to the liver in turkey poults (Nair and Kollanoor Johny, 2018).

Probiotic combinations have also been proposed to improve anti-pathogenic activity, such as a combination of *P. freudenreichii* JS, *L. rhamnosus* GG and LC705, and *B. breve* 99, which promoted the inhibition, displacement and competition with several pathogenic species (Collado et al., 2006). In another study, *P. freudenreichii* JS decreased the adhesion of *H. pylori* to Caco-2 intestinal cells when used individually, but also

inhibited membrane leakage, improved epithelial barrier function and modulated inflammatory cytokines when used in combination with *L. rhamnosus* and *B. breve* strains (Myllyluoma et al., 2008).

5.7. Anticancer properties

Anticancer properties have also been reported in this species. A study showed that *P. freudenreichii* ITGP18 and *P. freudenreichii* SI41 could induce apoptosis on cultured human colorectal carcinoma cell lines *in vitro* and this effect was mediated by short-chain fatty acids (SCFAs), such as propionate and acetate (Jan et al., 2002). Following up, it was further clarified that the effect of SCFAs was modulated by extracellular pH shifts; and in acidic pH, cell death mode changed from apoptosis to necrosis in human colon HT-29 cells (Lan et al., 2007b). These effects were confirmed *in vivo*, with the induction of apoptosis of colon cells in human microbiota-associated rats treated with 1,2-dimethylhydrazine (Lan et al., 2008).

Another strain, *P. freudenreichii* ITG P9, was also employed for the development of a fermented milk with anticancer properties, since it induced apoptosis in cultured HGT-1 human gastric cancer cells *in vitro* (Cousin et al., 2012b). Next, this fermented milk was proposed as an adjuvant in colorectal cancer therapy based on TNF-related apoptosis-inducing ligand (TRAIL), due to possible synergistic effect between the bacterium and TRAIL, which was confirmed with the enhancement of cytotoxic activity in HT-29 cells (Cousin et al., 2016). Another study investigated the crosstalk between bacterium and cancer cells: the later produce lactate as a result of the metabolic shift referred as “aerobic” glycolysis or “Warburg effect”; lactate may then be used by this bacterium as a carbon source, stimulating its production of SCFAs (Casanova et al., 2018).

5.8. Modulation of microbiota composition

Regarding the modulation of microbiota composition, in *P. freudenreichii* 7025 cultures, a bifidogenic growth stimulator was observed in cell-free filtrate and cellular methanol extract (Kaneko et al., 1994). Following analysis with the same strain allowed the purification of a bifidogenic growth stimulator component, the identification of its chemical structure (2-amino-3-carboxy-1,4-naphthoquinone, ACNQ) and the demonstration of its bifidogenic activity in the concentration of 0.1 ng/mL (Mori et al., 1997). Another strain, *P. freudenreichii* ET-3 was reported to produce 1,4-dihydroxy-2-naphthoic acid (DHNA) in concentrations of 10 µg/mL, which also stimulated the growth of bidobacteria (Isawa et al., 2002). The beneficial effect of DHNA was later confirmed *in vivo*, using mice with colitis induced by 2.0% dextran sodium sulphate (DSS). DHNA attenuated inflammation, through the modulation of intestinal bacterial microbiota and suppression of lymphocyte infiltration (Okada et al., 2006).

The bifidogenic growth stimulator derived from *P. freudenreichii* was also orally administrated to human patients in a pilot study, being promising for the treatment of ulcerative colitis (Suzuki et al., 2006). Subsequent studies included optimizations of the production of bifidogenic growth stimulators, including an increased production by switching to aerobic growth conditions (Furuichi et al., 2006) and the use of lactic acid as a carbon source in a bioreactor system with a filtration device (Kouya et al., 2007).

5.9. Immunomodulatory properties

There is mounting evidence both *in vitro* and *in vivo*, that *P. freudenreichii* exerts immunomodulatory effects by several mechanisms, in a strain-dependent manner. For example, a screening for IL-10 induction on human peripheral blood mononuclear cells (PBMCs) was performed in 10 strains of *P. freudenreichii*, resulting in the selection of the 2 most anti-inflammatory strains: *P. freudenreichii* ITG P20 (equivalent to CIRM-BIA129) and SI48 (Foligné et al., 2010). In the same study, the strain *P. freudenreichii* SI48 was further tested *in vivo*, in mice with TNBS-induced acute colitis, lowering significantly inflammatory and histological markers of colitis (Foligné et al., 2010). Another multi-strain study employed an integrative strategy encompassing comparative genomics, surface proteomics, transcriptomics, assays of cytokines induction and genes inactivation, in order to identify relevant proteins and strains specificities in immunomodulation (Deutsch et al., 2017). Remarkably, surface proteins were shown to be crucial in immunomodulation, but the immunomodulatory properties varied among strains due to complex combinations of molecular features (Deutsch et al., 2017). In a different subset of *P. freudenreichii* strains, the secretome was investigated in order to identify proteins that were exported in a strain-specific manner, such as surface proteins, adhesins and moonlighting proteins (Frohnmeyer et al., 2018). Additionally, acute colitis induced by dextran sodium sulfate (DSS) in rats was ameliorated by *P. freudenreichii* KCTC 1063, which stimulated in intestinal cells the expression of MUC2, a main component of mucus (Ma et al., 2020).

The roles of *P. freudenreichii* in the modulation of host immunological response became even more relevant when a human commensal strain was identified. *P. freudenreichii* UF1 was demonstrated to be a component of the gut microbiota of preterm infants that were fed with human breast milk and to mitigate intestinal inflammatory diseases (Colliou et al., 2017). Moreover, this strain modulated the intestinal immunity of mice against pathogen challenge, specifically against systemic *Listeria monocytogenes* infection, by regulating Th17 cells (Colliou et al., 2018). This beneficial effect was confirmed in newborn mice, which were susceptible to intestinal pathogenic infection, but had their defense enhanced by this strain, particularly by the increase in protective Th17 cells and regulatory T cells (Ge et al., 2019).

Regarding the bacterial factors involved in immunomodulation, evidence points out mainly to surface proteins (Table ***1***). The strain *P. freudenreichii* CIRM-BIA129 had its proteome investigated, with the identification of surface-exposed proteins and their role in induction of IL-10 and IL-6 release by peripheral blood mononuclear cells (PBMCs) (Le Maréchal et al., 2015). Among the identified proteins, there were cell wall-

remodeling proteins, transport proteins, moonlighting proteins and other proteins involved in interactions with the host (Le Maréchal et al., 2015). The multi-strain and multi-omics study conducted by Deutsch et al. (2017) clarified that cytoplasmic proteins might also be relevant in immunomodulation, but confirmed the key role of surface-layer proteins B (SlpB) and E (SlpE), particularly in strain *P. freudenreichii* CIRM-BIA 129 (Deutsch et al., 2017). SlpB was then showed to be crucial for bacterial adhesion to epithelial intestinal cells (do Carmo et al., 2017), and a mutation in its gene had pleiotropic effects, suggesting this protein could have a central role in cellular processes (do Carmo et al., 2018). Additionally, *in vivo* assays that were conducted in mice with mucositis induced by 5-Flourouracil (5-FU), showed that SlpB protein is crucial for cytokine modulation triggered by *P. freudenreichii* CIRM-BIA129 (do Carmo et al., 2019). Moreover, the glycosylated large surface layer protein A (LspA) of the commensal strain *P. freudenreichii* UF1 was showed to regulate the interaction with SIGNR1 receptor, which regulates dendritic cells and counteracts pathogenic-driven inflammation, maintaining gut homeostasis (Ge et al., 2020).

In addition to surface proteins, DHNA was also associated to immunomodulation. Beside its bifidogenic properties, DHNA inhibited the production of proinflammatory cytokines in intestinal macrophages of IL-10(-/-) mice treated with piroxicam (Okada et al., 2013). Moreover, DHNA was also described as an activator of aryl hydrocarbon receptor (AhR), which is involved in the detoxification of xenobiotics and inflammation regulation (Fukumoto et al., 2014; Cheng et al., 2017).

Table 1. Proteins from *P. freudenreichii* related to its immunomodulatory properties.

Strain	Protein	Name / Description	Accession	Evidence level	Reference
CIRM-BIA129 (ITG P20)		Ensemble of surface proteins		proteomic, *in vitro*	(Le Maréchal et al., 2015)
	GroL2	60 kDa chaperonin 2	CDP49125	genomic, proteomic	(Deutsch et al., 2017)
	HsdM3	Type I restriction-modification system DNA methylase	CDP48267	genomic, transcriptomic, mutant studies *in vitro*	(Deutsch et al., 2017)
	LacI1	arabinose operon repressor	CDP47860	transcriptomic	(Deutsch et al., 2017)
	MerA	Pyridine nucleotide-disulphide oxidoreductase	CDP48574	genomic, proteomic	(Deutsch et al., 2017)
	Pep	Hypothetical protein	CDP48241	genomic, mutant studies *in vitro*	(Deutsch et al., 2017)
	PFCIRM129_04790	Hypothetical protein	CDP48736	genomic, transcriptomic	(Deutsch et al., 2017)
	PFCIRM129_10785	Hypothetical protein	CDP49252	transcriptomic	(Deutsch et al., 2017)
	PFCIRM129_10930	Hypothetical protein	CDP48242	genomic, transcriptomic	(Deutsch et al., 2017)
	SlpB	Surface layer protein B	CDP48273	genomic, transcriptomic, proteomic, mutant studies *in vitro*	(Deutsch et al., 2017)
				mutant studies *in vitro* and *in vivo*	(do Carmo et al., 2019)
				mutant studies *in vitro*	(do Carmo et al., 2017)
	SlpE	Surface protein with SLH domain	CDP48858	genomic, transcriptomic, proteomic, mutant studies *in vitro*	(Deutsch et al., 2017)
	SlpF	Surface protein with SLH domain	CDP49687	proteomic, mutant studies *in vitro*	(Deutsch et al., 2017)
CIRM-BIA 121	Acn	Aconitase, Aconitate hydratase	CEG89374	transcriptomic	(Deutsch et al., 2017)
	DcuA	C4-dicarboxylate transporter	CEG91776	genomic	(Deutsch et al., 2017)
	Eno1	Enolase 1	CEG91483	proteomic, mutant studies *in vitro*	(Deutsch et al., 2017)
	HtrA4	Serine protease	CEG91080	genomic, transcriptomic, proteomic, mutant studies *in vitro*	(Deutsch et al., 2017)

	PFCIRM121_08235	Hypothetical protein / unknown function	CEG91253	genomic, mutant studies *in vitro*	(Deutsch et al., 2017)
	SlpC1	Surface layer protein C	CEG91216	genomic, transcriptomic	(Deutsch et al., 2017)
UF1	LspA	Large surface layer protein A	n.a.	proteomic, *in vivo*	(Ge et al., 2020)
	DlaT	dihydrolipoamide acetyltransferase	n.a.	proteomic, mutant studies *in vivo*	(Colliou et al., 2017)

Legend. n.a.: not available.

5.10. Functional food

Importantly, the immunomodulatory properties of *P. freudenreichii* were preserved when food matrices were used as delivery vectors, including cheese (Plé et al., 2015; Rabah et al., 2018a, 2018b, 2020) and fermented milk (Cousin et al., 2012a; Foligné et al., 2016; Moslemi et al., 2016), indicating a great potential for developing probiotic-based functional foods with immunomodulatory properties. As an example, a dairy product fermented by strain CIRM-BIA129 reduced the secretion of pro-inflammatory cytokines by colonic mucosa, improved food intake and growth of piglets (Cousin et al., 2012a). *P. freudenreichii* CIRMBIA-129 was also employed in the production of an immunomodulatory cheese, whose consumption by mice ameliorated colitis induced by trinitrobenzenesulphonic acid (TNBS) (Plé et al., 2015). In healthy piglets, the consumption of the same strain associated to a cheese matrix was crucial in the preservation or enhancement of the immunomodulatory properties of the bacterium, including the induction of Th2 and Treg phenotypes (Rabah et al., 2018a). The importance of the cheese matrix was also related to the protection of immunomodulatory protein SlpB against proteolysis in simulated gastrointestinal tract conditions (Rabah et al., 2018b). The strain *P. freudenreichii* CIRM-BIA129 was also used in combination with strains *Lactobacillus delbrueckii* CNRZ327 and *Streptococcus thermophilus* LMD-9 to produce an Emmental cheese that was effective in the improvement of inflammatory markers of DSS-induced colitis in mice (Rabah et al., 2020). These examples unveiled how appropriate food matrices protected or enhanced the beneficial properties of these traditional dairy propionibacteria, while establishing perspectives for the design of novel functional foods (Moslemi et al., 2016).

5.11. Safety assessments

The long term production of fermented food, such as Emmental cheese, and the bacterium status of “generally recognized as safe” (GRAS) and “qualified presumption of safety” (QPS) assure the safety of *P. freudenreichii* consumption (Rabah et al., 2017; Dudek-Wicher et al., 2020). However, additional assessments need to be conducted in different matrices and contexts. Probiotics included in humans trials are most frequently from genus *Lactobacillus* or *Bifidobacterium*; however, propionibacteria have also been tested (Dudek-Wicher et al., 2020). For example, two clinical studies evaluated *P. freudenreichii* ET-3 culture medium safety in human adult subjects, the first one reported no differences in gastrointestinal symptoms between the groups and the other one reported differences in hematological parameters, although within the normal ranges (Uchida et al., 2011).

Moreover, several clinical trials tested multispecies probiotic supplementation containing propionibacteria. A complex formula that included *P. freudenreichii* JS, together with *L. rhamnosus* GG*, L. rhamnosus* Lc705*, B. breve* 99, and galacto-

oligosaccharides prebiotics has been tested in several randomized, double-blind, placebo-controlled setups. The probiotic intervention was conducted in pregnant women and newborn infants, being safe and effective in the prevention of atopic eczema in children (Kukkonen et al., 2007), increased children resistance to respiratory infections (Kukkonen et al., 2008), protected Caesarean-delivered children from IgE-associated allergic disease (Kuitunen et al., 2009), restored microbiota composition in children treated with antibiotics or born by caesarean procedure (Korpela et al., 2018) and protected Caesarean-delivered children from allergic disease in a 13-year follow-up (Kallio et al., 2018). Finally, an integrative study analyzed adverse events associated with this probiotic combination in some of these trials, concluding that there was no association with adverse events in young and elderly subjects (Tapiovaara et al., 2016).

Importantly, probiotics supplementation is not recommended in cases of immunosuppression, such as during anticancer treatment (Dudek-Wicher et al., 2020). Moreover, their beneficial effects and safety are conditioned to a complex interplay between peculiarities of the host and of the probiotic strain or strains, which both encourages further research and suggests caution in some of its applications (Dudek-Wicher et al., 2020).

5.12. Postbiotics and beyond

As previously detailed, *P. freurenreichii* probiotic effect has been associated to several factors, including cytoplasmic and surface-exposed proteins (Le Maréchal et al., 2015; Deutsch et al., 2017; do Carmo et al., 2017, 2019), short chain fatty acids (Jan et al., 2002; Lan et al., 2007b), metabolites (Isawa et al., 2002; Okada et al., 2006; Kouya et al., 2007) and culture supernatants (Uchida et al., 2011; Nair and Kollanoor Johny, 2018) (Figure 15). These probiotic-derived factors, which exert a beneficial effect on the host, have been referred as postbiotics (Żółkiewicz et al., 2020).

Postbiotic is an emerging denomination that encompasses probiotic-derived cell-free metabolic products with health-promoting properties, including proteins, lipids, organic acids, vitamins, supernatants, among others (Tsilingiri and Rescigno, 2013; Nataraj et al., 2020; Żółkiewicz et al., 2020). The advantages of postbiotics over probiotics include purity, easy production and storage, industrial scalability, higher specificity in the mechanism of action and less adverse effects (Nataraj et al., 2020; Żółkiewicz et al., 2020).

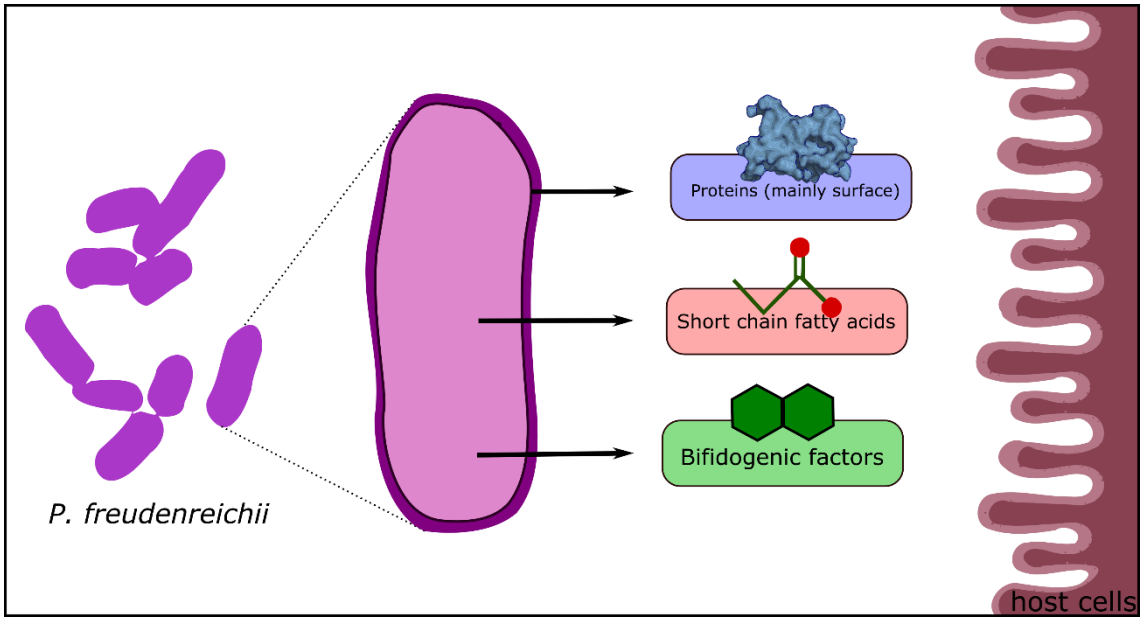

Figure 15. Schematic summary of *P. freudenreichii* probiotic traits at the molecular level. Based on results by Isawa et al. (2002); Jan et al. (2002), do Carmo et al. (2017, 2019), graphically summarized by Rodovalho (2021).

Another example that would fit into postbiotics definition and whose importance is increasing are extracellular vesicles (EVs). In probiotic bacteria, such as several *Lactobacillus* and *Bifidobacteriaum* strains, EVs have been reported as immunomodulatory (Molina-Tijeras et al., 2019). In the case of *P. freudenreichii*, we recently described the production of EVs by the strain CIRM-BIA129 (Rodovalho et al., 2020b), which has been the first report with physicochemical, proteomic and functional characterization of EVs in the species. We identified relevant proteins in their cargo and demonstrated their anti-inflammatory activity via the modulation of NF-κB pathway in cultured human intestinal epithelial cells *in vitro* (Rodovalho et al., 2020b).

Postbiotics, including EVs, hold promising perspectives for developing novel probiotic-derived products with enhanced safety and functionality (Molina-Tijeras et al., 2019). Moreover, yield and cargo loading optimization are promising for modulating bacterial EVs properties, enhancing their beneficial effect and biotechnological applications (Liu et al., 2018b). Finally, clinical trials should be conducted in the near future to assure the suitability of postbiotics, as well as probiotics themselves, in therapy and prophylaxis, since they might exert a great impact in human health (Molina-Tijeras et al., 2019; Dudek-Wicher et al., 2020; Rad et al., 2020).

Chapter 2 – Research question, objectives and strategy

1. Context and research question

EV production is now considered an ubiquitous phenomenon, although its identification in particular contexts was delayed due to technical limitations or misconceptions (Woith et al., 2019). That was the case of Gram-positive bacteria, whose thick peptidoglycan cell wall seemed an unsurmountable barrier (Brown et al., 2015). Similarly, EV research on beneficial bacteria is only in its infancy, if compared to pathogenic bacteria (Bitto and Kaparakis-Liaskos, 2017). Nevertheless, mounting evidence suggested that secreted factors mediated some of the beneficial effects associated to bacteria, sometimes at distant sites from where they are usually found in the human body, such as the brain (Haas-Neill and Forsythe, 2020). EVs could explain these observations, since they are natural vehicles of intercellular exchange, that promote the protection of their internal cargo, being able to cross the mucus layer in the gastrointestinal tract, migrate to other tissues and spread to distant sites (Bitto and Kaparakis-Liaskos, 2017; Molina-Tijeras et al., 2019).

Likewise, EVs are also technologically promising, with a great potential for cost-effective and large-scale production, long-term stability and safety (Bitto and Kaparakis-Liaskos, 2017). EVs were safely used as human-targeted vaccines and they are considered an appropriate alternative for probiotics in the case of immunocompromised individuals (Bitto and Kaparakis-Liaskos, 2017; Molina-Tijeras et al., 2019). Furthermore, the production of EVs derived from beneficial bacteria, as well as their properties, have also been optimized to fulfill specific criteria, such as yield improvement, and content and activity modulation (Nakayama-Imaohji et al., 2016; Pérez-Cruz et al., 2016; Hirayama and Nakao, 2020; Kuhn et al., 2020). All these features make of EVs a very interesting route of content delivery, both in natural and technological contexts.

With the improvement in purification methods and omics characterization, the tendency is a great increase in the reports of widespread EV occurrence and the characterization of their contents, with a special interest in long overlooked, yet very important, beneficial bacteria. One of such beneficial bacteria is *P. freudenreichii*, a Gram-positive dairy probiotic traditionally used in the production of Swiss-type cheese, organic acids and vitamin B12 (Rabah et al., 2017). As reviewed in detail in Chapter 1, *P. freudenreichii* was recognized as an important probiotic species due to several beneficial traits, including the adhesion to human epithelial cells, the anti-pathogenic activity, the production of bifidogenic factors that modulate microbiota composition, the anticancer properties mediated by short-chain fatty acids and immunomodulation. These effects are mediated by surface-exposed or secreted factors (Jan et al., 2002; Okada et al., 2013; do Carmo et al., 2017), and they were sometimes attributed to cell-free culture supernatants (Nair and Kollanoor Johny, 2018; Ma et al., 2020). Furthermore, a

secretome profiling of the species included TEM images of *P. freudenreichii* strain JS22 indicating the presence of membrane-bulging structures that resembled EVs (Frohnmeyer et al., 2018).

As EVs are being increasingly recognized as a general export system, including in Gram-positive and beneficial bacteria (Brown et al., 2015; Molina-Tijeras et al., 2019), we hypothesized that *P. freudenreichii* could produce EVs involved in the mediation of its beneficial roles, particularly the immunomodulation of human intestinal cells (Figure 1). Therefore, we formulated the following research question:

Does the probiotic *P. freudenreichii* produce EVs with immunomodulatory roles in different conditions?

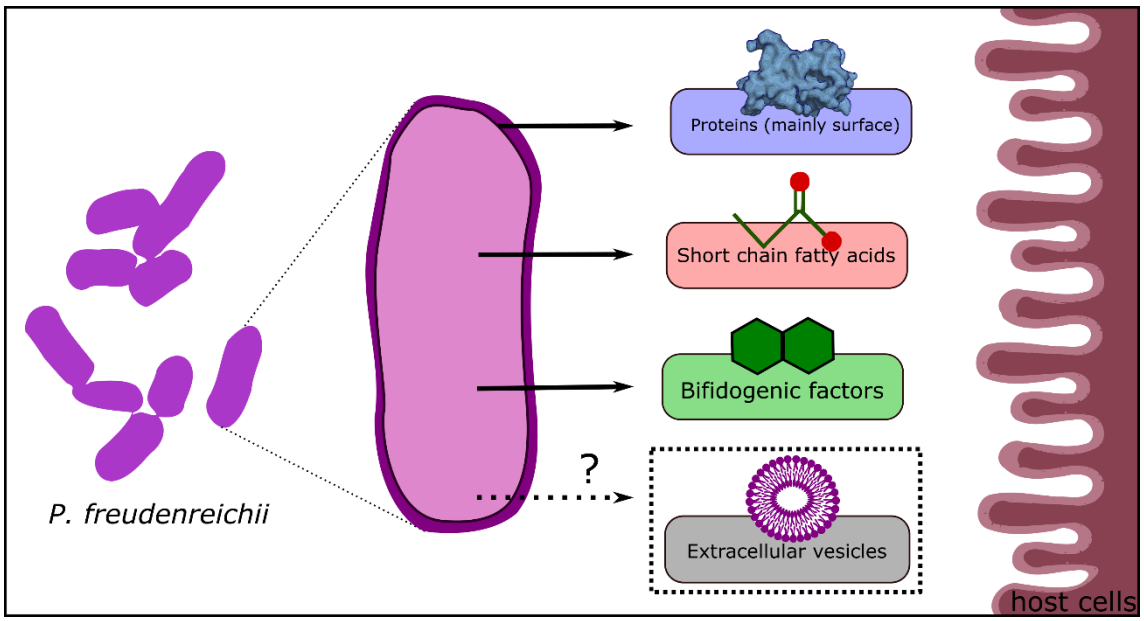

Figure 1. Schematic representation of the research question, concerning the hypothesis that, in addition to secreted short chain fatty acids and bifidogenic factors, as well as surface proteins, EVs could also mediate the probiotic effects of *P. freudenreichii* towards human cells. Based on results by Isawa et al. (2002); Jan et al. (2002), do Carmo et al. (2017, 2019) and the research question of this study, graphically summarized by Rodovalho (2021).

2. Objectives and strategy

The overall objective of this work was to determine whether *P. freudenreichii* produces EVs with immunomodulatory roles towards human cells. Therefore, an interdisciplinary approach was adopted, integrating nanoparticle characterization, proteomics, interactomics and functional assays to disclose the characteristics and roles of *P. freudenreichii*–derived EVs obtained in different conditions. In order to investigate this central question, it has been divided in the following specific objectives:

1. Determine if *P. freudenreichii* produces EVs in a standard growth condition, using size exclusion chromatography as a purification method.
2. Investigate the biophysical characteristics (size, concentration, morphology) of *P. freudenreichii*–derived EVs.
3. Investigate the protein content of *P. freudenreichii*–derived EVs.
4. Apply *in silico* approaches to predict interactions between proteins from *P. freudenreichii*–derived EVs and human proteins.
5. Study the immunomodulatory activity of *P. freudenreichii*–derived EVs towards human intestinal cellular models.
6. Characterize *P. freudenreichii*–derived EVs obtained from cultures in a different growth medium.
7. Compare growth conditions for the modulation of the properties of *P. freudenreichii*–derived EVs, including biophysical parameters, protein content, immunomodulatory activity and interactions patterns.
8. Characterize the properties of *P. freudenreichii*–derived EVs obtained by a different purification method: ultracentrifugation with density gradient.
9. Analyze the conservation of *P. freudenreichii*–derived EVs protein content across different growth conditions and purification methods.

Objectives 1-5 will be addressed in Chapter 3, which presents an original research article reporting the purification of *P. freudenreichii*–derived EVs from cultures in milk ultrafiltrate (UF) medium by size-exclusion chromatography (SEC). This work also presents the biophysical characteristics of these EVs, in addition to the investigation of their protein content, interaction network and immunomodulatory activity.

Objectives 6-7 will be addressed in Chapter 4, which presents an original research article reporting the purification of *P. freudenreichii*–derived EVs by SEC from cultures in either UF or yeast extract-lactate (YEL) medium. This work presents the possibility of modulating EVs properties, including protein content and immunomodulatory activity, through the optimization of culture conditions.

Objectives 8-9 will be addressed in Chapter 5, which introduces the characterization of *P. freudenreichii*–derived EVs obtained by ultracentrifugation (UC) with density gradient, from cultures in either UF or YEL medium. Then, the protein content of EVs derived from the different culture media and purification methods is compared.

A general discussion of the results, the conclusions and perspectives will be presented in Chapter 6.

Chapter 3 – Research article 1

Extracellular Vesicles Produced by the Probiotic *Propionibacterium freudenreichii* CIRM-BIA 129 Mitigate Inflammation by Modulating the NF-κB Pathway

Some aspects of the probiotic effects of *P. freudenreichii* have been attributed to surface-exposed or secreted factors and cell-free supernatants. Although the importance of specific proteins for the immunomodulation exerted by the species was demonstrated, it remained unclear if the functionality of those proteins was mediated exclusively by surface exposure or conventional export systems; or if there were other important export systems, such as EVs. Additionally, structures resembling EVs were previously identified in TEM images of this species. However, those structures were not purified or further characterized. Therefore, we aimed to investigate whether *P. freudenreichii* produced EVs, what were their properties and their functional roles, particularly regarding immunomodulation.

Accordingly, the anti-inflammatory strain *P. freudenreichii* CIRM-BIA129 was applied in this investigation, and hence grown in UF medium until the beginning of the stationary phase. Then, as a protocol for EV purification, the cultures were centrifuged and filtered, and the resulting supernatants were concentrated and applied to a column of SEC. The resulting samples were characterized by TEM and NTA, unveiling nano-sized EVs with typical spherical and cup-shaped morphology. A content investigation by shotgun proteomics showed that EVs contained a diverse set of proteins, including those of cytoplasmic origin, as reported for other Gram-positive bacteria. Some of the identified proteins, such as SlpB, were previously associated to the immunomodulatory roles exerted by *P. freudenreichii*. Furthermore, *in silico* interactions predictions revealed potential interactions of these vesicular proteins with a subunit of human transcription factor NF-κB, which is implicated in inflammatory processes. Therefore, the immunomodulatory activity of these EVs was evaluated in intestinal cellular models, with EV treatment reducing the release of pro-inflammatory cytokine IL-8 and the activation of NF-κB transcription factor, specifically when the cells were induced to an inflammatory state by bacterial LPS. This effect was partly mediated by SlpB protein, as the mutant produced EVs with a less intense anti-inflammatory effect.

Therefore, this chapter shows that not only *P. freudenreichii* CIRM-BIA129 produces EVs, but they have an anti-inflammatory role towards human epithelial cells, which is related to the immunomodulatory proteins carried by these EVs. This is the first comprehensive characterization of the EVs derived from *P. freudenreichii* and resulted in an original research article published in the journal *Frontiers in Microbiology* (DOI: 10.3389/fmicb.2020.01544), which is presented in the next pages.

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ORIGINAL RESEARCH
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Extracellular Vesicles Produced by the Probiotic *Propionibacterium freudenreichii* CIRM-BIA 129 Mitigate Inflammation by Modulating the NF-κB Pathway

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Extracellular vesicles (EVs) are nanometric spherical structures involved in intercellular communication, whose production is considered to be a widespread phenomenon in living organisms. Bacterial EVs are associated with several processes that include survival, competition, pathogenesis, and immunomodulation. Among probiotic Gram-positive bacteria, some *Propionibacterium freudenreichii* strains exhibit anti-inflammatory activity, notably via surface proteins such as the surface-layer protein B (SlpB). We have hypothesized that, in addition to surface exposure and secretion of proteins, *P. freudenreichii* may produce EVs and thus export immunomodulatory proteins to interact with the host. In order to demonstrate their production in this species, EVs were purified from cell-free culture supernatants of the probiotic strain *P. freudenreichii* CIRM-BIA 129, and their physicochemical characterization, using transmission electron microscopy and nanoparticle tracking analysis (NTA), revealed shapes and sizes typical of EVs. Proteomic characterization showed that EVs contain a broad range of proteins, including immunomodulatory proteins such as SlpB. *In silico* protein-protein interaction predictions indicated that EV proteins could interact with host proteins, including the immunomodulatory transcription factor NF-κB. This potential interaction has a functional significance because EVs modulate inflammatory responses, as shown by IL-8 release and NF-κB activity, in HT-29 human intestinal epithelial cells. Indeed, EVs displayed an anti-inflammatory effect by modulating the NF-κB pathway; this was dependent on their concentration and on the proinflammatory inducer (LPS-specific). Moreover, while this anti-inflammatory effect partly depended on SlpB, it was not abolished by EV surface proteolysis, suggesting possible intracellular sites of action for EVs. This is the first report on identification of *P. freudenreichii*-derived

EVs, alongside their physicochemical, biochemical and functional characterization. This study has enhanced our understanding of the mechanisms associated with the probiotic activity of *P. freudenreichii* and identified opportunities to employ bacterial-derived EVs for the development of bioactive products with therapeutic effects.

Keywords: extracellular vesicles, membrane vesicles, probiotic, propionibacteria, immunomodulation, anti-inflammatory, IL-8, NF-κB

INTRODUCTION

Intercellular communication is an essential biological process that involves several soluble biomolecules that may be secreted, surface-exposed or packed inside extracellular vesicles (EVs) (Gho and Lee, 2017; Toyofuku, 2019). EVs are lipid bilayer nanoparticles which range in size from 20 to 300 nm and are released by cells from all living kingdoms (Brown et al., 2015; Kim et al., 2015a; Liu et al., 2018a). They play a pivotal role in cell-to-cell communication through their ability to transport bioactive molecules (proteins, nucleic acids, lipids, metabolites) from donor to recipient cells. Bacterial EVs are implicated in virulence factor delivery, antibiotic resistance, competition, survival, and host cell modulation (Kim et al., 2015b; Toyofuku et al., 2019).

The participation of EVs in the beneficial roles of probiotic bacteria has been increasingly reported (Molina-Tijeras et al., 2019). The release of EVs by *Lactobacillus* species is well documented; *Lactobacillus reuteri* DSM 17938-derived EVs are associated with extracellular DNA-dependent biofilm formation (Grande et al., 2017) and EVs secreted by *Lactobacillus casei* BL23 have also been reported and shown to contain diverse biomolecules which include nucleic acids and proteins previously associated with its probiotic effects, such as p40 and p75 (Rubio et al., 2017). *Lactobacillus rhamnosus* GG-derived EVs have been associated with the apoptosis of hepG2 cancer cells (Behzadi et al., 2017), and *Lactobacillus plantarum* WCFS1-derived EVs modulated the response of human cells to vancomycin-resistant enterococci (Li et al., 2017). Moreover, EVs derived from other probiotic species, such as *Bifidobacterium longum* KACC 91563, impact host cell responses by inducing mast cell apoptosis, which has implications for the treatment of food allergies (Kim et al., 2016). Furthermore, probiotic strains of *Escherichia coli* release outer membrane vesicles (OMVs) that are involved in reinforcement of the gastrointestinal epithelial barrier (Alvarez et al., 2016), the regulation of inflammatory responses and intestinal homeostasis, via the NOD1-signaling pathway (Cañas et al., 2018).

Propionibacterium freudenreichii has also been regarded consistently as a probiotic species, mainly because of its immunomodulatory properties and protective effects against experimentally induced inflammation *in vivo* (Lan et al., 2007a, 2008; Foligne et al., 2010; Cousin et al., 2012; Rabah et al., 2018a; Do Carmo et al., 2019). *P. freudenreichii* is a Gram-positive, pleiomorphic, microaerophilic dairy bacterium that is generally recognized as safe (GRAS) and has a qualified presumption of safety (QPS) status (Loux et al., 2015; Deutsch et al., 2017; Rabah et al., 2017). This species is also known for its involvement in the ripening, texture, and flavor of cheese (Ojala et al., 2017) and in vitamin B12 synthesis (Deptula et al., 2017). A *P. freudenreichii* strain was recently isolated from the gut microbiota of a human breast milk-fed preterm infant, suggesting that this species could also be considered as a commensal inhabitant of the human digestive tract (Colliou et al., 2017).

As for the molecular mechanisms underlying its probiotic effects, some studies have focused on identifying the surface proteins of *P. freudenreichii* and their role in cytokine induction (Le Maréchal et al., 2015; Deutsch et al., 2017; Do Carmo et al., 2019). Notably, cell wall-related proteins, S-layer type proteins, moonlighting proteins and proteins related to interactions with the host have been identified as important actors in immunomodulation of *P. freudenreichii* strain CIRM-BIA 129 (Le Maréchal et al., 2015). Specifically, recent studies reported the role of surface-layer protein B (SlpB) from this strain in bacterial adhesion to intestinal HT-29 cells and immunomodulation (Do Carmo et al., 2017, 2019; Rabah et al., 2018b), as well as that of large surface layer protein A (LspA) from strain P. UF1 in the regulation of colonic dendritic cells during inflammation via SIGNR1 binding (Ge et al., 2020). As well as surface proteins, additional metabolites may contribute to the probiotic effect, such as 1,4-Dihydroxy-2-naphthoic acid (DHNA) from *P. freudenreichii* ET3, which is linked to AhR pathway activation (Fukumoto et al., 2014). DHNA has also been implicated in colitis regression (Okada et al., 2013). Moreover, short-chain fatty acids (SCFAs) from strains TL133 and TL142 have been demonstrated to play a role in inducing apoptosis of tumor cell lines (Lan et al., 2007b, 2008; Cousin et al., 2016).

In view of the fact that EVs are emerging as important carriers of biologically active cargos and that vesiculogenesis is a generally occurring phenomenon, we hypothesized that they might explain some of the probiotic properties of *P. freudenreichii.* For the first time, our findings have shown that this species produces EVs, and we have characterized their physiochemical, biochemical and functional features. We report that *P. freudenreichii* CIRM-BIA 129-derived EVs are implicated in its anti-inflammatory properties, via modulation of the NF-κB pathway, thus building on knowledge regarding this important probiotic bacterium.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Propionibacterium freudenreichii CIRM-BIA 129 (equivalent to the ITG P20 strain) was supplied, stored and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire,

INRAE, Rennes, France). *P. freudenreichii* CIRM-BIA 129 and its isogenic *P. freudenreichii* CIRM-BIA 129 Δ*slpB* mutant strain (Do Carmo et al., 2017) were cultured in cow milk ultrafiltrate (UF) supplemented with 100 mM sodium lactate and 5 g L^{-1} casein hydrolysate at 30°C and without agitation, until stationary phase (72 h of incubation, 2×10^9 CFU mL^{-1}), as reported previously (Cousin et al., 2012).

Purification of EVs

Cells were pelleted by the centrifugation (6000 *g*, 15 min, room temperature) of cultures in UF (500 mL) and the supernatant fraction was filtered using 0.22 μm Nalgene top filters (Thermo Scientific) to remove any remaining bacterial cells. The supernatant was then concentrated 1000 times using Amicon ultrafiltration units with a 100 kDa cut-off point in successive centrifugations at 2500 *g*. The concentrated suspension of EVs was recovered in TBS buffer (Tris-Buffered Saline, 150 mM NaCl; 50 mM Tris–HCl, pH 7.5) and further purified by size exclusion chromatography (qEV original 70 nm; iZON), as recommended by the manufacturer (Böing et al., 2014). Briefly, 0.5 mL of EV samples was applied to the top of the chromatographic column, followed by TBS buffer for elution. Then, fractions of 0.5 mL were recovered in separate tubes. Fractions 1–6 were discarded as void, EVs-containing fractions (fractions 7–9) were pooled together and the remaining fractions were discarded due to protein contamination or low EV content.

Negative Staining for Transmission Electron Microscopy

To characterize the shape of purified EVs, negative staining electron microscopy was conducted as previously described (Tartaglia et al., 2018). Briefly, a drop of EV solution was applied on a glow-discharged formvar-coated copper EM grid and blotted with a filter paper to remove excess solution. A drop of 2% uranyl acetate was applied to the EM grid, blotted again and finally dried before imaging under a Jeol 1400 transmission electron microscope (JEOL Ltd.) operating at 120 Kv.

Nanoparticle Tracking Analysis for EV Size and Concentration Assessment

To measure the size and concentration of EVs, nanoparticle tracking analysis (NTA) was performed at 25.0°C using a NanoSight NS300 instrument (Malvern Panalytical) with a sCMOS camera and a Blue488 laser (Mehdiani et al., 2015). Samples were applied in constant flux with a syringe pump speed of 50. For each measurement, 5×60-s videos were recorded with camera level 15. Other parameters were adjusted accordingly to achieve image optimization.

Proteomic Analysis

Three independent biological replicates of purified EVs from *P. freudenreichii* CIRM-BIA 129 (approximately 1 μg per sample) and the whole cell proteome were separated and visualized using 12% SDS-PAGE (Laemmli, 1970) and silver staining (Switzer et al., 1979). Next, EV proteins were hydrolyzed with trypsin for NanoLC-ESI-MS/MS analysis, as previously described (Gagnaire et al., 2015; Huang et al., 2016). Briefly, gel pieces were washed with acetonitrile and ammonium bicarbonate solution and dried under a vacuum. Next, in-gel trypsin digestion was performed overnight at 37°C and stopped with trifluoroacetic acid (Sigma-Aldrich). After digestion, the peptides were identified from the MS/MS spectra using X!TandemPipeline software (Langella et al., 2017) and searches were performed against the genome sequence of *P. freudenreichii* CIRM-BIA 129. The database search parameters were specified as follows: trypsin cleavage was used and the peptide mass tolerance was set at 10 ppm for MS and 0.05 Da for MS/MS. Methionine oxidation was selected as a variable modification. For each peptide identified, a maximum *e*-value of 0.05 was considered to be a prerequisite for validation. A minimum of two peptides per protein was imposed, resulting in a false discovery rate (FDR) of 0.15% for protein identification.

Proteomic data were further analyzed and visualized using Python libraries Pandas, NumPy, Matplotlib, and Seaborn. Functional annotations and Clusters of Orthologous Groups (COGs) were obtained using the eggNOG-mapper v2 web tool (Huerta-Cepas et al., 2017, 2019), while proteins and gene data were retrieved from NCBI and Uniprot (Bateman, 2019). Subcellular location prediction was performed with CELLO2GO (Yu et al., 2014) and the prediction of lipoproteins was conducted using PRED_LIPO (Bagos et al., 2008).

Prediction of Protein-Protein Interactions

In order to screen for potential biological functions of EVs, the prediction of interactions between EV proteins and human proteins was carried out. The reference human proteome was retrieved from Uniprot (UP000005640) and contained 74,788 protein sequences. For the first method of prediction, EVs and human proteins were submitted to the InterSPPI web server (Lian et al., 2019), a machine-learning-based predictor. For the second method of prediction, a interolog-based approach was used (Folador et al., 2014), establishing homology relationships with the interactions described in the String and Intact databases (Kerrien et al., 2012; Szklarczyk et al., 2017). The resulting interactions were filtered according to the prediction scores (intersppi: minimum score of 0.9765, for a specificity 0.99; interolog: minimum score of 500 out of 1000). Next, the dataset was reduced to a canonical representation, only retaining the human protein isoform appearing in highest-scoring interactions and removing non-reviewed human proteins. For the predicted interactions, the human counterpart was programmatically mapped to KEGG pathways in order to identify the associated functional modules. Data analysis and graphic representations were obtained using Python libraries Pandas, Seaborn, Matplotlib, Matplotlib_venn, and Cytoscape software (Shannon, 2003).

Culture of Eukaryotic Cells

HT-29 human epithelial cells were used for immunomodulation assays; either the parental lineage (HT-29, colon adenocarcinoma; ATCC HTB-38) or a lineage transfected with the secreted alkaline phosphatase (SEAP) reporter

gene for NF-kB activation monitoring (HT-29/kb-seap-25) (Lakhdari et al., 2010). The reporter HT-29/kb-seap-25 cells were cultured in RPMI-Glutamine medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Corning), 1% non-essential amino acids, 1% sodium pyruvate, 1% HEPES buffer (Thermofisher Scientific), and 1% penicillin-streptomycin (Lonza) according to Lakhdari et al. (2010). The parental HT-29 cells were cultured in high-glucose DMEM medium (Dominique Dutscher) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Do Carmo et al., 2017). For subcultures, cells were rinsed with DPBS (Thermofisher Scientific) and detached with a trypsin (0.05%) – EDTA (0.02%) solution (Sigma). Periodically, 100 μg/mL Zeocin (Invivogen) was applied to the HT-29/kb-seap-25 cell culture in order to maintain selective pressure on the cells containing the transfected plasmid.

NF-κB Modulation Assays

HT-29/kb-seap-25 cells were seeded on 96-well plates at 3×10^4 cells/well and incubated for 24 h at 37°C under 5% CO_2 prior to stimulation (Lakhdari et al., 2010). Monolayer confluence was checked under the microscope before and after every stimulation. TNFα (1 ng mL^{-1}; PeproTech), IL-1β (1 ng mL^{-1}; Invivogen), and LPS from *E. coli* O111:B4 (1 ng mL^{-1}; L3024-5MG, Sigma-Aldrich) were used to induce inflammation. The cells were stimulated with the samples (controls and EV preparations) and inflammation inducers for 24 h. The supernatants from all the wells were then revealed with Quanti-Blue™ reagent (Invivogen) to assess SEAP activity. Cell proliferation was evaluated under all conditions using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega), according to the manufacturer's instructions. Absorbance was read at 655 nm for the SEAP activity assay and at 490 nm for the MTS assay using a Xenius (SAFAS Monaco) microplate reader. For surface protein assays, 10^9 EV ml^{-1} were applied directly or after incubation at 37°C for 1 h in the presence or absence of proteinase K (20 μg mL^{-1}; Qiagen), in order to evaluate a possible role for EV surface proteins in immunomodulation.

ELISA Cytokine Assay

Enzyme-linked immunosorbent assay (ELISA) tests tests were performed under the same conditions as the NF-κB modulation assays using HT-29 parental cells. The human IL-8/CXCL8 DuoSet (R&D Systems) kit was used to evaluate cell culture supernatants after stimulation, according to the manufacturer's instructions. Absorbance was read at 450 nm using a Xenius (SAFAS Monaco) microplate reader.

Statistical Analysis

All experiments were conducted independently and in triplicate at least, and the results are expressed as means ± standard deviations of biological replicates. For absorbance measurements, the values were normalized by the control condition. The differences between groups were verified using one-way ANOVA followed by Tukey's multiple comparisons test with GraphPad Prism (GraphPad Software, San Diego, CA, United States).

RESULTS

Propionibacterium freudenreichii Produces Extracellular Vesicles

In order to determine whether *P. freudenreichii* produced EVs, strain CIRM-BIA 129 was cultured in cow milk UF medium and EVs were purified from the cell-free supernatants of stationary phase cultures. As a control, we checked that EVs were absent from the UF medium before being used for bacterial culture. Visualization by electron microscopy revealed that *P. freudenreichii* strain CIRM-BIA 129 produced EVs of a typical shape, i.e., spherical cup-shaped structures (**Figure 1A**). Size characterization by NTA showed that the EVs presented a monodisperse profile with modal diameter of 84.80 ± 2.34 nm (**Figure 1B**).

P. freudenreichii-Secreted EVs Contain a Functionally Diverse Set of Proteins, Including Immunomodulatory Proteins

Cargo proteins associated with *P. freudenreichii*-secreted EVs were determined by Nano LC-ESI-MS/MS analysis from three biological replicates of EVs. A total of 319 proteins was identified consistently in EVs derived from UF medium cultures (**Supplementary Table S1**), which corresponds to 11% of the whole theoretical proteome of *P. freudenreichii* CIRM-BIA 129. **Figure 2A** confirmed a much more complex proteome in the whole cell *P. freudenreichii* extract than in the EV extract.

The proteins associated with these EVs were distributed between most of the COG categories (**Figure 2B**). The majority of proteins could be assigned to COGs related to the general category of "metabolism," e.g., energy production and conversion (C, 14.8%), amino acid transport and metabolism (E, 10.4%), and carbohydrate transport and metabolism (G, 8%). The most common COGs in the general category of "information, storage and processing" were translation, ribosomal structure and biogenesis (J, 7.1%) and transcription (K, 4.2%). Finally, cell wall/membrane/envelope biogenesis (M, 5.3%) and post-translational modifications, protein turnover and chaperones (O, 5%), were the most frequently counted COGs in the general category of "cellular processes and signaling." Interestingly, several proteins previously identified as important actors in immunomodulation were packed within EVs: enolase (Eno1, PFCIRM129_06070), aconitase (Acn, PFCIRM129_04640), glutamine synthetase (GlnA1, PFCIRM129_11730), glucose-6-phosphate isomerase (Gpi, PFCIRM129_10645), triosephosphate isomerase (Tpi1, PFCIRM129_11290), the surface-layer proteins SlpB (PFCIRM129_00700) and SlpE (PFCIRM129_05460), the BopA solute binding protein (PFCIRM129_08120), internaline A (InlA, PFCIRM129_12235), the hypothetical protein PFCIRM129_10785 and the GroL2 chaperonin (PFCIRM129_10100) (Le Maréchal et al., 2015; Deutsch et al., 2017; Do Carmo et al., 2017).

Regarding predictions of the subcellular localization of the proteins, they were mainly predicted to be cytoplasmic (n = 239), but some membrane (n = 51), and extracellular (n = 29) proteins were also identified (**Figure 2C**). Lipoprotein

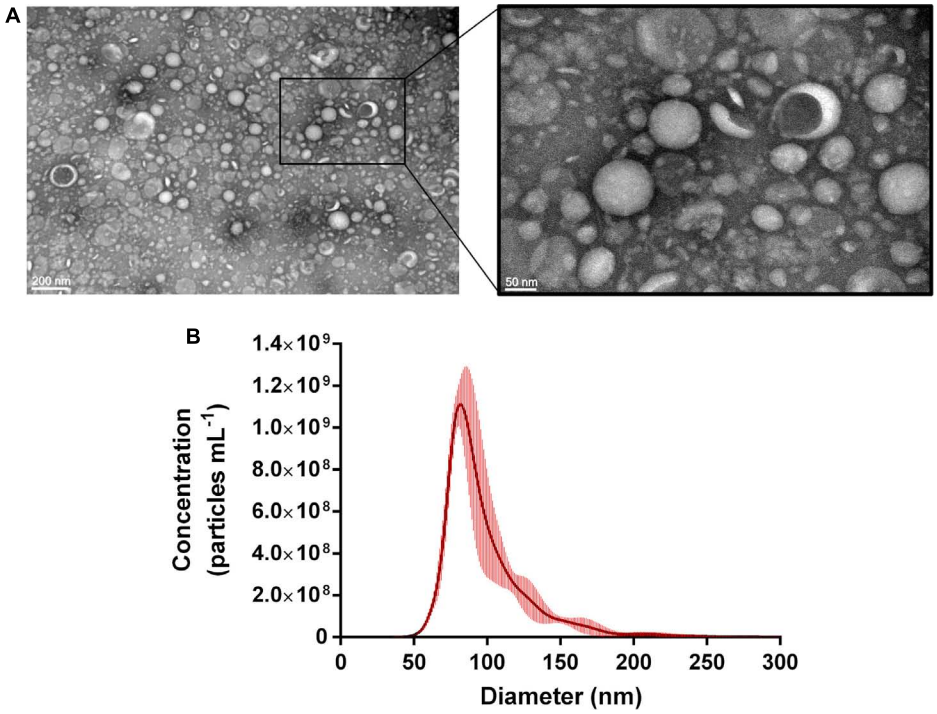

FIGURE 1 | *P. freudenreichii* CIRM-BIA 129 secretes extracellular vesicles. **(A)** Transmission electron microscopy image after the negative staining of *P. freudenreichii*-secreted EVs purified from UF culture supernatants. **(B)** Size distribution (diameter) of purified EVs as measured by nanoparticle tracking analysis (NTA). Concentration data are expressed as mean ± standard deviation from three independent biological replicates.

signal peptides were also predicted in a small fraction of the proteins (n = 22). Other proteins were predicted to contain a secretory signal peptide I (n = 22) and transmembrane motifs (n = 34) (**Figure 2D**). Regarding the protein abundance index (Ishihama et al., 2005), it displayed a non-normal distribution with a tail of highly expressed proteins, including immunomodulatory SlpB, 60 kDa chaperonin 2 (GroL2) and enolase 1 (Eno1), as well as cold shock-like protein CspA, iron/manganese superoxide dismutase (SodA), cysteine synthase 2 (Cys2), alkyl hydroperoxide reductase subunit C (AhpC), and malate dehydrogenase (Mdh) (**Figure 2E**).

The Proteins From *P. freudenreichii*-Secreted EVs Potentially Interact With Human Immunomodulatory Proteins

The proteins found in *P. freudenreichii*-derived EVs were tested against the human proteome *in silico* in order to predict their interactions. Machine learning-based (intersppi) and homology-based (interolog) methods were employed for this task. There was a considerable difference regarding the number of predicted interactions which depended on the method used. Intersppi predicted 117,513 interactions, while the interolog method predicted 2,890 interactions; there were 143 common interactions between the two methods (**Figure 3A** and **Supplementary Tables S2–S4**). Regarding interacting bacterial proteins, 115 proteins appeared exclusively in intersppi interactions, 51 proteins appeared exclusively in interolog interactions and 90 proteins appeared in interactions predicted by both methods (**Figure 3B** and **Supplementary Table S5**). As for interacting human proteins, the majority was predicted by the intersppi method only (6,883 proteins), whereas 747 proteins appeared exclusively in interolog interactions and 611 proteins appeared in interactions shared by both methods of prediction (**Figure 3C** and **Supplementary Table S6**).

The predicted interactions mapped to diverse KEGG terms, including metabolism, signal transduction, infectious diseases and the immune system (**Supplementary Figure S1**). Interestingly, the nuclear factor NF-κB p105 subunit (NFKB1, P19838) was the most frequent interacting human protein considering common and intersppi-exclusive interactions. The subnetwork of interactions mapping to the KEGG NF-κB signaling pathway (**Figure 3D**) included both interactions with adapter molecules (e.g., TICAM1, TICAM2, TRAF6) and Toll-like receptors (e.g., TLR1, TLR4, TLR5, TL6), as well as NFKB1 itself (**Supplementary Figures S2, S3**). These results therefore indicated some interesting potential roles for EV proteins in immunomodulation that need to be verified experimentally.

P. freudenreichii-Secreted EVs Modulate the NF-κB Pathway in a Dose and Inducer-Dependent Manner

In view of the finding that *in silico* predictions showed interactions between EV proteins and human

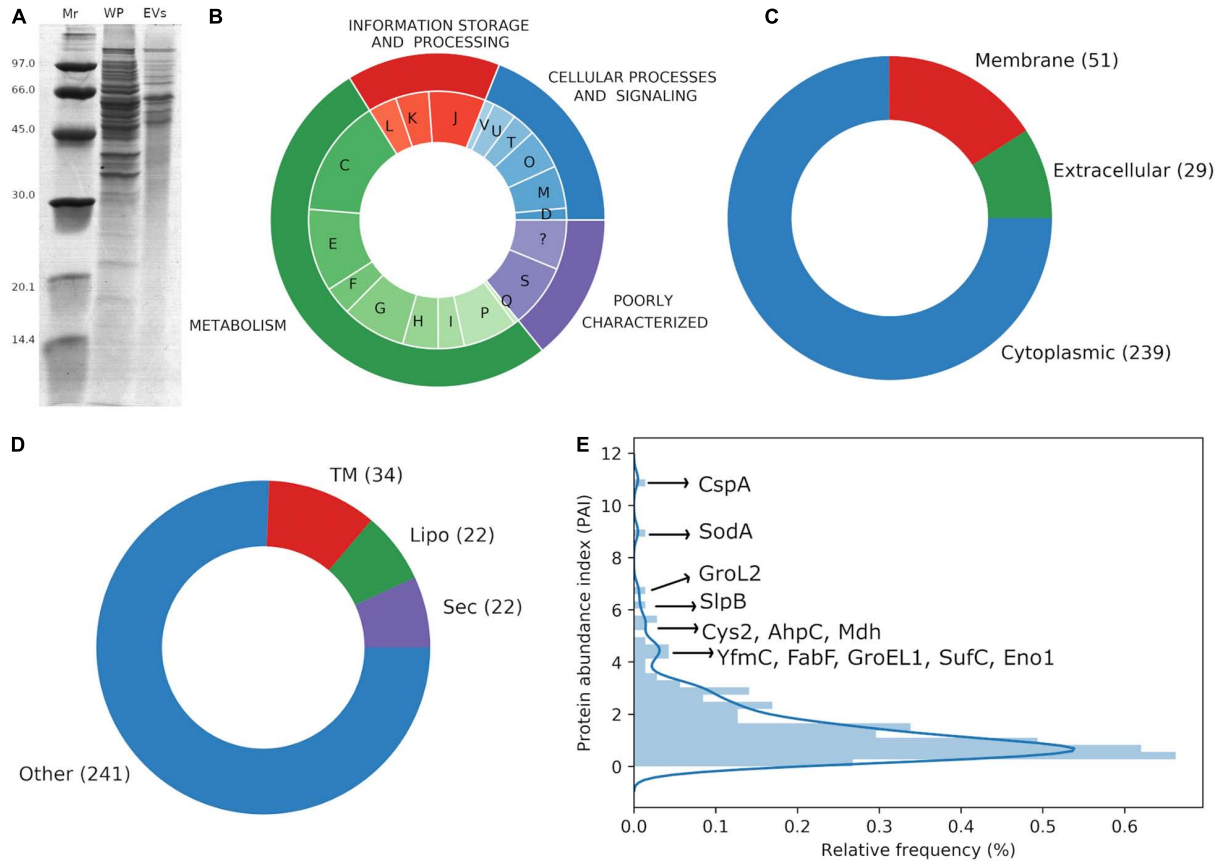

FIGURE 2 | *P. freudenreichii*-secreted EVs contain proteins involved in interactions with the host. **(A)** SDS-PAGE showing the protein profile of the whole proteome (WP) and vesicular proteome (EVs). Molecular weight standards are indicated on the left (Mr, kDa). **(B)** Functional annotation of EV proteins by the prediction of COG categories: D – Cell cycle control, cell division, chromosome partitioning, M – Cell wall/membrane/envelope biogenesis, O – Post-translational modification, protein turnover, and chaperones, T – Signal transduction mechanisms, U – Intracellular trafficking, secretion, and vesicular transport, V – Defense mechanisms, J – Translation, ribosomal structure and biogenesis, K – Transcription, L – Replication, recombination and repair, C – Energy production and conversion, E – Amino acid transport and metabolism, F – Nucleotide transport and metabolism, G – Carbohydrate transport and metabolism, H – Coenzyme transport and metabolism, I – Lipid transport and metabolism, P – Inorganic ion transport and metabolism, Q – Secondary metabolites biosynthesis, transport, and catabolism, S – Function unknown, ? – Not predicted. **(C)** Subcellular localization of EV proteins as predicted by Cello2GO. **(D)** Prediction of lipoproteins by PRED-LIPO: Sec: secretion signal peptide, Lipo: lipoprotein signal peptide, TM: transmembrane, Other: no signals found. **(E)** Protein abundance index (PAI) frequency distribution for EV proteins, highlighting the most frequently expressed proteins.

immunomodulatory proteins (and particularly NFKB1), together with previous evidence of immunomodulatory roles for *P. freudenreichii*, we investigated this potential *in vitro*. A cellular reporter system regarding modulation of the regulatory activity of the NF-κB transcription factor (HT-29/kb-seap-25) was therefore employed (Lakhdari et al., 2010). Cells were kept in contact with proinflammatory inducers (LPS, TNF-α or IL-1β) and EV preparations, in order to test the ability of EVs to attenuate the induced inflammatory response.

Among cells that were not treated with proinflammatory inducers, their exposure to EVs did not affect the activity of the reporter, keeping a basal level of NF-κB activity (**Figure 4A**). When HT-29/kb-seap-25 cells were treated with LPS in the absence of EVs, NF-κB activation increased, thus showing LPS-dependent induction of the NF-κB pathway. With the addition of EVs at increasing concentrations, a dose-dependent reduction of NF-κB activation was observed. With the highest EV concentration tested (1.0×10^9 EVs ml^{-1}), NF-κB activation was comparable to that of untreated control cells. Furthermore, when the EV concentration was kept at a constant level, NF-κB modulation was also dependent on the pathway inducer. In the presence of EVs, there was a significant reduction in NF-κB activation in LPS-treated cells, but not in cells treated with other inducers (TNF-α and IL-1β) (**Figure 4B**).

P. freudenreichii-Secreted EVs Also Modulate IL-8 Release in a Dose and Inducer-Dependent Manner

To further investigate the anti-inflammatory role suggested by the reduction in NF-κB activity, release of the proinflammatory chemokine IL-8 by HT-29 cells was determined in the presence of various EV concentrations and proinflammatory inducers.

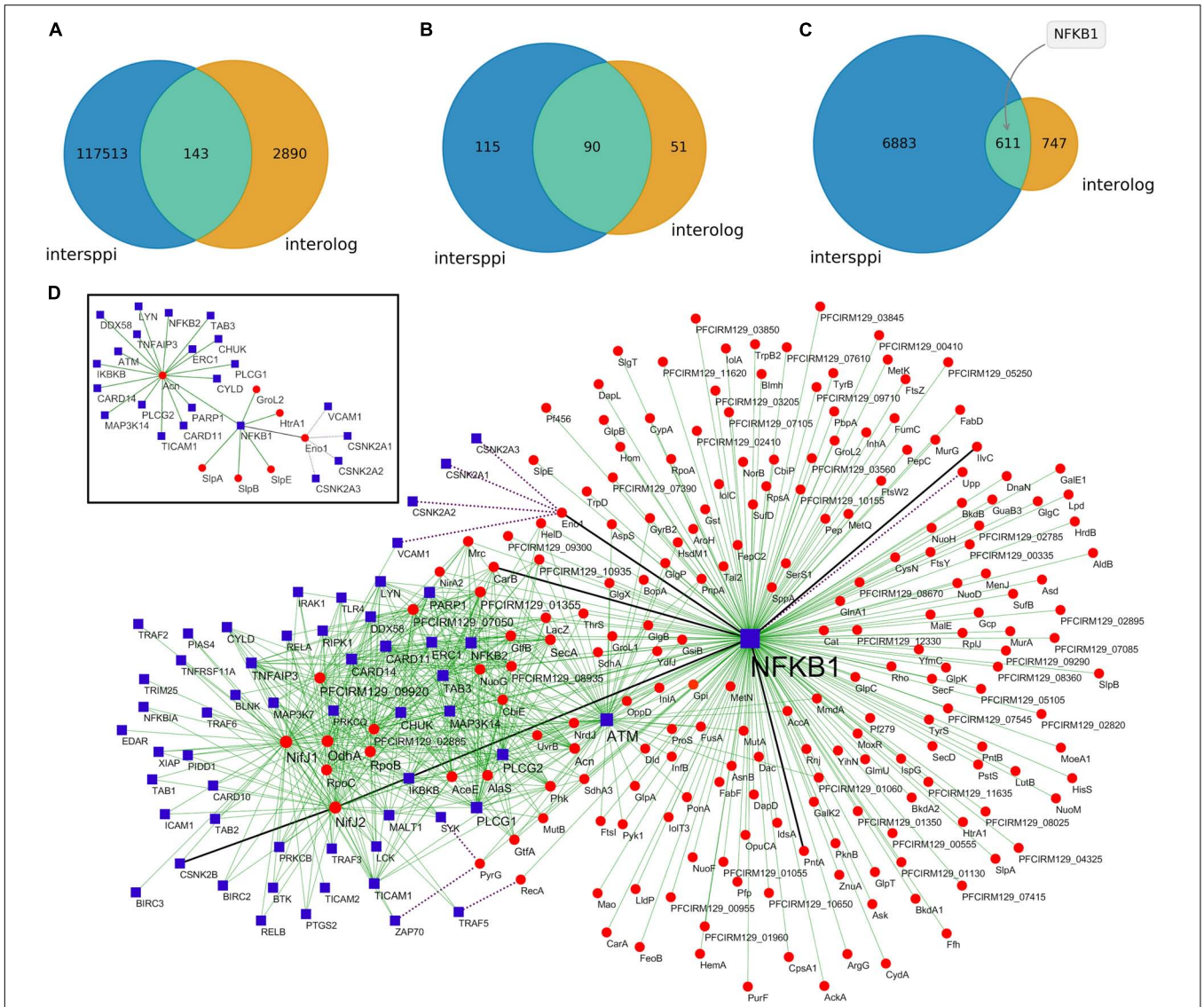

FIGURE 3 | Predictions of protein-protein interactions suggest a modulation of the NF-κB signaling pathway by *P. freudenreichii*-secreted EVs. **(A)** Number of interactions predicted by each methodology. **(B)** Number of bacterial proteins predicted to interact as a function of the prediction method. **(C)** Number of human proteins predicted to interact as a function of the prediction method, highlighting the top-interacting NFKB1 protein. **(D)** Subnetwork of predicted interactions mapping to the KEGG NF-κB pathway. Blue square nodes represent human proteins, red round nodes represent bacterial proteins, full thin green lines represent intersppi-predicted interactions, dotted purple lines represent interolog-predicted interactions and full thick black lines represent interactions shared by both methods. Inset: selected interactions involving bacterial proteins previously associated with immunomodulatory roles.

In the absence of proinflammatory inducers, EVs had no effect on IL-8 release from HT-29 cells (**Figure 5A**). In the TBS buffer control group, LPS-treated HT-29 cells displayed an increase in IL-8 release when compared to untreated cells, reflecting the LPS-induced proinflammatory effect. In the presence of EVs, a significant and dose-dependent decrease of IL-8 release by LPS-stimulated cells was observed. At the highest concentration (1.0×10^9 EVs ml^{-1}), IL-8 release was reduced down to a level comparable to that seen in untreated control cells.

Moreover, as in the case of NF-κB activity, the EV-mediated reduction in IL-8 release was specific to treatment with the LPS proinflammatory inducer (**Figure 5B**). When cells were treated with TNF-α or IL-1β, no effect of EVs could be detected on IL-8 release by HT-29 cells. Taken together, these results showed that *P. freudenreichii*-derived EVs were endowed with anti-inflammatory properties that depended on the concentration and inflammatory stimulus.

P. freudenreichii-Secreted EVs Are Not Cytotoxic Against Intestinal Epithelial Cells

In order to ensure that reductions in NF-κB activation and IL-8 release were associated with regulatory

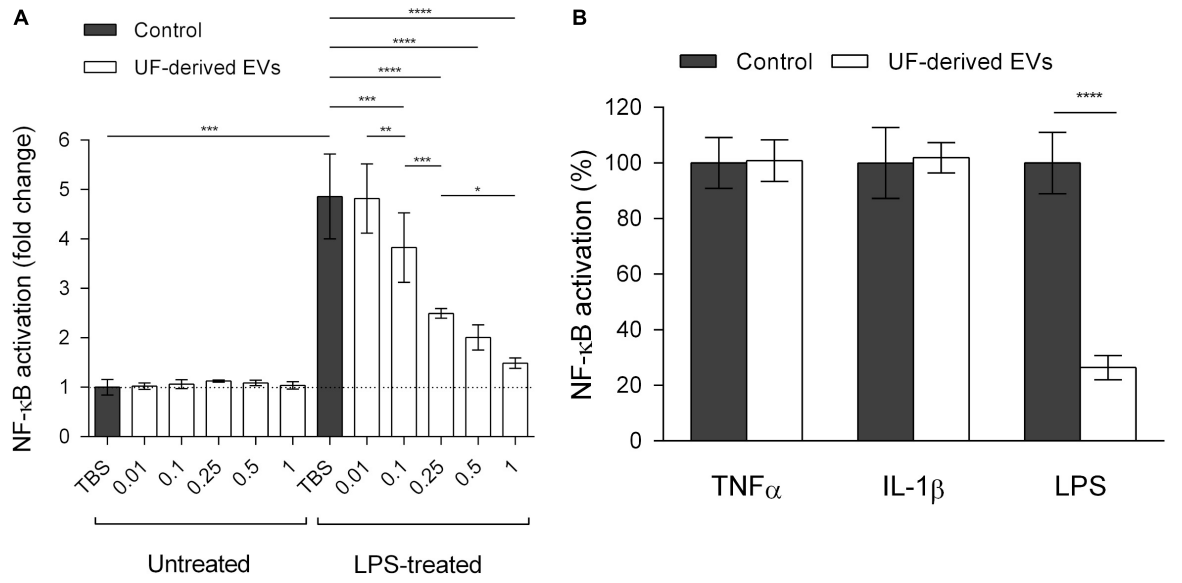

FIGURE 4 | *P. freudenreichii*-secreted EVs specifically mitigate LPS-induced NF-κB activation in intestinal epithelial cells. **(A)** Measure of regulatory activity of the NF-κB transcription factor in HT-29 intestinal epithelial cells untreated or treated with LPS (1 ng mL^{-1}) in the presence of TBS buffer control or different concentrations of EVs purified from the supernatants of *P. freudenreichii* CIRM-BIA 129 culture in UF medium. EV concentrations: 0.01 = 1.0 × 10^7 EVs ml^{-1}, 0.1 = 1.0 × 10^8 EVs ml^{-1}, 0.25 = 2.5 × 10^8 EVs ml^{-1}, 0.5 = 5.0 × 10^8 EVs ml^{-1}, 1 = 1.0 × 10^9 EVs ml^{-1}. **(B)** Percentage NF-κB activation in HT-29 intestinal epithelial cells after stimulation by LPS (1 ng mL^{-1}), TNF-α (1 ng mL^{-1}) or IL-1β (1 ng mL^{-1}) inducers, in the presence or absence of UF-derived EVs (1.0 × 10^9 EVs ml^{-1}). The values are normalized by the control conditions (stimulation by the inducer in the absence of EVs). ANOVA with the Tukey's multiple comparison test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

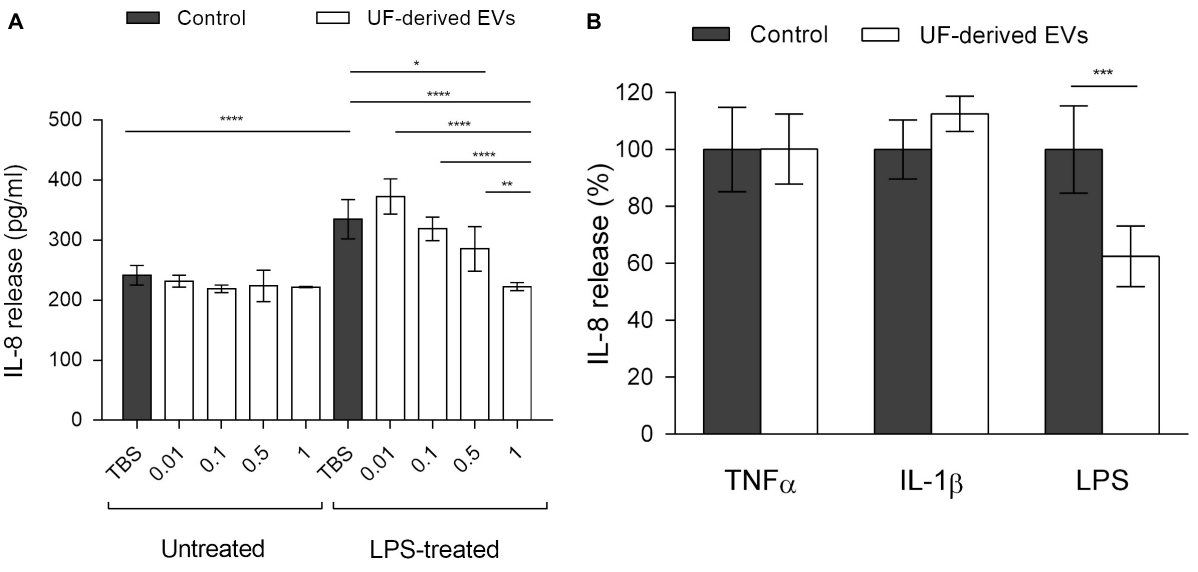

FIGURE 5 | *P. freudenreichii*-secreted EVs mitigate the release of IL-8 induced by LPS. **(A)** Concentration of IL-8 (pg ml^{-1}) released by HT-29 intestinal epithelial cells untreated or treated with LPS (1 ng mL^{-1}) in the presence of different concentrations of EVs purified from the supernatants of *P. freudenreichii* CIRM-BIA 129 culture in UF medium. EV concentrations: 0.01 = 1.0 × 10^7 EVs ml^{-1}, 0.1 = 1.0 × 10^8 EVs ml^{-1}, 0.5 = 5.0 × 10^8 EVs ml^{-1}, 1 = 1.0 × 10^9 EVs ml^{-1}. **(B)** Percentage of IL-8 released by HT-29 intestinal epithelial cells after stimulation by LPS (1 ng mL^{-1}), TNF-α (1 ng mL^{-1}) or IL-1β (1 ng mL^{-1}) inducers, in the presence or absence of UF-derived EVs (1.0 × 10^9 EVs ml^{-1}). The values are normalized by the control conditions (stimulation by inducer in the absence of EVs). One-way ANOVA followed by Tukey's (left panel) or Dunnett's (right panel) multiple comparisons test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

activity and not simply with cell death, an MTS cell proliferation assay was also performed (**Figure 6**). This assay showed that there was no significant difference in cell viability between the control and test groups in terms of both parental HT-29 and HT-29/kb-seap-25 reporter cells.

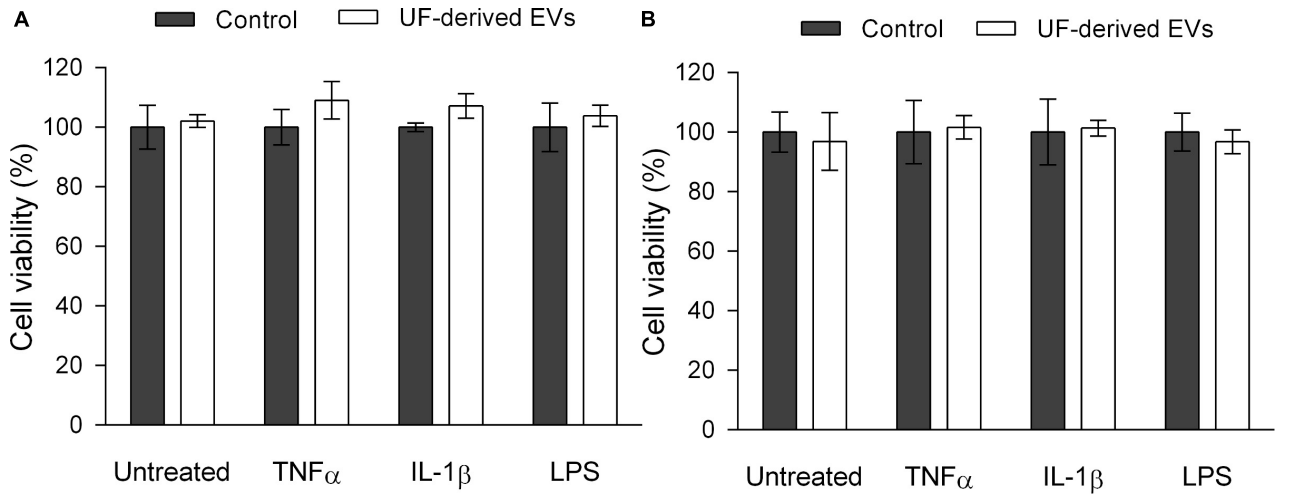

FIGURE 6 | *P. freudenreichii*-secreted EVs are not cytotoxic against intestinal epithelial cells. Viability percentage of HT-29 intestinal epithelial cells **(A)** and HT-29/kb-seap-25 cellular reporter cell line **(B)** before or after stimulation by LPS (1 ng mL^{-1}), TNF-α (1 ng mL^{-1}) or IL-1β (1 ng mL^{-1}) inducers, in the presence or absence of UF-derived EVs (1.0×10^9 EVs ml^{-1}). The values are normalized by the control conditions (control buffer or stimulation by an inducer in the absence of EVs). There are no statistically significant differences between group means as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

Influence of Surface Proteins on NF-κB Modulation

Further tests were performed to determine whether the modulation of NF-κB by *P. freudenreichii* EVs was influenced by a surface-layer protein (SlpB), recognized as being immunomodulatory in the studied strain (Deutsch et al., 2017; Do Carmo et al., 2017; **Figure 7**). EVs derived from an isogenic mutant *P. freudenreichii* CIRM-BIA 129 Δ*slpB* and proven not to produce this specific protein, displayed a partial reduction of NF-κB activation, when compared to wild type-derived EVs. This suggested that SlpB plays a fundamental role in EV modulation of the NF-κB pathway, but it is likely that other important effectors also need to be considered.

Furthermore, in order to determine whether NF-κB pathway modulation by EVs is dependent on proteins that might be exposed on the EV surface, they were treated with proteinase K prior to the cellular assays (**Figure 7**). The incubation of EVs at 37°C for 1 h, in the presence or absence of proteinase K, did not result in significant differences in NF-κB activation, when compared to the wild-type untreated control. This suggested that these immunomodulatory effectors may be inside EVs and not at their surface.

DISCUSSION

Although research on bacterial EVs focused initially on Gram-negative and pathogenic bacteria, a major increase in the number of studies involving Gram-positive and probiotic bacteria has been seen during the past decade (Liu et al., 2018b; Molina-Tijeras et al., 2019). That is in line with the growing recognition of the widespread occurrence and diverse functions of EVs (Brown et al., 2015; Liu et al., 2018a,b). In the case of *P. freudenreichii*, a Gram-positive dairy probiotic bacterium, a recent study indicated the presence of an extracellular structure resembling a potential EV, which bulged out from the membrane of *P. freudenreichii* strain JS22 (Frohnmeyer et al., 2018), suggesting that this species might produce EVs. Nevertheless, ours is the first complete report to have identified the occurrence of EVs in *P. freudenreichii* strain CIRM-BIA 129, and included their physicochemical, biochemical and functional characterization. The EVs thus identified displayed the basic features of extracellular prokaryotic membrane vesicles, i.e., a nanometric size range, a cup-shaped morphology and a spherical structure (Raposo and Stoorvogel, 2013; Liu et al., 2018b; Tartaglia et al., 2018).

CIRM-BIA 129 EVs carry a diverse set of proteins that represent a broad range of biological functions. More than half of these proteins are related to metabolism, so that, initially, they may not appear to exert specific functions outside bacterial cells. However, the transport of metabolism-related proteins by EVs may represent a mechanism of functional exchange and complementation in the context of bacterial communities. For example, members of the *Bacteroides* genus share enzymes with the microbiome through OMVs, so as to contribute to the degradation of complex polysaccharides (Elhenawy et al., 2014; Rakoff-Nahoum et al., 2014; Lynch and Alegado, 2017). On the other hand, some of these metabolism-related proteins, from either *P. freudenreichii* CIRM-BIA 129, or from other strains in the species, have been implicated in interactions with the host. This is the case of enolase (Eno1, PFCIRM129_06070) and aconitase (Acn, PFCIRM129_04640) (Deutsch et al., 2017). Others, such as glutamine synthetase (GlnA1, PFCIRM129_11730), glucose-6-phosphate isomerase (Gpi, PFCIRM129_10645) and triosephosphate isomerase (Tpi1, PFCIRM129_11290), have also been described as moonlighting proteins, with adhesin functions, in other species (Kainulainen et al., 2012;

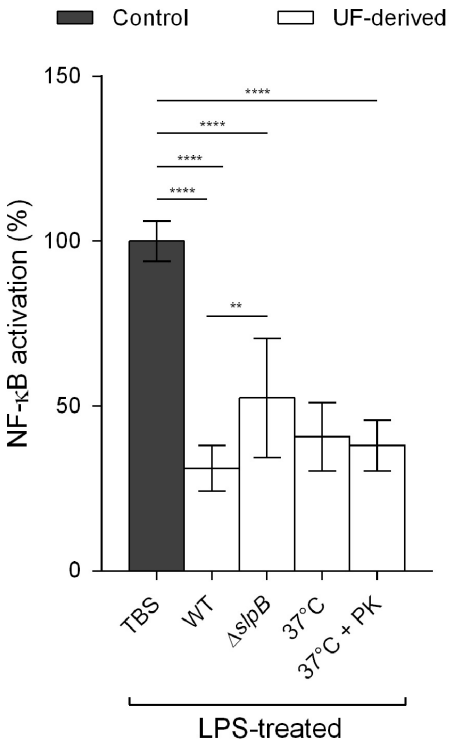

FIGURE 7 | SlpB is required to achieve the effective mitigation of NF-κB activation in human intestinal epithelial cells. Percentage of NF-κB activation in HT-29 intestinal epithelial cells after stimulation by LPS (1 ng μL^{-1}) in the presence or absence of UF-derived EVs (1.0×10^9 EVs ml^{-1}). WT: wild type *P. freudenreichii* CIRM-BIA 129-derived EVs, Δ*SlpB*: isogenic mutant *P. freudenreichii* CIRM-BIA 129 Δ*SlpB*-derived EVs, 37°C: wild type *P. freudenreichii* CIRM-BIA 129-derived EVs incubated at 37°C for 1 h, 37°C + PK: wild type *P. freudenreichii* CIRM-BIA 129-derived EVs incubated at 37°C for 1 h in the presence of proteinase K. The values are normalized by the control conditions (stimulation by an inducer in the absence of EVs). One-way ANOVA followed by Tukey's multiple comparisons test: $^{**}P < 0.01$, $^{****}P < 0.0001$.

Rodríguez-Bolaños and Perez-Montfort, 2019). As well as metabolism-related proteins, other proteins packed into EVs are also related to interactions between *P. freudenreichii* and the host: SlpB (PFCIRM129_00700) and SlpE (PFCIRM129_05460) surface-layer proteins, the BopA solute binding protein (PFCIRM129_08120), internaline A (InlA, PFCIRM129_12235), the hypothetical protein PFCIRM129_10785 and the GroL2 chaperonin (PFCIRM129_10100) (Le Maréchal et al., 2015; Deutsch et al., 2017; Do Carmo et al., 2017). It has been shown that SlpB mediates the adhesion of *P. freudenreichii* CIRM-BIA 129 to intestinal epithelial HT29 cells (Do Carmo et al., 2017), reduces LPS-induced IL-8 expression in HT-29 cells (Do Carmo et al., 2019) and participates in the induction of anti-inflammatory cytokines such as IL-10 in human peripheral blood mononuclear cells, mesenteric lymph nodes cells and epithelial HT29 cells (Foligne et al., 2010; Le Maréchal et al., 2015; Deutsch et al., 2017; Rabah et al., 2018a). Also, inactivation of the gene encoding SlpE suppresses IL-10 induction by *P. freudenreichii* CIRM-BIA 129 (Deutsch et al., 2017). It is interesting to note that SlpB, Eno1 and GroL2 were found to be some of the most abundant EV proteins, which may be a further indication of the potentially beneficial roles exerted by CIRM-BIA 129 EVs on host cells. This is in accordance with the potential immunomodulatory role suggested for these proteins in a multi-strain and multi-omics study (Deutsch et al., 2017).

In order to investigate potential roles for EVs in the context of host-microorganism interactions, we conducted *in silico* predictions of interactions between the bacterial proteins identified in EVs and human proteins. We employed a machine learning-based method (intersppi), which relies on protein sequences and network properties, to identify patterns of classification into groups of interacting or non-interacting protein pairs (Lian et al., 2019). We also used a homology-based prediction of interacting pairs (interolog) that relies on the mapping of similarities to interaction databases and interaction transfers among homologs (Folador et al., 2014). Our results revealed a considerable difference between the methods in the number and nature of the interactions predicted. This difference was mainly due to the fact that the interolog methodology depends on data availability in interaction databases, such as STRING and INTACT, from which homology mapping is performed (Wang et al., 2012). The data may therefore have been biased by experimental work focused on specific aspects and organisms, as well as the conservation of proteins, which may affect homology identification. For example, metabolism-related proteins tend to be more conserved, so they are therefore overrepresented in interactions predicted using the interolog methodology. On the other hand, intersppi aims to capture more general patterns of protein binding, enabling *ab initio* predictions that are not reliant on the availability of prior data. However, it is still susceptible to training data bias and a certain probability of false positives (Keskin et al., 2016).

The predicted interactions mapped to several immunology-related KEGG terms, such as signal transduction, infectious diseases and the immune system, thus shedding light on a possible immunomodulatory role for *P. freudenreichii* EVs. These predictions corroborate some previous findings which associated the *P. freudenreichii* bacterium with immunomodulatory roles (Foligne et al., 2010; Deutsch et al., 2017; Do Carmo et al., 2017; Frohnmeyer et al., 2018; Rabah et al., 2018a). Interestingly, the predicted data also suggested that this immunomodulation could involve the NF-κB pathway, since the nuclear factor NF-κB p105 subunit (NFKB1, P19838) was the most frequent interacting human protein according to the intersppi predictions and also the interactions shared between the two methods. Regarding bacterial proteins previously reported as being immunomodulatory, Acn, Eno1, GroL2, HtrA1, SlpA, SlpB, and SlpE were predicted to interact directly with NFKB1 using the intersppi method. In addition, the interaction between Eno1 and NFKB1 was also predicted by interolog. Other proteins in the NF-κB pathway were also predicted to interact with Acn: the inhibitor of nuclear factor kappa-B kinase subunits alpha (IKKA) and beta (IKKB), as well as TIR domain-containing adapter molecule 1 (TICAM1). Moreover, interactions with toll-like receptors (e.g., TLR1, TLR4, TLR5, TL6) suggested that the immunomodulatory role of EVs might also occur at the receptor

level. Briefly, PPI data (i.e., highly interacting NFKB1 protein, interactions involving bacterial proteins previously demonstrated to be immunomodulatory and interactions mapping to KEGG terms related to the immune response) suggested an ability of EVs produced by *P. freudenreichii* CIRM-BIA 129 to exert an immunomodulatory effect via the NF-κB pathway, which then needed to be confirmed *in vitro*.

When tested on HT-29 intestinal epithelial cells, we found that EVs exert an inhibitory effect on LPS-induced IL-8 secretion, as was previously observed with intact *P. freudenreichii* CIRM-BIA 129 cells (Do Carmo et al., 2019). Their effect was dose-dependent and unrelated to side effects of EVs on cell viability. This immunomodulatory response was mediated through modulation of the regulatory activity of the NF-κB transcription factor. The NF-κB pathway leads to the upregulation of proinflammatory genes, being targeted by diverse pathogens and probiotic bacteria (Hayden and Ghosh, 2012; Mitchell et al., 2016). Therefore, some probiotics can downregulate the production of these proinflammatory cytokines, acting at different steps along the NF-κB pathway: *L. rhamnosus* GG and *Lactobacillus delbrueckii* subsp. *bulgaricus* downregulated p38 and IkB expression, respectively (Giahi et al., 2012), *L. plantarum* LM1004 and *Lactobacillus casei* DN-114 001 modulated the nuclear translocation of NF-κB (Tien et al., 2006; Lee et al., 2019), *Bacteroides thetaiotaomicron* promoted the nuclear export of RelA (Kelly et al., 2004), VSL#3 inhibited proteasome degrading activity (Petrof et al., 2004), and *Bifidobacterium breve* C50 decreased the phosphorylation of p38-MAPK and IkappaB-alpha (Heuvelin et al., 2009). Probiotic strains were also shown to exhibit immunoregulatory effects via the modulation of TLR negative regulators of the NF-κB pathway (Lakhdari et al., 2011; Kanmani and Kim, 2019). Specifically, *Lactobacillus helveticus* SBT2171 was reported to inhibit NF-κB activation by inducing A20 expression via TLR2 signal in LPS-stimulated peritoneal macrophages (Kawano et al., 2019). *Lactobacillus acidophilus* was shown to regulate the inflammatory response induced by enterotoxigenic *E. coli* K88 in piglets, notably through the increased expression of Tollip, IRAK-M, A20, and Bcl-3 (Li et al., 2016). *Lactobacillus paracasei* was associated to the inhibition of pro-inflammatory cytokines production by monocyte-macrophages, via the induction of A20, SOCS1, SOCS3, and IRAK3 (Sun et al., 2017).

The precise mechanism by which EVs produced by *P. freudenreichii* CIRM-BIA 129 modulate NF-κB activity still needs to be elucidated, but our work has provided some clues. EVs do not exert an immunomodulatory effect when the NF-κB pathway is stimulated by other inducers (such as TNF-α and IL-1β), indicating that EVs mitigate the activation of NF-κB via the LPS signaling pathway. These different ligands bind to specific receptors at the cell surface in order to activate the NF-κB pathway. Once bound, they use the same signal transduction mechanisms to activate the pathway. Therefore, EVs probably act at a level of the NF-κB pathway that is not common to the three inducers: TLR4, CD14, LBP, MD-2 and the TICAM1 and TICAM2 TIR domain-containing adaptor proteins. It is interesting to note that *in silico* prediction of protein-protein interactions predicted interactions with TLR4, the cell-surface receptor for LPS, but not with the TNF-α and IL-1β receptors. Furthermore, proteinase-treated EVs conserved their immunomodulatory properties. Therefore, the EV-triggered inhibition of NF-κB does not signal through binding between EV surface exposed proteins and the LPS receptor (i.e., LBP, TLR-4, CD14, MD-2). One can suppose that non-proteinaceous inhibitors may also be involved. However, to date, the immunomodulatory properties of *P. freudenreichii* have been associated with proteins. Moreover, EVs produced by a Δ*slpB P. freudenreichii* mutant partly lost their immunomodulation effects, indicating that several proteins, including SlpB, play a direct or indirect role in the anti-inflammatory response mediated by EVs. These results also suggest that these immunomodulatory proteins are packed into EVs and are not surface exposed. It cannot be excluded that immunomodulatory proteins interact directly with LPS receptors after their release from the lysis of EVs in the vicinity of cells, but it does seem more likely that they target specific intercellular components of the LPS-induced NF-κB pathway after EV uptake by membrane fusion or endocytosis (Mulcahy et al., 2014; Jefferies and Khalid, 2020).

It is now well recognized that EVs act as proxies of their parental cells. Accordingly, *P. freudenreichii* and its EVs share common features, notably their immunomodulatory effects mediated by SlpB. Whether *P. freudenreichii* also signals through the same pathway as its EVs constitutes a basis for further studies. Likewise, whether *P. freudenreichii* EVs exert immunomodulatory effects *in vivo,* as has been shown for the bacterium (Foligne et al., 2010; Rabah et al., 2018a; Do Carmo et al., 2019) is a challenging question that should be addressed in future research and with respect to potential EV-based probiotic applications. In sum, this study reflects efforts to demonstrate the widespread occurrence and functional diversity of EVs, particularly in a group of emerging EVs research, such as Gram-positive probiotic bacteria. It also contributes to a clearer understanding of the mechanisms associated with the probiotic traits of *P. freudenreichii* while opening up possibilities of employing bacterial-derived EVs for functional cargo delivery and for the development of novel probiotic products.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found here: https://data.inrae.fr/dataset.xhtml?persistentId=doi:10.15454/Q6PPXY.

AUTHOR CONTRIBUTIONS

VR, GJ, YL, VC, and EG conceived and designed the experiments. VR, VB-B, JJ, BL, and HR performed the experiments. VR, EF, AN, JJ, and EG analyzed the data. FC, AN, JJ, VB-B, HB, NL, GJ, and EG gave practical suggestions to perform experiments. VC, YL, and EG contributed to funding acquisition. VR and EG wrote the original draft. All authors contributed to data interpretation, drafting the manuscript, critically revising the manuscript, and approving its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01544/full#supplementary-material

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Chapter 4 – Research article 2

Environmental conditions modulate the protein content and immunomodulatory activity of extracellular vesicles produced by the probiotic *Propionibacterium freudenreichii*

As demonstrated in Chapter 3, *P. freudenreichii* CIRM-BIA129 cultured in UF medium produced EVs with anti-inflammatory activity towards human epithelial cells. The biophysical, biochemical and functional properties of those UF-derived EVs were comprehensively characterized. However, mounting evidence suggests that several factors, including growth media, growth phase, salts, pH and exposure to antibiotics, can modulate the properties of bacteria-derived EVs. Therefore, we aimed next to investigate the influence of growth media in the modulation of *P. freudenreichii*-derived EVs properties.

Accordingly, *P. freudenreichii* CIRM-BIA129 was grown in either UF or YEL medium until the beginning of stationary phase. Then, the same protocol for EV isolation was applied. That consisted in culture centrifugation and filtration to recover cell-free supernatants, concentration and application in a SEC column. The resulting samples were characterized by NTA measurements, immunomodulatory activity assays, qualitative and quantitative proteomics. These analyses revealed differences between UF and YEL-derived EVs in abundance, diameter, anti-inflammatory activity, protein identities and abundances. Moreover, it pinpoints how the different abundances of EV-associated proteins could affect the pattern of interactions between bacterial and human proteins, thus affecting immunomodulatory activities.

Therefore, this comparative study allowed a better comprehension of how the optimization of bacterial growth conditions could be applied to modulate EVs properties, even in modulating their content and enhancing their anti-inflammatory properties. This chapter introduces the second original research article derived from this thesis, published in the journal Applied and Environmental Microbiology (DOI: 10.1128/AEM.02263-20), which is presented in the next pages.

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Environmental Conditions Modulate the Protein Content and Immunomodulatory Activity of Extracellular Vesicles Produced by the Probiotic *Propionibacterium freudenreichii*

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ABSTRACT *Propionibacterium freudenreichii* is a probiotic Gram-positive bacterium with promising immunomodulatory properties. It modulates regulatory cytokines and mitigates the inflammatory response *in vitro* and *in vivo*. These properties were initially attributed to specific bacterial surface proteins. Recently, we showed that extracellular vesicles (EVs) produced by *P. freudenreichii* CIRM-BIA129 mimic the immunomodulatory features of parent cells *in vitro* (i.e., modulating NF-κB transcription factor activity and interleukin-8 release), which underlies the role of EVs as mediators of the probiotic effects of the bacterium. The modulation of EV properties, and particularly of those with potential therapeutic applications, such as the EVs produced by the probiotic *P. freudenreichii*, is one of the challenges in the field to achieve efficient yields with the desired optimal functionality. Here, we evaluated whether the culture medium in which the bacteria are grown could be used as a lever to modulate the protein content and, hence, the properties of *P. freudenreichii* CIRM-BIA129 EVs. The physical, biochemical, and functional properties of EVs produced from cells cultivated on laboratory yeast extract lactate (YEL) medium and cow milk ultrafiltrate (UF) medium were compared. UF-derived EVs were more abundant and smaller in diameter, and they displayed more intense anti-inflammatory activity than YEL-derived EVs. Furthermore, the growth media modulated EV content in terms of both the identities and abundances of their protein cargos, suggesting different patterns of interaction with the host. Proteins involved in amino acid metabolism and central carbon metabolism were modulated, as were the key surface proteins mediating host-propionibacterium interactions.

IMPORTANCE Extracellular vesicles (EVs) are cellular membrane-derived nanosized particles that are produced by most cells in all three kingdoms of life. They play a pivotal role in cell-cell communication through their ability to transport bioactive molecules from donor to recipient cells. Bacterial EVs are important factors in host-microbe interactions. Recently, we have shown that EVs produced by the probiotic *P. freudenreichii* exhibited immunomodulatory properties. We evaluate here the impact of environmental conditions, notably culture media, on *P. freudenreichii* EV production and function. We show that EVs display considerable differences in protein cargo and immunomodulation depending on the culture medium used. This work offers new perspectives for the development of probiotic EV-based molecular delivery systems and reinforces the optimization of growth conditions as a tool to modulate the potential therapeutic applications of EVs.

KEYWORDS EV, NF-κB, anti-inflammatory, comparative proteomics, growth conditions, immunomodulation, membrane vesicle, protein-protein interactions

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Probiotic organisms are increasingly being used in medical and technological contexts because of their health benefits (1, 2). Among these organisms, dairy bacteria such as *Propionibacterium freudenreichii* are of particular value because of their long-term safe consumption and economic interest (3, 4). *P. freudenreichii* is a Gram-positive bacterium that is used traditionally as a Swiss-type cheese starter (5) but has also been studied with respect to the production of vitamin B_{12} (6, 7) and organic acids (8, 9). Furthermore, this bacterium can survive for a considerable period *in vitro* and under cheese-making conditions (10). It can also survive and adapt metabolically to animal and human gastrointestinal tracts (11–13) and has been identified in the fecal samples from a discrete cohort of human preterm breastfed infants (14, 15).

P. freudenreichii has long been studied for its probiotic properties, such as modulating the composition of the microbiota, antitumor activity, and immunomodulation (3). This species mainly modulates the composition of the microbiota through a bifidogenic effect, promoted by abundantly produced metabolites such as 1,4-dihydroxy-2-naphthoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) (16–19). Its antitumor activity is linked to the production of short-chain fatty acids such as propionate and acetate (20–24). Regarding immunomodulation, some proteins in this species, and particularly surface layer protein B (SlpB), have been associated with a reduction in the proinflammatory cytokines released and/or an increase in the release of anti-inflammatory cytokines both *in vitro* and *in vivo* (19, 25–29).

In addition to bacterial surface exposure, we recently showed that some immunomodulatory *P. freudenreichii* proteins are found associated with extracellular vesicles (EVs) (30). EVs are spherical nanometric structures produced by cells in the three domains of life (31). They are composed of lipid bilayers and an internal functional cargo, implicated in several biological processes that include interactions with host cells (31–34). The production of EVs has been demonstrated in several probiotic bacteria species (35–41). In the case of *P. freudenreichii*, EVs produced by the CIRM-BIA129 strain had a proteome predicted to interact with key protein components of the human inflammatory response, which was reinforced by the ability of EVs to reduce both the activity of the transcription factor NF-κB and interleukin-8 (IL-8) release in an intestinal epithelial cell model (30). The modulation exerted by ultrafiltrate (UF)-derived EVs occurred when the inflammatory response was induced by bacterial lipopolysaccharide (LPS) but not by other inducers, such as tumor necrosis factor alpha (TNF-α) and IL-1β. In addition, the EV-borne SlpB protein was shown to play a role in this immunomodulation, because EVs secreted by a Δ*slpB* mutant displayed reduced anti-inflammatory activity (30).

As well as their functional role in modulating interactions with the host, bacterial EVs are themselves modulated by specific conditions. In other words, environmental shifts impact the production and functional properties of EVs (42). For example, exposure to antibiotics induces vesiculation in several *Staphylococcus aureus* strains (43) and modifies the protein content of EVs secreted by *Acinetobacter baumannii* DU202 (44) and *Campylobacter jejuni* 81-176 (45). Iron-limiting conditions were shown to be associated with quantitative changes to the EV proteome of pathogenic and probiotic *Escherichia coli* strains (46, 47), of *Helicobacter pylori* 60190 (48), and of *Mycobacterium tuberculosis* H37Rv (49). Salt stress was also shown to be linked to drastic changes in the protein content and proinflammatory activity of EVs secreted by *Listeria monocytogenes* 10403S (50, 51). The EV proteome or its activity also changed in line with growth phases in *Bacillus subtilis* 168 (52), *Rhizobium etli* CE3 (53), *Pseudomonas aeruginosa* PAO1 (54), and *H. pylori* 26695 (55) and with the composition of media in *Gallibacterium anatis* 12656-12 (56) and *Pseudomonas putida* KT2440 (57). The bacterial properties of EVs were also affected by pH shifts (58) and exposure to epibromohydrin (59) or cannabidiol (60) and to other environmental conditions (61).

The impact of environmental conditions on EV production and function still remains poorly documented in probiotic bacteria, although it may represent a tool to modulate the properties of EVs and have potential therapeutic applications. Therefore, our aim was to investigate whether different growth conditions impact the production of

P. freudenreichii-derived EVs as well as modulate their properties. In the present study, we have shown that *P. freudenreichii* CIRM-BIA129 cultured in milk UF or yeast extract-lactate (YEL) growth medium produced EVs with distinct physicochemical, biochemical, and functional properties. In particular, UF- and YEL-derived EVs displayed considerable differences in protein cargo and immunomodulation. This study is a comparative analysis of the properties of the EVs produced by *P. freudenreichii* under various growth conditions and will contribute to understanding how probiotic traits are affected by different contexts. It also suggests interventional opportunities for the engineering of EV content and activity, enabling improvements to their potential technological and therapeutic roles.

RESULTS

The production of EVs by *P. freudenreichii* is dependent on growth conditions. During this work, we tried to determine whether environmental conditions, i.e., different growth media, affected the production of EVs by *P. freudenreichii* CIRM-BIA129. For that purpose, the sizes and concentrations of EVs purified from UF and YEL culture media were evaluated. Both conditions yielded EVs with a monodisperse size distribution, although YEL-derived EVs were less abundant than UF-derived EVs (Fig. 1A). They also displayed differences in diameter: UF-derived EVs had a modal size of 80.06 ± 2.606 nm and a mean size of 100.9 ± 3.204 nm, whereas YEL-derived EVs were significantly larger ($P < 0.05$), with a modal size of 89.72 ± 6.324 nm and a mean size of 117.9 ± 7.856 nm (Fig. 1B and C). The total concentration of EVs was significantly higher ($P < 0.01$) from UF ($2.164 \times 10^{12} \pm 1.383 \times 10^{11}$ EVs ml^{-1}) than from YEL ($9.394 \times 10^{11} \pm 1.846 \times 10^{11}$ EVs ml^{-1}) medium (Fig. 1D). Finally, the EV relative yield of CIRM-BIA129 (i.e., the amount of recovered EVs normalized by the amount of bacterial cells at sampling time) was more than three times higher from UF than from YEL medium (Fig. 1E).

The biological activity of *P. freudenreichii*-derived EVs is dependent on growth conditions. EVs derived from the two growth conditions were also evaluated in terms of their biological activity *in vitro*, i.e., NF-κB activation and IL-8 release. UF-derived *P. freudenreichii* CIRM-BIA129 EVs had previously been shown to exert an immunomodulatory effect on HT-29 human intestinal epithelial cells via the NF-κB pathway, i.e., modulation of transcription factor NF-κB activity and IL-8 release (30). To determine whether growth conditions also modulate EV activity, we compared the ability of the EVs produced from UF and YEL media to modulate the regulatory activity of the NF-κB transcription factor and the release of IL-8 from human intestinal epithelial cells. For that purpose, NF-κB/SEAP HT-29 cells, which are designed to measure NF-κB regulatory activity (62), and HT-29 parental cells were induced to an inflammatory state by different proinflammatory inducers and treated with EV preparations. Whatever the growth medium, EVs exerted an inhibitory effect on the regulatory activity of the NF-κB transcription factor only when the inflammation pathway was induced by LPS (Fig. 2A). Nevertheless, EV-mediated reduction of the LPS-induced proinflammatory effect was growth medium dependent. Indeed, the anti-inflammatory activity of EVs was significantly more intense with UF-derived than with YEL-derived EVs ($P < 0.0001$) (Fig. 2A). The specific LPS-induced anti-inflammatory effect of EVs was confirmed by the evaluation of IL-8 release from the HT-29 parental cell line. However, in this case, only UF-derived EVs were able to reduce LPS-induced IL-8 release (Fig. 2B). Finally, UF- and YEL-derived EVs had no cytotoxic effect on the two HT-29 cell lines, indicating that reductions in NF-κB activation and IL-8 release were not associated with cell death (Fig. 2C and D).

EVs produced under different growth conditions contain shared and exclusive proteins. To identify potential factors involved in the differences in biological activity between UF- and YEL-derived EVs, we characterized the protein content of EVs produced under the two growth conditions (see Table S4 in the supplemental material). Qualitative proteomics analysis enabled the identification of 391 proteins, 32 of which were exclusively detected in UF-derived EVs, one was exclusively detected in YEL-

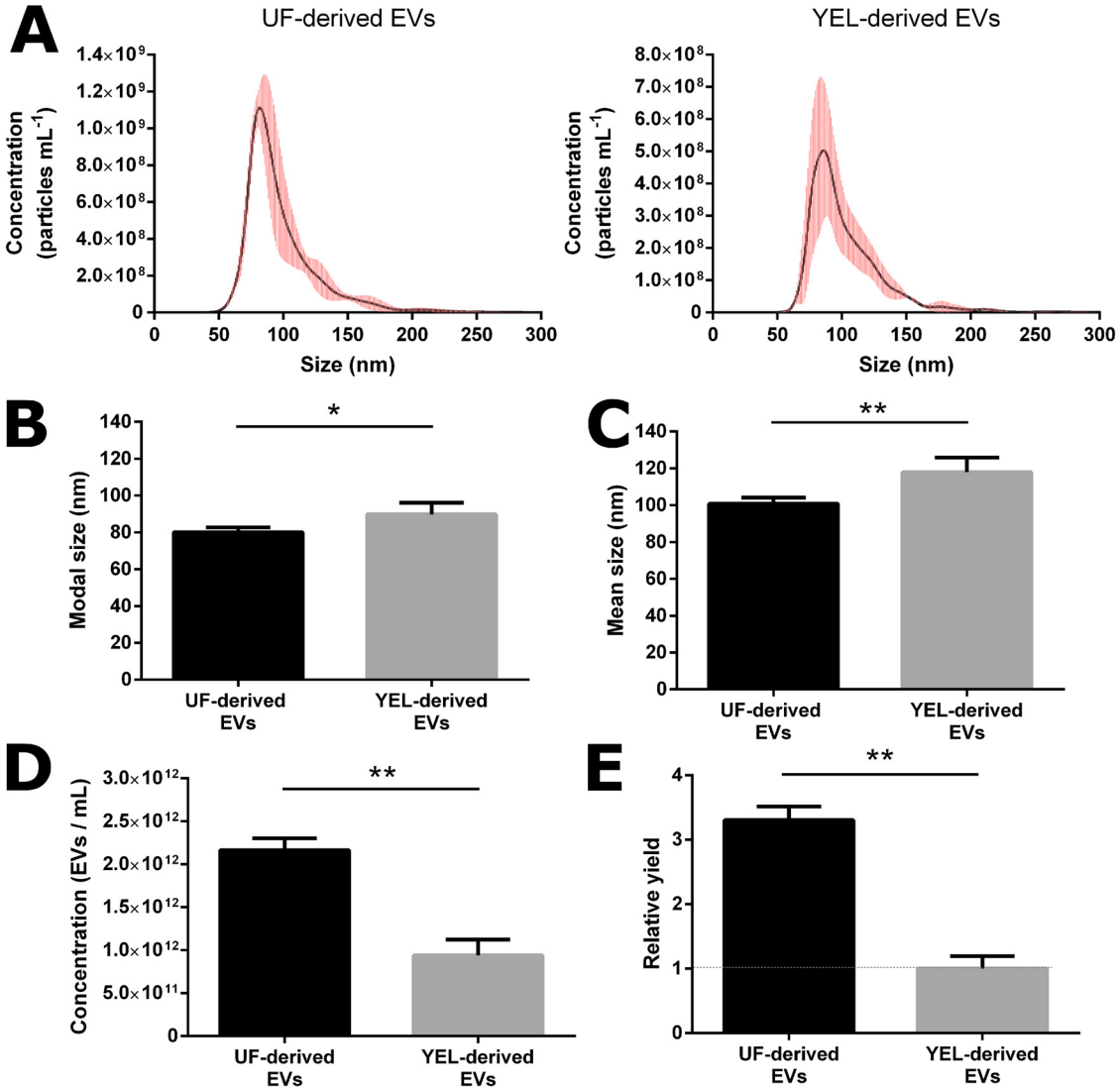

FIG 1 Characteristics of *P. freudenreichii*-secreted EVs under different growth conditions. (A) Size distribution of EVs derived from UF (left) and YEL (right) culture media. (B and C) Mode (B) and mean (C) diametric sizes of EVs produced from UF and YEL culture media. (D) Total concentrations of EVs produced from UF and YEL culture media. (E) Relative yields of EVs (normalized by the amount of bacterial cells in each culture medium). Size and concentration measurements were acquired using nanoparticle tracking analysis (NTA). Data are expressed as means ± standard deviations of values obtained from at least three independent biological replicates. Asterisks indicate statistical significance as evaluated by the Mann-Whitney test: **, $P \leq 0.01$; *, $P \leq 0.05$.

derived EVs, and 358 were common to both growth conditions (Fig. 3A). Concerning subcellular localization, all exclusive proteins (UF and YEL exclusive) were predicted to be cytoplasmic. The shared proteins were mainly cytoplasmic ($n = 256$), but some were also predicted to be membrane proteins ($n = 65$) or extracellular proteins ($n = 37$) (Fig. 3B). Predicted lipoproteins were only identified among proteins found under both conditions ($n = 25$) (Fig. 3C). The frequencies of Clusters of Orthologous Groups (COG) categories were well distributed among shared proteins, mainly being related to metabolism and information processing. UF-exclusive proteins were mainly linked to carbohydrate or amino acid metabolism and DNA processing, while the only YEL-exclusive protein had an unknown function (Fig. 3D).

A comparison of growth conditions reveals several differentially abundant EV proteins. Qualitative proteomic analysis pinpointed proteins specific to YEL- and UF-derived EVs. Further, to better grasp the impact of growth conditions on EV content, the relative abundance of the proteins was analyzed under each condition. Quantitative proteomics showed that the relative abundance of EV proteins could vary

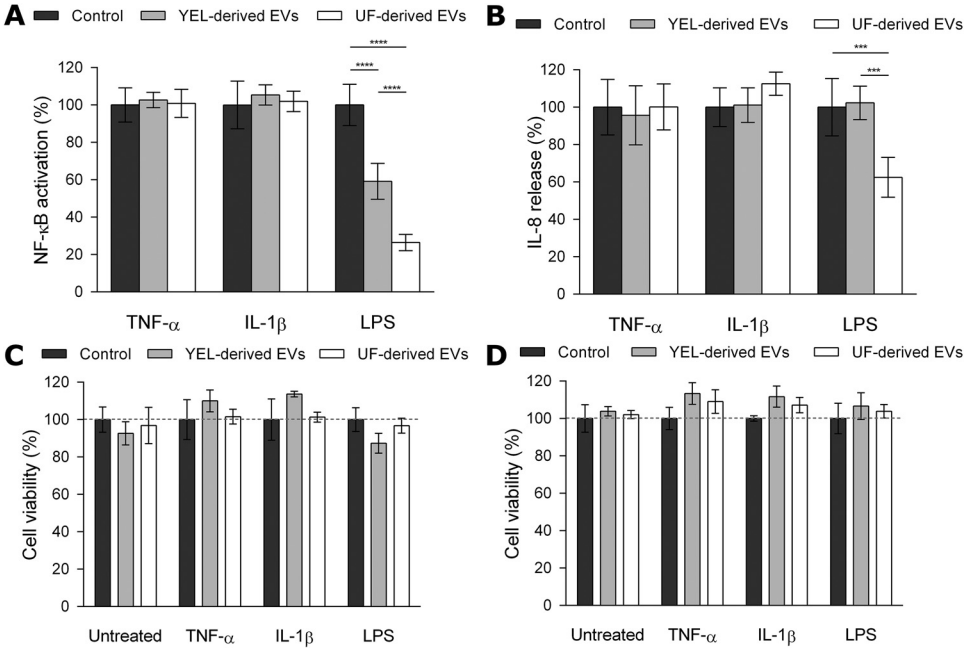

FIG 2 *P. freudenreichii*-secreted EVs play different biological roles depending on growth conditions. (A) Percent NF-κB transcription factor activity in HT-29/kb-seap-25 cells left untreated or treated with the inflammatory inducer TNF-α (1 ng ml^{-1}), IL-1β (1 ng ml^{-1}), or LPS (1 ng ml^{-1}) in the presence or absence of UF- and YEL-derived EVs (1.0×10^9 EVs ml^{-1}). The values are normalized by the control conditions (control TBS buffer or stimulation by the inducer in the absence of EVs). (B) Percentage of IL-8 released by HT-29 intestinal epithelial cells after stimulation by LPS (1 ng ml^{-1}), TNF-α (1 ng ml^{-1}), or IL-1β (1 ng ml^{-1}) inducer in the presence or absence of UF- and YEL-derived EVs (1.0×10^9 EVs ml^{-1}). The values are normalized to the control conditions (control TBS buffer or stimulation by inducer in the absence of EVs). (C and D) Percent viability of HT-29/kb-seap-25 reporter (left) and HT-29 parental (right) cells before or after stimulation by LPS (1 ng ml^{-1}), TNF-α (1 ng ml^{-1}), or IL-1β (1 ng ml^{-1}) inducer in the presence or absence of UF- and YEL-derived EVs (1.0×10^9 EVs ml^{-1}). The values are normalized by the control conditions (control TBS buffer or stimulation by an inducer in the absence of EVs). Data are expressed as mean ± standard deviation of values obtained from at least three independent biological replicates. Asterisks indicate statistical significance as evaluated by two-way ANOVA with Tukey's multiple-comparison test: ****, $P \leq 0.0001$; ***, $P \leq 0.001$.

as a function of culture medium (Fig. S1). Among the total of 391 identified proteins, the abundances of 164 proteins did not differ between YEL- and UF-derived EVs ($P > 0.05$), 127 proteins were significantly more abundant in UF-derived EVs ($P \leq 0.05$, log [fold change] > 0), and 100 proteins were significantly more abundant in YEL-derived EVs ($P \leq 0.05$, log[fold change] < 0) (Fig. 4A). Among the proteins more abundant in UF-derived EVs, the most significant included those related to carbohydrate (LacZ, IolC, and IolE1), amino acid (AroH and Hom), and energy (NirA2) metabolism. Among the proteins more abundant in YEL-derived EVs, the most significant were mainly ribosomal proteins (RplC, RplV, RplT, RplB, and RpsG). Differentially abundant proteins under each condition also included those of unknown function or poorly characterized (PFCIRM129_01355, PFCIRM129_01765, PFCIRM129_02410, and PFCIRM129_10740) (Fig. 4B). Interestingly, among EV proteins undergoing significant changes to their abundance as a function of the culture medium, we identified several that had reportedly been associated with a stress response (Fig. 4C) or immunomodulatory properties (Fig. 4D) in *P. freudenreichii* or other bacterial species. Functional enrichment analysis (Tables S5 and S6) showed that proteins that were more abundant in UF-derived EVs were mainly related to energy, carbohydrate, amino acid, and sulfur metabolism KEGG pathways, together with COG category C (energy production and conversion). On the other hand, proteins more abundant in YEL-derived EVs were mainly related to glycolysis/gluconeogenesis and ribosome KEGG pathways, together with COG category J (translation, ribosomal structure, and biogenesis) (Fig. 4E).

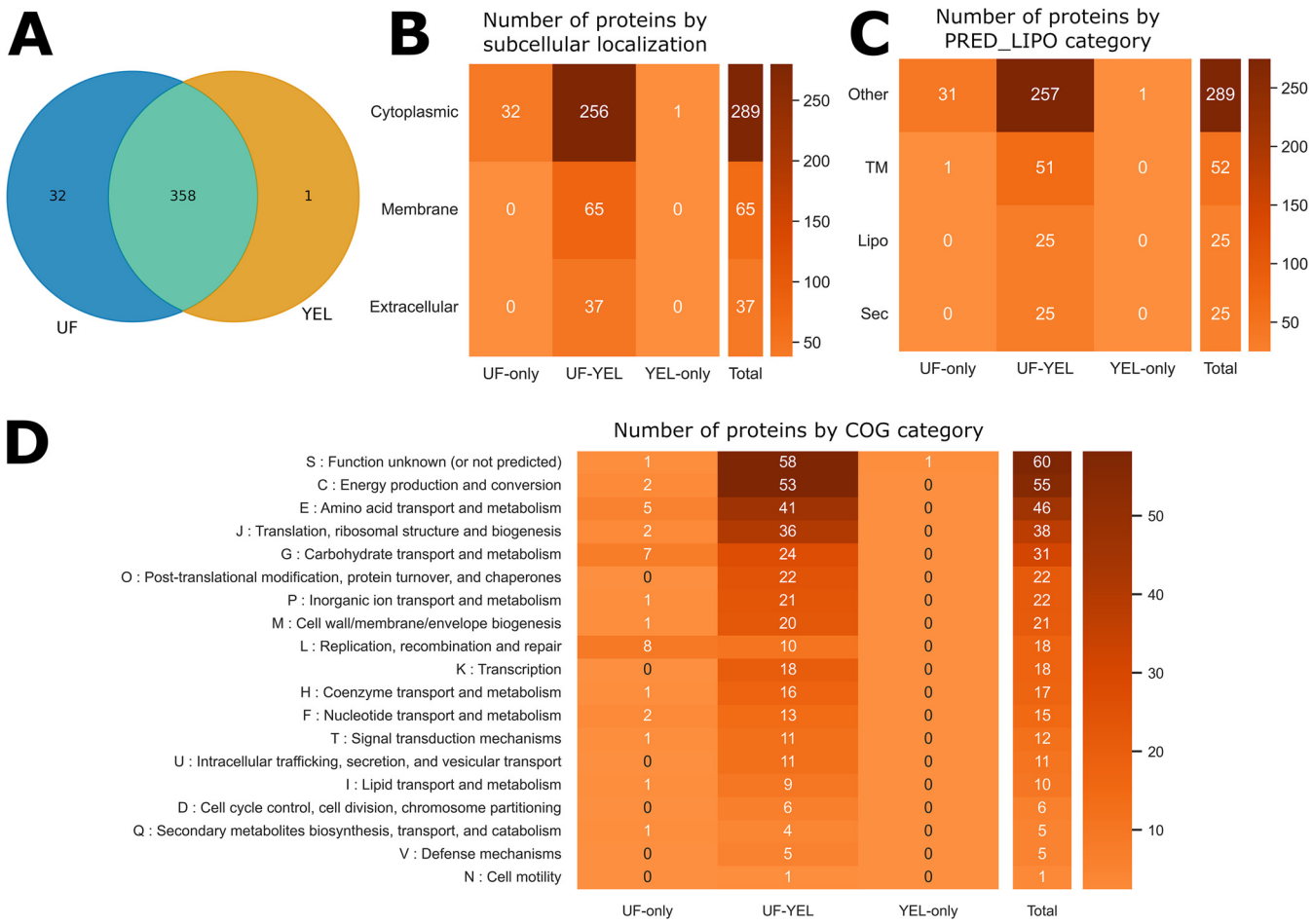

FIG 3 Qualitative proteomics can reveal differences in EV protein content as a function of growth conditions. (A) Venn diagram showing the number of shared and specific proteins for EVs under the two growth conditions. (B to D) Heatmaps showing the distribution of proteins as a function of growth condition categories and functional predictions. Columns show growth condition categories (UF-only, proteins identified exclusively in UF-derived EVs; YEL-only, proteins identified exclusively in YEL-derived EVs; UF-YEL, proteins identified under both growth conditions). Rows show subcellular localization prediction (cytoplasmic, membrane, or extracellular) (B), PRED-LIPO prediction (Sec, secretion signal peptide; Lipo, lipoprotein signal peptide; TM, transmembrane; Other, no signals found) (C), and COG functional categories (D).

Predictions of interactions highlight the relevant bacterial proteins potentially implicated in immunomodulation. The qualitative and quantitative differences in the protein content of EVs revealed factors that might vary with growth conditions. Our next aim was to investigate whether this variation, when translated into different patterns of interaction, might offer insights into the mechanisms underlying the differential activity of EVs. Therefore, 101 human proteins from the NF-κB pathway and 391 bacterial proteins found in EVs were submitted to the prediction of protein-protein interactions. A total of 893 interactions were considered to be valid (minimum score of 0.9765), involving 49 human and 266 bacterial proteins (Table S7). Next, we performed progressive filtering procedures to select meaningful interactions according to experimental criteria. First, in view of the fact that the anti-inflammatory activity of EVs was LPS specific, we filtered out interactions involving human proteins that were not related to the LPS-induced part of the NF-κB pathway, which resulted in a subnetwork of 357 interactions (Fig. 5A). A further filtering was then performed to retain only human proteins that were specific to the LPS-induced NF-κB pathway, i.e., not related to TNF-α or IL-1β induction. This resulted in 23 interactions, involving just two human proteins: TCAM1 and TLR4 (Fig. 5B). A final filtering was performed in bacterial proteins to retain only those whose differential expression was considered significant ($P \leq 0.05$) according to quantitative proteomics (Fig. 5C). Therefore, TLR4 and TCAM1 human proteins were identified within the NF-κB signaling pathway as potential

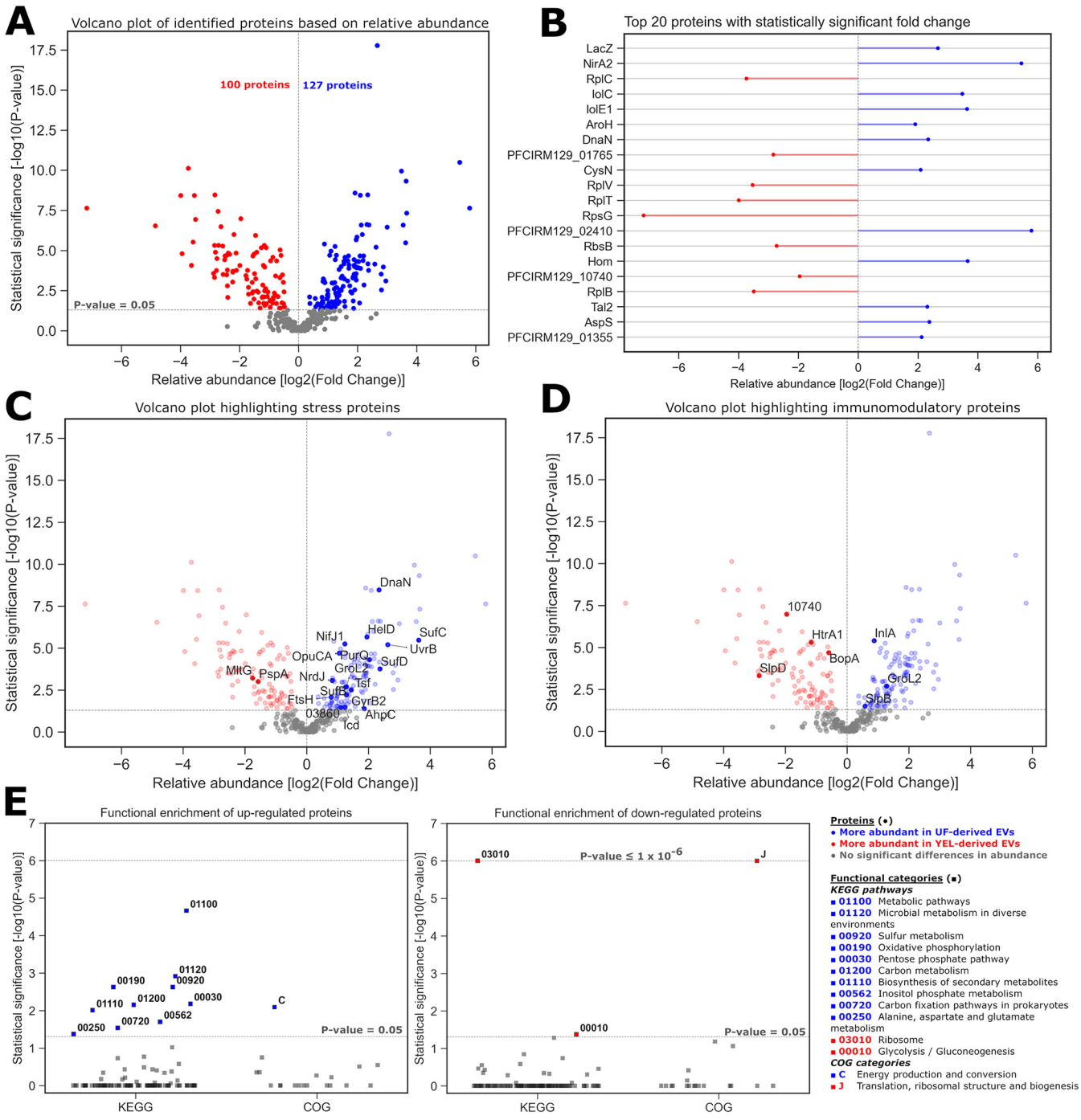

FIG 4 EVs derived from different growth conditions carry proteins with different relative abundances. (A) Volcano plot showing the relative abundance of proteins from UF-derived EVs versus YEL-derived EVs (fold change UF/YEL) as well as the significance of their fold changes. The results are presented according to a logarithmic scale, with positive fold change ratios shown in blue, negative fold change ratios shown in red, and nonsignificant ratios shown in gray. The horizontal dotted line shows the threshold of significance, and the vertical line indicates a logarithmic fold change of zero. (B) Relative abundance of proteins with the highest significant UF/YEL fold changes. (C and D) Volcano plots highlighting subsets of proteins reportedly associated with a stress response (C) or immunomodulation (D) in *P. freudenreichii* or other bacterial strains. (E) Enrichment analysis of KEGG pathways and COG categories for the upregulated (left) and downregulated (right) proteins. Significant results are shown in blue for categories enriched in upregulated proteins, in red for categories enriched in downregulated proteins, and in gray for categories that are not statistically enriched. The lower dotted line represents the minimal threshold of significance, and the higher dotted line indicates a cap for categories with *P* values of 1×10^{-6} or lower.

targets for immunomodulation. Moreover, novel bacterial proteins were suggested to be responsible for the differential intensity of the anti-inflammatory response resulting from various growth conditions: pyruvate synthase/pyruvate-flavodoxin oxidoreductase (NifJ1), the NADH-quinone oxidoreductase chain G (NuoG), an uncharacterized protein

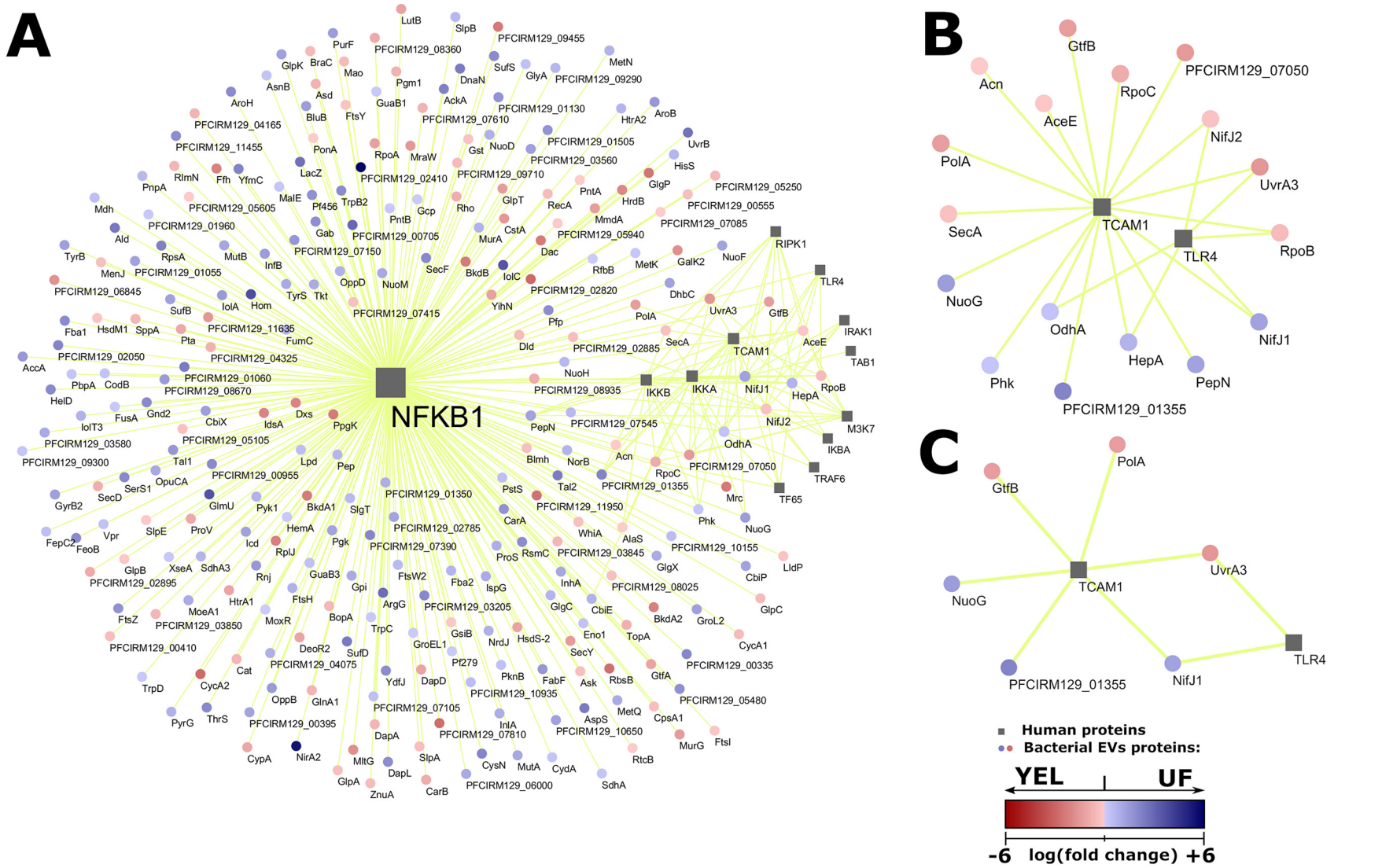

FIG 5 Prediction of protein-protein interactions highlights relevant bacterial proteins. (A to C) A network representation of predicted interactions filtered by human proteins that correspond to the LPS-induced part of the NF-κB pathway (A), by uman proteins involved in the LPS-induced NF-κB pathway but not in induction by TNF-α or IL-1β (B), and by bacterial proteins whose abundance was considered significant by quantitative proteomic analysis (C). Human proteins are represented by gray squares, and bacterial proteins are represented by red or blue circles. Predictions of interactions are represented by yellow lines connecting squares and circles. The relative abundance of bacterial proteins is seen according to the scale in the bottom right corner (logarithm of fold change), where positive values (blue) indicate a higher abundance in UF-derived EVs and negative values (red) indicate a higher abundance in YEL-derived EVs.

(PFCIRM129_01355), the GtfB glycosyltransferase (GtfB), a putative DNA polymerase I (PolA), and UvrABC system protein A (UvrA3).

DISCUSSION

Several studies have demonstrated that the production, properties, and roles of bacterial EVs are affected by environmental conditions. Thus, monitoring the environmental conditions under which the bacteria are grown offers a tool to modulate the production and properties of EVs and may have some therapeutic applications (42). We recently reported the production of EVs by the probiotic *P. freudenreichii* CIRM-BIA129, and our study included their physicochemical and functional characterization (30). In particular, we showed that they displayed anti-inflammatory effects throughout modulation of the regulatory activity of the NF-κB transcription factor and IL-8 release in human epithelial cell models. However, whether the production and immunomodulatory effects of *P. freudenreichii*-secreted EVs could be modulated by environmental conditions still needed to be determined.

Here, we compared the properties of *P. freudenreichii* EVs purified from two growth media that are routinely used for *P. freudenreichii* cultures: cow's milk ultrafiltrate (UF) medium and yeast extract-lactate (YEL) medium (29, 63, 64). YEL is the gold-standard laboratory medium for propionibacteria. This medium, in addition to yeast extract (providing the necessary growth factors) and sodium lactate (the preferred carbon and energy source), contains peptone (as a nitrogen source) (65). This medium was developed to mimic the growth conditions of propionibacteria in Swiss-type cheeses after fermentation of the cheese curd by lactic acid bacteria. We later developed a milk UF medium to mimic the growth of propionibacteria in fermented milk (63, 66). This medium represents the aqueous phase of cow milk, added with casein peptone and sodium lactate. UF and YEL were chosen to compare EV properties because they differentially impact the physiology of the bacterium, notably its growth parameters (biomass and generation time), the pH of the extracellular medium at the end of stationary phase, and cell viability after stress challenges (63, 64).

We demonstrated that culture media affect the physical, biochemical, and biological properties of *P. freudenreichii*-secreted EVs. Notably, compared to EVs recovered from YEL cultures, markedly more were recovered from UF cultures. In some bacterial species, an increase in EV production has been linked to an adaptation to stressful conditions (67–69). In the case of *P. freudenreichii*, UF could be considered a more stressful growth condition than YEL, since the bacterium accumulated higher proportions of trehalose during growth in UF than in YEL (64), with this sugar being involved in the response to stress, notably during acid adaptation and osmoadaptation (64, 70, 71). Moreover, some of the proteins that we found more abundant in UF than YEL-derived EVs were associated in the parent cells with the adaptation of the bacterium to stress (Fig. 4C). Among these, chaperonin GroL2 (GroL2), pyruvate synthase/pyruvate-flavodoxin oxidoreductase (NifJ1), and alkyl hydroperoxide reductase protein C22 (AhpC) were notably associated with the response to acid stress in this bacterium (72). Taken together, these results suggested that EV production in *P. freudenreichii* is affected by stress conditions and showed that the control of growth conditions might offer a lever to modulate EV production in this probiotic bacterium.

As mentioned above, in addition to impacting the physical properties of EVs, the culture medium also modulates EV content in terms of both the identities and abundances of its protein cargo. When the protein contents were compared, modest differences were observed between YEL- and UF-derived EVs. Indeed, more than 90% ($n = 358$) of EV proteins were found to be common to the YEL and UF media. Only one protein with an unknown function was specific to YEL, and 32 of the predicted cytoplasmic proteins were found to be exclusive to UF-derived EVs. This notably included several proteins with functions assigned to the COG categories of "replication, recombination, and repair" (L, $n = 8$), "carbohydrate transport and metabolism" (G, $n = 7$), and "amino acid transport and metabolism" (E, $n = 5$). The most important impact of the

culture medium concerned the abundance of proteins packed into EVs. The abundance of approximately 60% of EV proteins changed significantly as a function of culture medium. In the case of YEL-derived EVs, the more abundant proteins were mainly related to ribosomes, glycolysis, and gluconeogenesis, which may have been linked to the higher growth rate of *P. freudenreichii* in YEL medium (63, 64). In contrast, UF-derived EVs were more enriched in proteins related to pentose phosphate, sulfur, secondary metabolites, inositol phosphate, amino acids, and energy metabolism, which are probably related to stress responses. Although the rules for the cargo selection and sorting into bacterial EVs remain elusive, especially in Gram-positive bacteria (73), it is often thought that the content of EVs reflects the physiology, metabolic status, and biological properties of the cells producing them (32, 52). Therefore, modulation of both the presence and abundance of proteins in EVs as a function of culture medium might be related to the level of their synthesis by cells. Indeed, it seems consistent that the relative abundance of a protein in a whole cell would affect its availability to be packed into EVs in the absence of, or in addition to, a selective process (34, 74). Accordingly, several proteins associated with the adaptation of the bacterium to UF or YEL medium were identified as being more abundant in UF- or YEL-derived EVs, respectively. For example, some of the proteins specifically present or found to be more abundant in UF-derived EVs were involved in the assimilation of the main carbon sources of UF, including beta-galactosidase (LacZ) (which hydrolyzes lactose into glucose and galactose) and inositol assimilation proteins (IolE1, IolC, IolT3, and IolA). However, one cannot exclude that, in addition to the level of synthesis by cells, some of these proteins are also purposely packed into EVs to perform specific functions, such as host cell interactions (75). To date, the molecular mechanisms that drive the recruitment of proteins into bacterial EVs have remained unclear. Determining which proteins are selected, and how, is of crucial value to identifying those purposely packed into EVs, notably to better understand their biological roles. Recently, we showed that abundance, charge, and subcellular localization could influence the protein availability of the vesicle cargo in *S. aureus* (74). Whether these rules are also applicable to the EVs secreted by *P. freudenreichii* still needs to be investigated, but our data may offer an additional opportunity to uncover the mechanisms governing the selection of proteins into EVs.

Finally, the culture medium in which bacteria are grown also modulates the biological activity of CIRM-BIA129 EVs. Indeed, UF- and YEL-derived EVs presented remarkable differences in terms of immunomodulation. We showed that EVs from both origins reduced the activity of NF-κB, a key mediator of inflammatory responses, but at different intensities. This reduction occurred specifically when inflammation was induced by bacterial LPS and was achieved in roughly 74% for UF-derived EVs but only 41% for YEL-derived EVs. This differential EV-mediated modulation of NF-κB activity has profound consequences on the cellular inflammatory response. Indeed, LPS-induced IL-8 release by HT-29 cells was significantly reduced by UF-derived EVs but not by YEL-derived EVs. To date, the bacterial effectors involved in the immunomodulatory activity of *P. freudenreichii*-secreted EVs are still unknown. We recently showed that SlpB participates in EV-mediated anti-inflammatory effects *in vitro*, since EVs derived from a Δ*slpB* mutant displayed a partial reduction of NF-κB activation compared to wild-type-derived EVs (30). However, it is not known whether the role of SlpB is direct or indirect. Among *P. freudenreichii*-associated immunomodulatory proteins, SlpB, internaline A (InlA), and the chaperonin GroL2 were more abundant in UF- than in YEL-derived EVs. In contrast, surface-layer protein D (SlpD), the solute binding protein of the ABC transport system (BopA), trypsin-like serine protease (HtrA1), and a protein of unknown function, PFCIRM129_10740, were more abundant in YEL-derived EVs. The differences in abundance of at least one of these recognized immunomodulatory proteins may explain the differential anti-inflammatory activity seen for UF- and YEL-derived EVs (27–29, 76).

One can also suppose that other differentially abundant proteins are implicated in the differential activity of EVs triggered by culture media. These include those bacterial

proteins predicted to interact with the human proteins Toll-like receptor 4 (TLR4) and TIR domain-containing adapter molecule 1 (TICAM-1), which are of particular interest. Indeed, these two proteins are involved in the NF-κB signaling pathway induced by LPS but not by TNF-α or IL-1β; therefore, they are good candidates as molecular targets for immunomodulation mediated by *P. freudenreichii*-derived EVs with LPS-specific induction. Consequently, the abundances of their potential bacterial interacting partners might also explain the differences in EV activity as a function of culture medium. Among the predicted TLR4- and TICAM1-interacting bacterial proteins, six displayed a different abundance pattern between UF- and YEL-derived EVs. These included NifJ1, the NADH-quinone oxidoreductase chain G (NuoG), and an uncharacterized protein (PFCIRM129_01355), which were more abundant in UF-derived EVs, and GtfB glycosyltransferase (GtfB), a putative DNA polymerase I (PolA), and the UvrABC system protein A (UvrA3), which were more abundant in YEL-derived EVs. These proteins have not previously been characterized as being immunomodulatory, but our *in silico* network predictions suggest they can interact with human proteins with key roles in the inflammatory response; their abundances also could explain why UF- and YEL-derived EVs display distinct patterns of biological activity. Whether these proteins as well as other differentially abundant immunomodulatory proteins participate in the modulation of EV biological activity as a function of culture medium certainly deserves further investigation.

To sum up, our study showed that the probiotic *P. freudenreichii* CIRM-BIA129 produced EVs with distinct properties depending on the culture medium in which the bacterium was grown and reinforced the importance of environmental conditions to the properties of EVs. Although little is known about the mechanisms that underpin the modulation of Gram-positive bacterial EV production, content, and activity, they respond definitively to environmental stimuli in the case of *P. freudenreichii*. Specifically, in the context of probiotic bacteria, the optimization of medium composition might represent a tool to improve the beneficial activity of EVs and enable the development of EV-based therapeutic applications, such as functional foods with improved properties or pure EV formulations. Comprehensive studies might elucidate the relationships between specific medium components and the vesicular export of immunomodulatory proteins, enabling an improvement to the anti-inflammatory properties of EVs.

MATERIALS AND METHODS

Bacterial cultures. *P. freudenreichii* CIRM-BIA129 (equivalent to the ITG P20 strain, provided by CNIEL) was supplied, stored, and maintained by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRAE, Rennes, France). The bacteria were grown under two conditions: in yeast extract-lactate medium (YEL) or cow milk ultrafiltrate medium (UF), which was further supplemented with 100 mM sodium lactate and 5 g liter^{-1} casein hydrolysate, as previously described (63, 65). For both conditions, incubation was at 30°C, without agitation, until the start of stationary phase (2×10^9 bacteria ml^{-1} for UF and 3×10^9 bacteria ml^{-1} for YEL), as reported elsewhere (63). The culture starting volume was 500 ml for each biological replicate.

Purification of EVs. Bacterial cultures (500 ml) were centrifuged at room temperature (6,000 $\times$ *g*, 15 min) and the supernatants filtered through 0.22-μm top filters (Nalgene, Thermo Scientific). The supernatants were then concentrated 1,000-fold using 100-kDa ultrafiltration units (Amicon, Merck Millipore) by successive centrifugations (2,500 $\times$ *g*). The concentrated suspension of EVs was recovered in TBS buffer (Tris-buffered saline; 150 mM NaCl, 50 mM Tris-Cl, pH 7.5) and further purified by size exclusion chromatography (qEV original, 70 nm; iZON) as recommended by the manufacturer (30, 77). The resulting fractions containing EVs in TBS buffer were pooled (1.5 ml) and further concentrated to achieve approximately 50 μl using 10-kDa centrifugal filter units (Amicon, Merck Millipore). The aliquots were stored at −20°C or used immediately.

Characterization of EVs. The characterization of EVs in terms of size and concentration was performed by nanoparticle tracking analysis using a NanoSight NS300 (Malvern Panalytical), equipped with a sCMOS camera, Blue488 laser, and NTA 3.3 Dev Build 3.3.104 software. The experiments were conducted at 25°C, with a camera level of 15 and a syringe pump speed of 50. Concentration, focus, and other parameters were adjusted accordingly for each experimental group. A detection threshold of 3 was employed to determine valid particles. EV homogeneity and integrity were also confirmed by negative staining electron microscopy (data not shown).

Evaluation of EV activity *in vitro*. Two HT-29 human colon adenocarcinoma cell lines were employed to characterize the activity of EVs *in vitro*, as previously reported (30). Briefly, the parental HT-

29 intestinal epithelial cells (ATCC HTB-38) were used to measure IL-8 release (IL-8/CXCL8 DuoSet; R&D Systems), and the lineage transfected with the secreted alkaline phosphatase (SEAP) reporter system (HT-29/kb-seap-25) was used to monitor NF-κB activity (Quanti-Blue reagent; Invivogen) (62). HT-29/kb-seap-25 cells were cultured in RPMI-glutamine medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Corning), 1% nonessential amino acids, 1% sodium pyruvate, 1% HEPES buffer (Thermofisher Scientific), and 1% penicillin-streptomycin (Lonza) (62). The parental HT-29 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Dominique Dutscher) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (76). The cells were treated with TBS buffer as a control or the EV preparations (1.0×10^9 EVs ml^{-1}) purified from UF or YEL cultures. To induce inflammation, the cells were also treated with TNF-α (1 ng ml^{-1}; PeproTech), IL-1β (1 ng ml^{-1}; Invivogen), LPS from *Escherichia coli* O111:B4 (1 ng ml^{-1}; Sigma-Aldrich), or the TBS control. After seeding in 96-well plates (3×10^4 cells/well) and stimulation with the samples, the cells were incubated at 37°C, under 5% CO_2, for 24 h. Cell confluence was verified under the microscope before and after stimulation. Cell viability after stimulation was investigated using the CellTiter 96 AQueous one-solution cell proliferation assay (MTS; Promega). Absorbance was measured with a Xenius (SAFAS Monaco) microplate reader at 655 nm for the SEAP (NF-κB) activity assay, 450 nm for the IL-8 enzyme-linked immunosorbent assay, and 490 nm for the MTS cell viability assay.

Identification and quantification of proteins using mass spectrometry. One microgram of EV was dissolved in SDS and subjected to SDS-PAGE for a short period, allowing the entry of the protein into 2 to 3 mm of separating gel. The gel pieces were subjected to in-gel trypsinolysis followed by peptide extraction, as described previously (30, 72, 78). After digestion, the peptides were stored at −20°C until further analysis. Nano-liquid chromatography tandem mass spectrometry (LC-MS/MS) experiments were performed as previously described (30, 79). The peptides were identified from MS/MS spectra using X! TandemPipeline software (80), and searches were performed against the genome sequence of *P. freudenreichii* CIRM-BIA129 (GenBank accession no. NZ_HG975455). The database search parameters were specified as the following: trypsin cleavage was used, and peptide mass tolerance was set at 10 ppm for MS and 0.05 Da for MS/MS. Methionine oxidation was selected as a variable modification. For each peptide identified, an E value lower than 0.05 was considered a prerequisite for validation. A minimum of two peptides per protein was imposed, resulting in a false discovery rate (FDR) of <0.15% for protein identification. Each peptide identified by tandem mass spectrometry was quantified using the free MassChroQ software (81) before data treatment and statistical analysis with R software (R 3.4; Project for Statistical Computing). A specific R package, called MassChroqR (v0.4.3), was used to automatically filter dubious peptides and group the peptide quantification data into proteins. Two different and complementary analytical methods were used, based on peak counting or XIC (extracted ion current). For peak counting, variance analysis was performed on proteins with a minimum peak ratio of 1.5 between both culture conditions. Proteins with an adjusted *P* value of <0.05 were considered significantly different. For XIC-based quantifications, normalization was performed to take account of possible global quantitative variations between LC-MS runs. Peptides shared between different proteins were automatically excluded from the data set, as were peptides present in fewer than two of the three biological replicates. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein. Analysis of variance was used to determine proteins whose abundance differed significantly between growth conditions.

Proteomic analysis. Protein subcellular localizations were predicted by Cello2GO (82); lipoproteins were predicted by PRED-LIPO (83) and Clusters of Orthologous Groups (COG) categories, and KEGG Pathways were predicted by eggNOG-mapper v2 (84, 85). The Venn diagram, heatmaps, and volcano plot were conceived using Python's Matplotlib-venn 0.11.5, Seaborn 0.10.0, and Bioinfokit 0.7 packages, respectively.

Functional enrichment analysis. For functional enrichment analysis, the lists of up- and downregulated proteins were submitted to the g:Profiler web server (86, 87), together with an ortholog-based Gene Matrix Transposed (GMT) file (see Files S1 and S2 in the supplemental material) constructed from KEGG pathways and COG whole-proteome annotation categories obtained from eggNOG-mapper v2 (84, 85). Protein lists were ordered by decreasing relative level of abundance, and a significance threshold (adjusted *P* value) of 0.05 was adopted. For visualization, adjusted *P* values of 1×10^{-6} or lower were capped to this value.

Prediction of protein-protein interactions. Human and bacterial protein sequences were retrieved from the UniProt and NCBI databases, respectively. Bacterial sequences identified in EVs derived from at least one of the growth conditions, as well as the human sequences corresponding to KEGG's NF-κB pathway, were submitted to the InterSPPI web server for the prediction of interactions (88). Only interactions with a score of 0.9765 or higher were considered valid, with a specificity of 0.99. These interactions were gradually filtered so that human counterparts corresponded to, or were specific to, the LPS-induced portion of the NF-κB pathway, and/or bacterial proteins displayed a significant fold change in their expression according to quantitative proteomics. The lists of human proteins used for filtering are available in Tables S1 to S3. Predicted interactions and protein annotations were processed using the Pandas Python package (89). Visualization of the interaction network was achieved using Cytoscape software (90).

Statistical analysis. All experiments were performed independently and at least in triplicate. The results are presented as means ± standard deviations. Absorbance measurements were normalized to the control conditions. The differences between experimental groups were analyzed using the Mann-Whitney test or two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test.

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, California, USA).

Data availability. The mass spectrometry proteomics data can be found at https://doi.org/10.15454/Q6PPXY.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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V.R.R., G.J., Y.L.L., V.A.C.A., and E.G. conceived and designed the experiments. V.R.R., V.B.-B., J.J., and B.S.R.L. performed the experiments. V.R.R., A.N., J.J., and E.G. analyzed the data. F.L.R.C., A.N., J.J., V.B.-B., G.J., and E.G. gave practical suggestions regarding performance of the experiments. V.A.C.A., Y.L.L., and E.G. contributed to funding acquisition. V.R.R. and E.G. wrote the original draft. All authors contributed to data interpretation, drafting the manuscript, critically revising the manuscript, and approving its final version.

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Chapter 5 – Additional results

The extracellular vesicles from *Propionibacterium freudenreichii* obtained by different purification methods and growth conditions share a large core proteome

The studies presented in the previous chapters showed that *P. freudenreichii* produced EVs with anti-inflammatory roles and that their properties depended on whether the bacteria were grown in UF or YEL culture media. Therefore, the optimization of bacteria growth conditions represented a tool for the modulation of EVs properties, including their anti-inflammatory activity towards human epithelial cells. In these previous studies, EVs were obtained by size exclusion chromatography (SEC), which is a size-based purification method. Nevertheless, in spite of the increasing recognition of SEC as a high-quality straightforward method for EV purification, the most widely used methods for bacteria EV purification are still those based on ultracentrifugation (UC). Therefore, we aimed to investigate if *P. freudenreichii*-derived EVs purified by this method would present similar properties, particularly regarding protein content.

P. freudenreichii was grown in UF and YEL media until the beginning of the stationary phase, when the cultures were centrifuged, filtered and the supernatant were concentrated. Next, purification was conducted with a series of ultracentrifugation steps, including one with a sucrose density gradient. Microscopic and size characterization confirmed EV purification. Shotgun proteomics allowed the identification of a diverse set of proteins, also depending on bacteria growth conditions. A comparative analysis of the protein content of UC- and SEC-derived EVs, isolated from cultures in both UF and YEL showed that, although some proteins were specific of each condition, 308 were shared among all conditions. This core proteome was enriched in proteins related to energy and carbon metabolism, ribosomal structure and biogenesis, quorum sensing, protein export and peptidoglycan biosynthesis.

Therefore, these results offer some clues of the functions conserved in *P. freudenreichii*-derived EVs, eliminating growth conditions and purification biases. They are presented in detail in the next pages.

1. Introduction

Extracellular vesicles (EVs) are nano-sized membranous particles that transport biomolecules implicated in intercellular communication (Brown et al., 2015; Woith et al., 2019; Dagnelie et al., 2020; Nagakubo et al., 2020). EVs were consistently reported as an export system in species from all kingdoms of life, including bacteria (Deatheragea and Cooksona, 2012; Woith et al., 2019; Nagakubo et al., 2020). A diverse set of functions was attributed to bacterial EVs, including quorum sensing (Mashburn and Whiteley, 2005), biofilm formation (Flemming et al., 2016; Caruana and Walper, 2020), competition (Li et al., 1998), nutrition (Elhenawy et al., 2014; Prados-Rosales et al., 2014), defense (Manning and Kuehn, 2011; Lee et al., 2013b), pathogenesis (Pathirana and Kaparakis-Liaskos, 2016; Cecil et al., 2019) and probiosis (Bitto and Kaparakis-Liaskos, 2017; Molina-Tijeras et al., 2019). Among beneficial bacteria, EVs with anti-inflammatory activity were reported in several species, including *Akkermansia muciniphila* (Ashrafian et al., 2019), *Bacteroides fragilis* (Shen et al., 2012), *Echerichia coli* Nissle 1917 (Fábrega et al., 2017), *Bifidobacterium bifidum* (López et al., 2012), *Lactobacillus* species (Seo et al., 2018; Vargoorani et al., 2020) and *Propionibacterium freudenreichii* (Rodovalho et al., 2020b, 2020a).

Research on bacterial EVs relies greatly on the available purification methods, since the culture media used to grow bacteria are complex and heterogeneous, containing EVs in varying concentrations, bacterial cells, spent medium components, and secreted molecules, such as proteins, lipids and metabolites (Klimentová and Stulík, 2015; Orench-Rivera and Kuehn, 2016). After separation from bacterial cells and concentration, EVs must be further purified to deplete medium contaminants by methods such as ultracentrifugation (UC) and size-exclusion chromatography (SEC) (Brown et al., 2015; Dauros Singorenko et al., 2017; Mol et al., 2017; McNamara and Dittmer, 2020). UC-based methods use high-speed centrifugation to differentially sediment EVs and contaminants (Klimentová and Stulík, 2015; Monguió-Tortajada et al., 2019a; McNamara and Dittmer, 2020), whereas SEC is based on the differential elution of EVs and contaminants through a size-selective porous polymeric matrix (Nordin et al., 2015; Benedikter et al., 2017; Mol et al., 2017; Monguió-Tortajada et al., 2019a, 2019b). Although SEC is an approach of rising relevance, UC is the most used method for bacterial EV purification to date (Dauros Singorenko et al., 2017; Monguió-Tortajada et al., 2019b, 2019a; Zhang et al., 2019a).

Our previous study demonstrated that the Gram-positive probiotic *P. freudenreichii* CIRM-BIA129 produces EVs with anti-inflammatory activity via NF-κB pathway modulation towards cultured human intestinal epithelial cells, together with EVs biophysical and proteomic characterization (Rodovalho et al., 2020b). We then showed that the properties of *P. freudenreichii*-derived EVs, including their anti-inflammatory activity and protein content, depended on the culture media in which the bacterium was grown (either UF, milk ultrafiltrate; or YEL, yeast extract-lactate) (Rodovalho et al., 2020a). In both studies, EVs were purified by SEC. However, as UC is the standard method for the purification of bacteria-derived EVs, we aimed to analyze whether this method is also suitable for the purification of *P. freudenreichii*-derived EVs and to compare their biophysical properties and protein content.

Here, we show that EVs were successfully obtained by the application of UC to the concentrated supernatants of *P. freudenreichii* CIRM-BIA129 cultures both in UF and YEL media. The comparison with SEC-purified EVs showed that their proteomic content varied, although it

was possible to identify a vesicular core proteome of 308 proteins, indicating that some functions are conserved in *P. freudenreichii*-derived EVs obtained in four different conditions. These conserved functions included carbon metabolism, peptidoglycan biosynthesis, ribosome, protein export, quorum sensing and immunomodulation. In addition to broadening the toolset for the purification of *P. freudenreichii*-derived EVs, this study identifies EV proteins that are conserved across different conditions of EV isolation, which could be relevant for the identification of biomarkers of purification quality and could also provide insights into mechanisms of EV biogenesis and cargo sorting.

2. Material and Methods

2.1. Culture conditions

The strain *Propionibacterium freudenreichii* CIRM-BIA 129 (ITG P20) was supplied by the CIRM-BIA Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRAE, Rennes, France). *P. freudenreichii* was cultured either in cow milk ultrafiltrate (UF) supplemented with 100 mM sodium lactate and 5 g L^{-1} casein hydrolysate or in yeast extract-lactate (YEL), both at 30°C, without agitation, until the beginning of the stationary phase (2 x 10^9 bacteria mL^{-1} for UF and 3 x 10^9 bacteria mL^{-1} for YEL) (Malik et al., 1968; Cousin et al., 2012c).

2.2. Purification of EVs

Bacterial cultures were centrifuged (6000 g, 15 min) and filtered (0.22 mm, Nalgene top filters, Thermo Scientific) at room temperature. Cell-free supernatants were then concentrated in successive centrifugations using Amicon ultrafiltration units (100-fold, 100 kDa, 2500 g). The concentrated supernatants were then submitted to a series of 3 ultracentrifugation rounds: 1) the first one to pellet EVs and discard the supernatant containing contaminant proteins (150,000xg, 120 min, 4°C), 2) the second one for a higher-quality density-based separation, with the application of the resuspended pellets onto the top of a discontinuous sucrose gradient (8-68%) (100,000xg, 150 min, 4°C), and 3) the third one to eliminate the excess of sucrose from pooled EV-containing fractions (150,000xg, 120 min, 4°C), a washing step using TBS buffer (150 mM NaCl; 50 mM Tris-Cl, pH 7.5) (Tartaglia et al., 2018, 2020). The final samples were then resuspended in TBS buffer and used immediately or stored at -20°C.

2.3. Biophysical characterization of EVs

The size and concentration of EVs were evaluated by nanoparticle tracking analysis (NTA), using a NanoSight NS300 instrument (Malvern Panalytical), equipped with a sCMOS camera and a Blue488 laser. All measures were performed at 25°C, in constant flux, with a syringe pump speed of 50. For each sample, 5 videos of 60 s were recorded, under camera level 15. Other parameters were adjusted accordingly to achieve image optimization (Vestad et al., 2017; Rodovalho et al., 2020b). Transmission electron microscopy (TEM) was performed to evaluate the morphology, homogeneity and integrity of EVs, as previously described (Tartaglia et al., 2018; Rodovalho et al., 2020b). Briefly, glow-discharged formvar-coated copper EM grids were used for the application of a drop of EV solution and the negative staining was conducted with

the application of 2% uranyl acetate to the grid. Between these steps, the grids were blotted with filter paper to remove the excess of solution. After drying, the grids were imaged using a Jeol 1400 transmission electron microscope (JEOL Ltd.) operating at 120 Kv.

2.4. Mass spectrometry and protein identification

The identification of proteins in EV samples was performed by in-gel trypsinolysis, peptide extraction and nano liquid chromatography tandem mass spectrometry (nano-LC-ESI MS/MS) analysis, as previously described (Gagnaire et al., 2015; Huang et al., 2016a; Gaucher et al., 2020a; Rodovalho et al., 2020b). The software X! TandemPipeline was used to identify peptides from MS/MS spectra (Langella et al., 2017) and the searches were performed against the genome of *P. freudenreichii* CIRM-BIA129 (Accession: NZ_HG975455). The database search parameters included trypsin cleavage, peptide mass tolerance set at 10 ppm for MS and 0.05 Da for MS/MS, and methionine oxidation as a variable modification. The E-value threshold for peptide identification was set to 0.05 and a minimum of two peptides was required for protein identification, resulting in a false discovery rate (FDR) of < 0.15%.

2.5. Bioinformatics analysis

Protein sequences were retrieved from NCBI GenBank (Accession: NZ_HG975455). Ortholog-based annotation was obtained with eggNOG-mapper (Huerta-Cepas et al., 2017, 2019), including the assignment to Clusters of Orthologous Groups (COG) categories and KEGG Pathways terms. Subcellular localization and lipoprotein signals were predicted with Cello2GO (Yu et al., 2014) and PRED-LIPO (Bagos et al., 2008), respectively. Venn diagrams, heatmaps and donut plots were constructed with Python libraries Pandas, Seaborn, Matplotlib_venn and Venn. Proteomic data for SEC-purified EVs were retrieved from our previous publication (Rodovalho et al., 2020a). Functional enrichment analysis were performed with g:profiler (Raudvere et al., 2019; Reimand et al., 2019), as previously described (Rodovalho et al., 2020a), using KEGG terms and adopting a significance threshold (adjusted P-value) of 0.05. Enrichment results were represented as an enrichment network, where enriched pathways were represented as the nodes and the overlaps among them (common proteins) as edges. The enrichment network was constructed from g:profiler results and visualized with EnrichmentMap (Merico et al., 2010) and Cytoscape (Shannon et al., 2003). For the network construction, a node FDR q-value threshold of 0.05 was applied for functional category filtering and a threshold of 0.375 was applied for the representation of the similarity between functional categories as edges.

2.6. Statistical analysis

All experiments were performed at least in triplicate and the numerical results are expressed as mean ± standard deviation, unless specified otherwise. For significance evaluation, Mann Whitney tests or one-way ANOVA followed by Tukey's multiple comparisons tests were performed.

3. Results

3.1. UC is a suitable method for the purification of *P. freudenreichii*-derived EVs

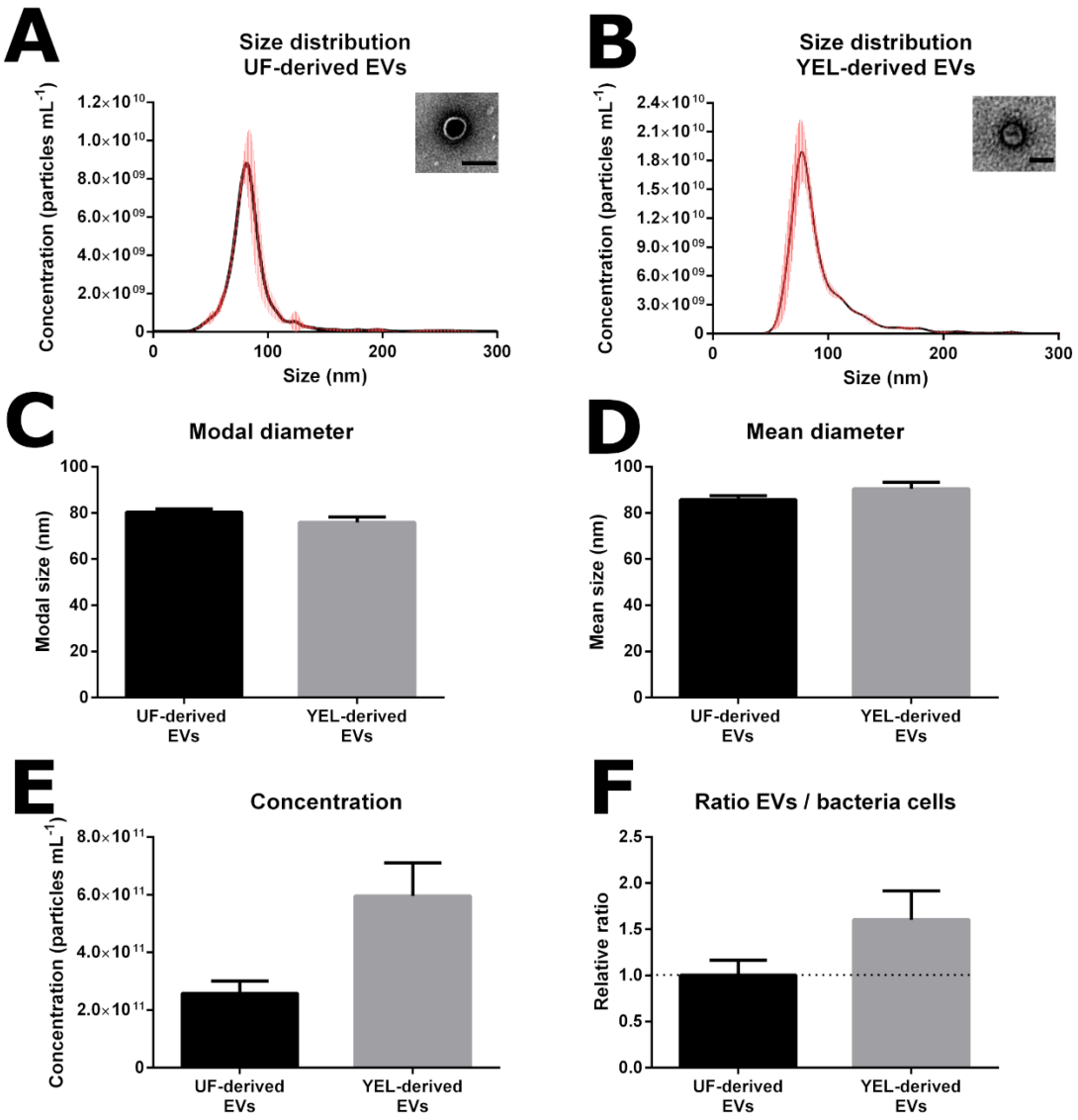

Figure 1. Biophysical characteristics of UC-purified EVs. A-B) Size distribution of EVs derived from A) UF and B) YEL media, as acquired from NTA measurements. Inset: TEM images of individual EVs, scale bar: 100 nm. C) Modal and D) mean diameter of UF- and YEL-derived EVs. E) Concentration of EVs recovered from cultures in UF and YEL media. F) Ratio between the amount of recovered EVs (obtained from NTA measurements) and bacterial cells (CFU counting) at sampling time, normalized relative to UF-derived EVs. Mann Whitney tests were performed, but no significative differences were found.

In this study, we evaluated whether *P. freudenreichii*-derived EVs could be purified by UC. Therefore, a series of UC steps were applied and the resulting samples were analyzed by NTA and TEM. The analysis confirmed the purification of EVs with typical characteristics. Regarding size distribution, both UF- and YEL-derived EVs presented a monodisperse profile (**Figure 1A-B**). Regarding morphology and integrity, TEM images showed intact and spherical structures (**Figure 1A-B, inset**). EV modal diameter did not varied significatively between UF-derived EVs (80.40 ± 1.308 nm) and YEL-derived EVs (75.87 ± 2.458 nm) (**Figure 1C**). A similar tendency was verified

for EV mean diameter, with 85.83 ± 1.704 for UF-derived EVs and 90.43 ± 2.875 for YEL-derived EVs (**Figure 1D**). Regarding EV recovery, although no statistically significative differences were verified, the concentration of was higher for YEL-derived EVs (5.950 x 10^{11} ± 1.6 x 10^{11}) than UF-derived EVs (2.587 x 10^{11} ± 4.1 x 10^{10}) (**Figure 1E**) and the relative ratio between the amount of recovered EVs and bacterial cells was 1.6 times higher for YEL-derived EVs, relative to UF-derived EVs (**Figure 1F**).

3.2. UC-purified EVs contain a diverse set of proteins

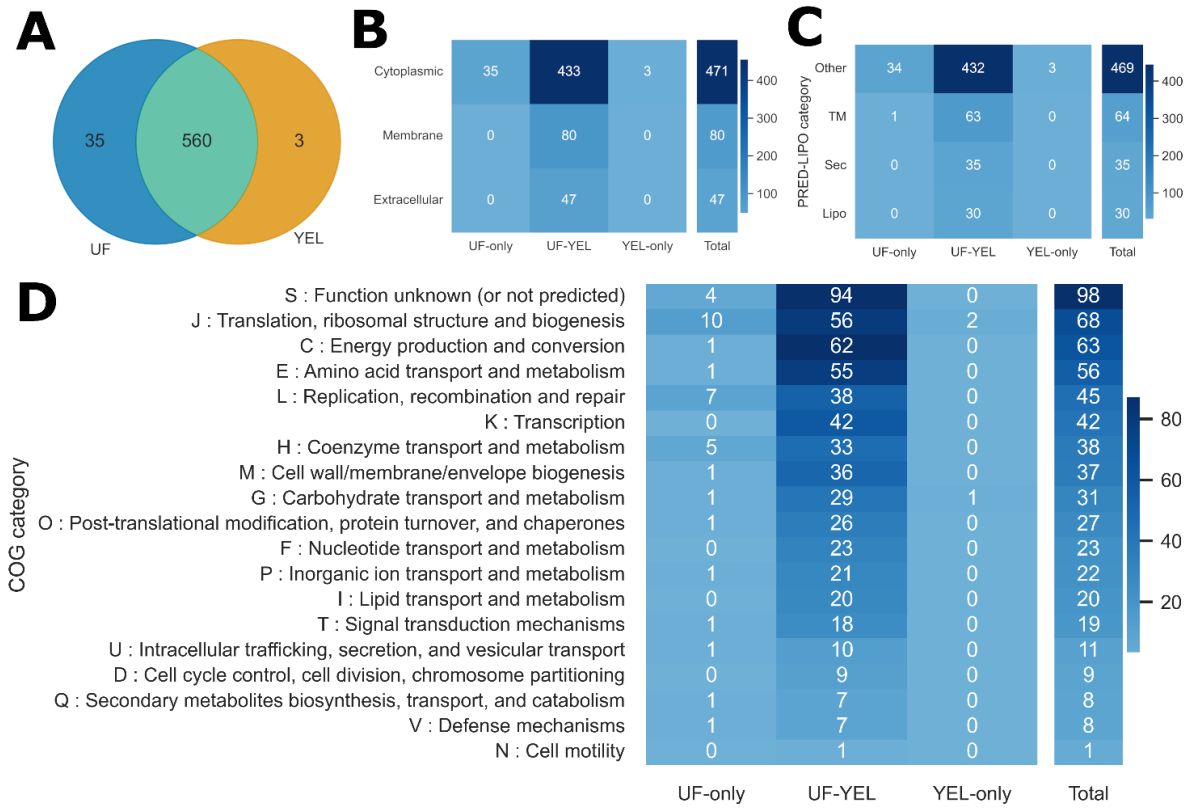

Figure 2. Proteomic profile of UC-purified EVs, according to culture media. A) Venn diagram presenting the number of proteins identified exclusively in UF- or YEL-derived EVs, or common to both conditions. B) Proteins subcellular localization, as predicted by Cello2GO. C) Lipoproteins prediction by LIPO-PRED. Sec: secretion signal peptide. Lipo: lipoprotein signal peptide. TM: transmembrane. Other: no signals found. D) COG categories assignment. B-D) UF-only: proteins exclusive to UF-derived EVs, YEL-only: proteins exclusive to YEL-derived EVs, UF-YEL: proteins common to both conditions.

UC-purified EVs were then biochemically analyzed, regarding their protein content. A total of 598 proteins were identified in UC-purified EVs, 560 of which were shared among UF- and YEL-derived EVs. Furthermore, 35 proteins were identified exclusively in UF-derived EVs, whereas 3 proteins were identified exclusively in YEL-derived EVs (**Figure 2A**). Regarding subcellular localization, the exclusive proteins of UF- and YEL-derived EVs were predicted to be cytoplasmic, whereas the proteins that were common to both conditions were mainly cytoplasmic (n=433), but also included some membrane (n=80) and extracellular (n=47) proteins (**Figure 2B**). Lipoprotein signals were identified only in proteins that were common to both conditions (UF- and YEL-derived EVs) (n=30) (**Figure 2C**). Regarding COG categories, YEL-

exclusive proteins related to translation (n=2) and carbohydrate metabolism (n=1), whereas UF-exclusive proteins were distributed among several categories, most notably translation (n=10), replication (n=7) and coenzyme metabolism (n=5). The proteins common to UF- and YEL-derived EVs were well distributed among COG categories, including mainly metabolism, translation and unknown functions (**Figure 2D**).

3.3. UC-purified EVs are less abundant than SEC-purified EVs

The biophysical properties of UC-purified EVs were then compared with those of SEC-purified EVs, of our previous study. Size distributions presented a monodisperse profile, although there were differences in modal sizes and particles concentration, particularly for SEC_UF condition (**Figure 3A**). Modal size for SEC_YEL group was higher than the others (**Figure 3B**). Particle concentration (**Figure 3C**) and relative EV yield (**Figure 3D**) presented similar tendencies, with SEC-purified EVs being more abundant than UC-purified EVs. SEC_UF condition resulted in a particularly large abundance of EVs, being drastically different from the other conditions (**Figure 3C-D**).

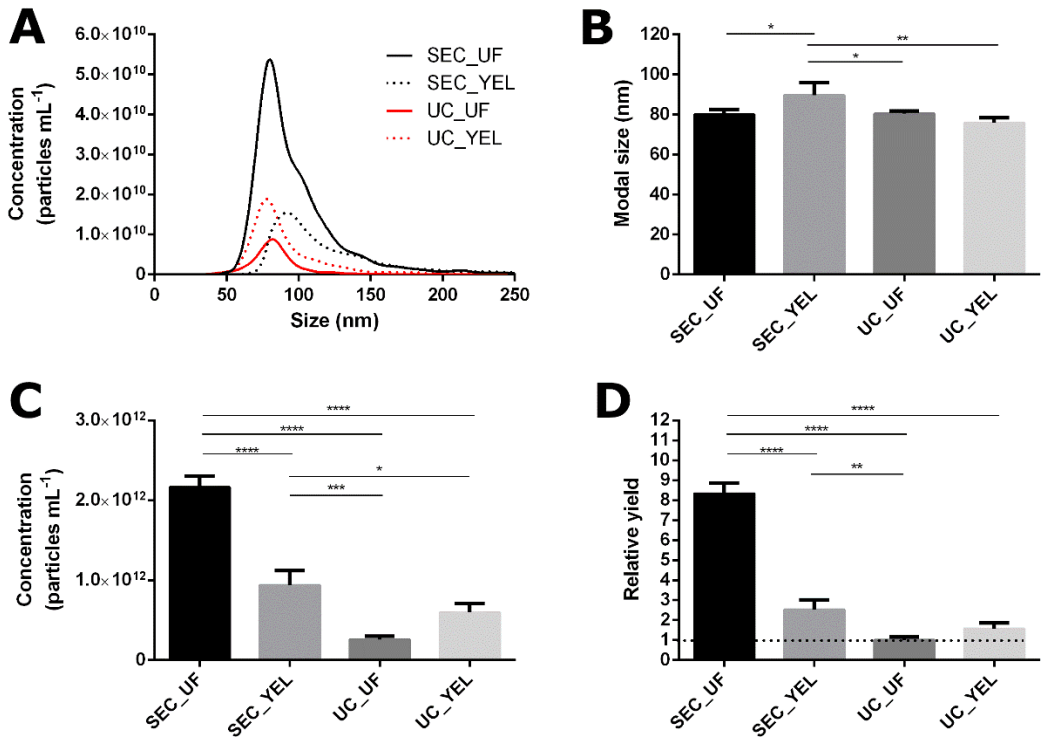

Figure 3. Comparison of the biophysical properties of UC- and SEC-purified EVs. **A**) Size (diameter) distribution of the particles in each condition. **B**) Modal diameter of the particles in each condition. **C**) Particles total concentration in the end of each purification condition. **D**) Particles relative yield in the end of each purification condition. For relative yield calculation, the concentration was divided by CFUs at starting cultures and normalized relative to UC_UF. **A-D**) UC_UF: UF-derived EVs purified by UC. UC_YEL: YEL-derived EVs purified by UC. SEC_UF: UF-derived EVs purified by SEC. SEC_YEL: YEL-derived EVs purified by SEC. Data were acquired from NTA measurements in this study (UC-purified EVs) and our previous study (SEC-purified EVs). Asterisks indicate statistical significance as evaluated by one-way ANOVA followed by Tukey's multiple comparisons tests: ****, $P \leq 0.0001$; ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$.

3.4. UC- and SEC-purified EVs share a core proteome of hundreds of proteins

Next, we compared the proteomic content of UC-purified EVs from this study with our previous analysis of the same strain, when EVs were purified by SEC. Therefore, we compared 4 conditions: UF-derived EVs purified by UC (UC_UF), YEL-derived EVs purified by UC (UC_YEL), UF-derived EVs purified by SEC (SEC_UF) and YEL-derived EVs purified by SEC (SEC_YEL). We found out that although some proteins were exclusive to each condition or common to some conditions, 308 proteins were identified in all 4 conditions, comprising the core proteome of the EVs derived from *P. freudenreichii* CIRM-BIA129 (**Figure 4**). Likewise, 302 proteins were identified in more than one condition, but not in all conditions, comprising the accessory proteome (**Figure 4B**). Notably, it included 273 growth-condition independent proteins that were specific to the EV purification method (46 for SEC and 227 for UC). Finally, 42 proteins were restricted to one of the conditions, comprising the exclusive proteome (**Figure 4B**).

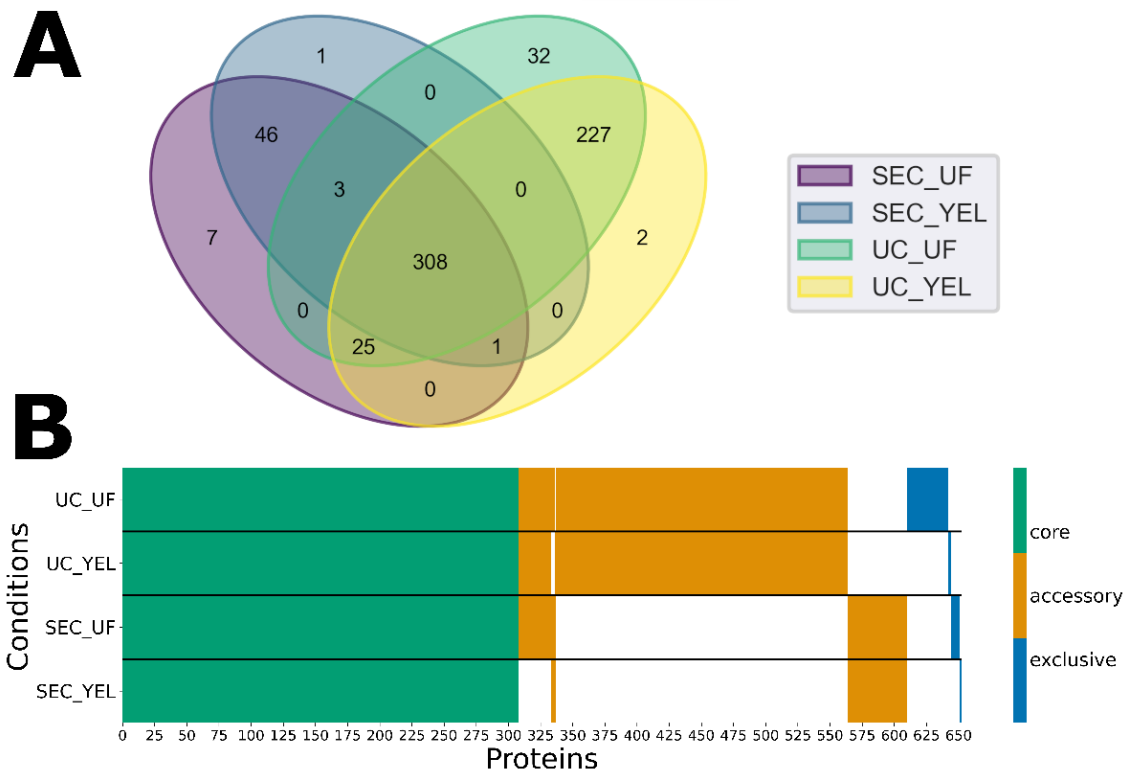

Figure 4. Proteins distribution according to condition of EV obtention. A) Venn diagram presenting the number of proteins per condition and intersections. B) Heatmap representing the presence (non-white) or absence (white) of all the analyzed proteins in each condition of EV obtention. Core: core proteome, proteins present in all 4 conditions. Accessory: accessory proteome, proteins present in 2 or 3 conditions. Exclusive: exclusive proteome, proteins present in only 1 condition. A-B) UC_UF: UF-derived EVs purified by UC. UC_YEL: YEL-derived EVs purified by UC. SEC_UF: UF-derived EVs purified by SEC. SEC_YEL: YEL-derived EVs purified by SEC.

3.5. The core proteome of *P. freudenreichii*-derived EVs is mainly related to metabolic functions

In order to understand what are the conserved features of the protein content of the EVs derived from *P. freudenreichii* CIRM-BIA129, we further analyzed their core proteome. The assignment of COG categories showed that almost half of the proteins were related to metabolic processes, although some were related to cellular processes, like cell envelope biogenesis; and information storage and processing, including replication, transcription and translation (**Figure 5A**). Regarding subcellular localization, almost three quarters of the proteins were predicted to be cytoplasmic (70.5%), although membrane (17.5%) and extracellular (12%) proteins were also identified (**Figure 5B**). Lipoprotein signals were present in only 8.1% of the proteins, whereas transmembrane (12.7%) and secretion (7.5%) signals were also identified (**Figure 5C**). Functional enrichment analysis with KEGG terms demonstrated these proteins were mainly related to central carbon metabolism, but also implicated in peptidoglycan biosynthesis, ribosome, protein export and quorum sensing (**Figure 5D**).

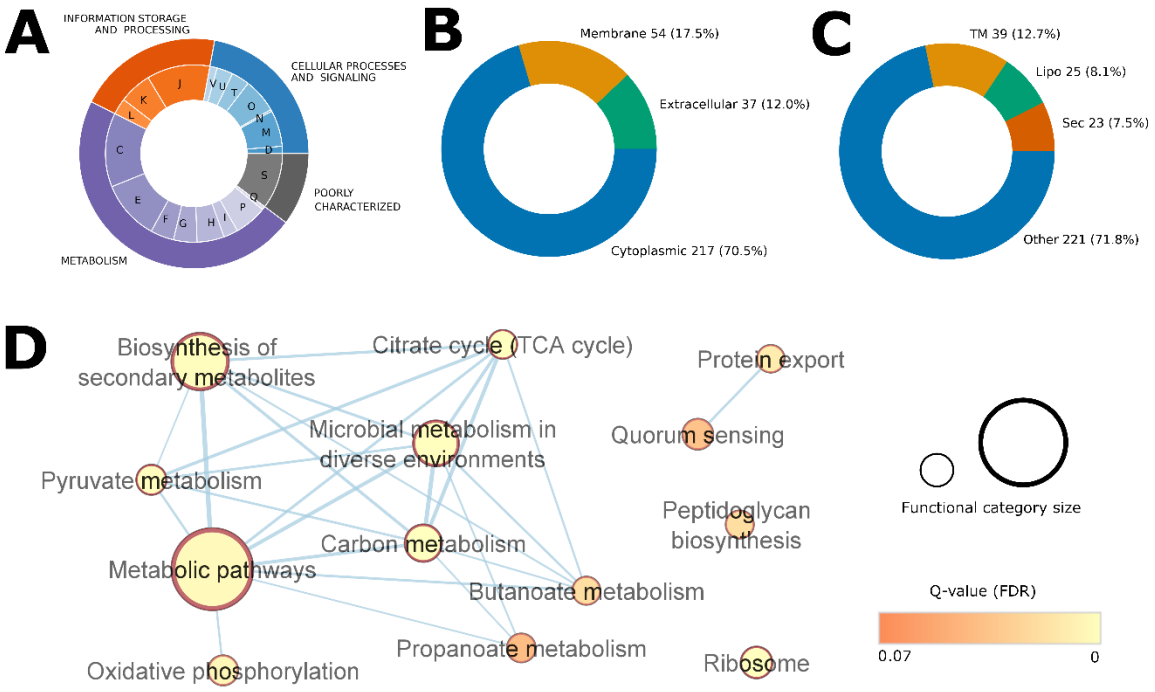

Figure 5. Functional and spatial features of the core proteome. A) Distribution of COG categories. B) Protein subcellular localizations, as predicted by Cello2GO. C) Lipoproteins prediction by LIPO-PRED. Sec: secretion signal peptide. Lipo: lipoprotein signal peptide. TM: transmembrane. Other: no signals found. D) Enrichment map obtained from functional enrichment analysis of KEGG Pathways terms. Node sizes are proportional to functional category set size. Edges indicate shared proteins among the linked sets. Color is related to statistical significance (Q-value).

Furthermore, some of the proteins identified in the EV core proteome were previously identified as immunomodulatory in studies with strains of *P. freudenreichii* (**Table 1**).

Table 1. Proteins from the EV core proteome that were identified as immunomodulatory in other studies with strains of *P. freudenreichii*.

Protein ID	GI ID	Accession	Description	COG	Localization	Lipoprotein
Eno1	659917660	CDP48974.1	Enolase 1	G	Cytoplasmic	Other
Acn	659918109	CDP48708.1	Aconitase	C	Cytoplasmic	Other
GroL2	659917458	CDP49125.1	60 kDa chaperonin 2	O	Cytoplasmic	Other
SlpE	659917805	CDP48858.1	Surface layer protein E	O	Extracellular	Sec
SlpB	659918413	CDP48273.1	Surface layer protein B	O	Extracellular	Sec
PFCIRM129_10785	659917415	CDP49252.1	Hypothetical protein	?	Membrane	Lipo

Legend: Sec: secretion signal peptide, Lipo: lipoprotein signal peptide, TM: transmembrane, Other: no signals found, ?: unknown. References: Deutsch et al., 2017; Do Carmo et al., 2017, 2019.

3.6. The accessory proteome of *P. freudenreichii*-derived unveils proteins exclusive to each purification method

In addition to the core proteome, we also evaluated the accessory proteome, which correspond to proteins present in more than one, but not all conditions. We focused on proteins that were found exclusively on UC-purified or SEC-purified EVs, in order to evaluate differences related to purification methods. Regarding COG categories, the proteins exclusive to both conditions of SEC-purified EVs were mostly related to metabolism, particularly energy, carbohydrate and amino acids metabolism (**Figure 6A**). UC-purified EVs were more distributed among other COG categories (**Figure 6A**). Furthermore, there were proportionally more cytoplasmic proteins in UC-purified EVs and more membrane proteins in SEC-purified EVs (**Figure 6B-C**).

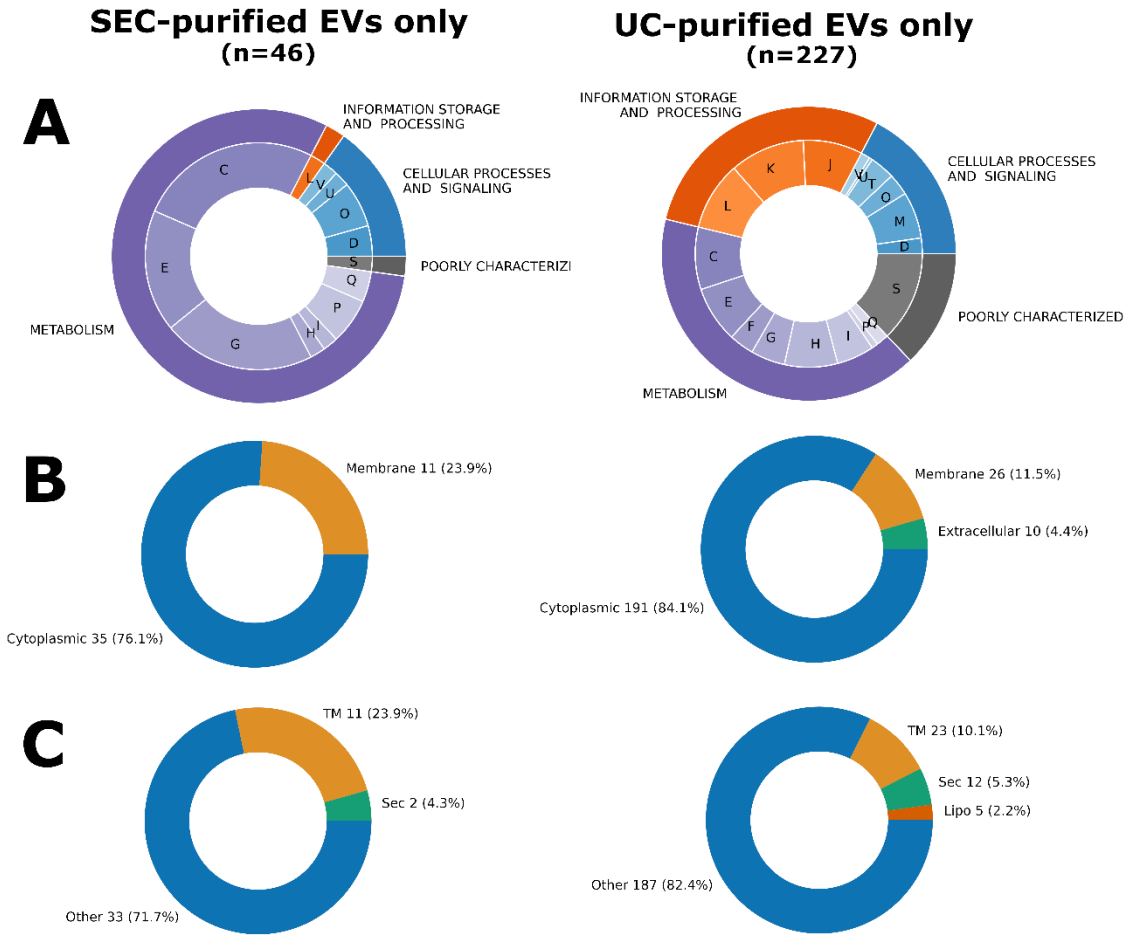

Figure 6. Characteristics of the proteins exclusive of SEC-purified (left panel) and UC-purified (right panel) EVs. A) Distribution of COG categories. B) Protein subcellular localizations, as predicted by Cello2GO. C) Lipoproteins prediction by LIPO-PRED. Sec: secretion signal peptide. Lipo: lipoprotein signal peptide. TM: transmembrane. Other: no signals found.

4. Discussion

We previously purified *P. freudenreichii*-derived EVs from cultures in UF and YEL by SEC method (Rodovalho et al., 2020b, 2020a). In this study, we report their purification using UC method, both from cultures in UF and YEL media. UC-based purification uses high-speed centrifugation to separate EVs and contaminants by differential sedimentation, whereas SEC is based on the differential elution of EVs and contaminants through a porous polymeric matrix with specific molecular weight cut-off (Klimentová and Stulík, 2015; Monguió-Tortajada et al., 2019a; McNamara and Dittmer, 2020). SEC is an approach of rising relevance, scalable and fast, that preserves EV structure and activity, although it has limitations in sample volume and co-purification of particles of similar sizes, such as viruses (Nordin et al., 2015; Benedikter et al., 2017; Mol et al., 2017; Monguió-Tortajada et al., 2019a; McNamara and Dittmer, 2020). At the same time, UC is time-consuming and operator-dependent, with risks of inducing EV aggregation and damaging, although it results in reliable high-purity samples, being the most used approach for bacterial EVs purification (Mol et al., 2017; Monguió-Tortajada et al., 2019a). Each method presents advantages and drawbacks, and its application should account for the complex tradeoff

between higher EV yields and less amounts of contaminants (Dauros Singorenko et al., 2017). Anyhow, as confirmed by TEM images and NTA measurements, it is possible to recover EVs of typical nanometric sizes and spherical cup-shaped morphology using UC-based purification, similarly to what we previously described for SEC-based purification. Therefore, UC is also a suitable method for the purification of *P. freudenreichii*-derived EVs.

For biophysical properties of UC-purified EVs, there was no difference between UC_UF and UC_YEL EVs regarding modal diameter and only a subtle difference regarding EV abundance relative to bacterial cells, yet not significative. Contrastingly, our study with SEC-purified EVs showed that SEC_YEL EVs were larger and less abundant than SEC_UF EVs (Rodovalho et al., 2020a). Therefore, important differences were identified between EVs purified by different methods, regarding EV abundance. SEC-purified EVs were more abundant than UC-purified EVs, particularly for UF-derived EVs. The differences in abundances between the two purification methods might be a direct consequence of the efficiency of each method for EV recovery, considering that lower EVs yield may be a downside of the efforts for minimization of contaminant proteins in EV preparations. The differences in sizes for SEC_UF condition could be due to factors such as the purification of different EVs subpopulations by each method, with distinct biophysical and biochemical properties (Dauros Singorenko et al., 2017; Gho and Lee, 2017), the modulation of EVs properties triggered by UF culture medium, or a combination of both factors. Another possibility is that the purification methodologies could trigger physical deformations, particularly for UC-based methods (Mol et al., 2017; Monguió-Tortajada et al., 2019a), thus altering EVs sizes and abundances. Although the purification methods impacted on EVs biophysical properties, there were no abnormal differences and EVs with typical biophysical properties were retrieved in all the four analyzed conditions.

Similar to what we previously verified for SEC-purified EVs, bacteria growth conditions also modulated the protein content of UC-purified EVs. There were 35 proteins exclusive to UC_UF EVs and 3 proteins exclusive to UC_YEL EVs, although the majority of 560 proteins were common to both conditions of EV preparation. In our previous study with SEC-purified EVs, 32 proteins were exclusive to SEC_UF EVs and 1 protein was exclusive to SEC_YEL EVs, with 358 proteins common to both conditions (Rodovalho et al., 2020a). Therefore, although a similar distribution was verified among exclusive and common proteins, 50% more proteins were identified in UC-purified EVs, in comparison to SEC-purified EVs. The proteins exclusively found associated to UC-purified EVs did not show apparent functional particularities regarding COG categories, but were predicted to be of cytoplasmic origin in a higher proportion than SEC-purified EVs. Again, the reason could be the purification of distinct EVs subpopulations by the two methods, which would result in protein content variations (Dauros Singorenko et al., 2017; Gho and Lee, 2017). Another possibility is that UC-based purification could be less efficient for *P. freudenreichii*-derived EVs, thus resulting in the co-purification of more contaminant proteins relative to SEC-based purification (Mol et al., 2017), as suggested by the increased number of cytoplasmic proteins, which could be a result of cell lysis. However, cell lysis also triggered EV biogenesis in bacteria (Turnbull et al., 2016; Toyofuku et al., 2017), which hinders such comparisons. Further studies with the optimization of parameters such as molecular weight cutoff for SEC and density gradient composition for UC, as well as single EV characterization, could elucidate the occurrence of EVs subpopulations and critically evaluate the purity of the samples (Dauros Singorenko et al., 2017; Gho and Lee, 2017; Mol et al., 2017).

Nonetheless, 308 proteins were consistently identified in EVs from both purification methods and growth conditions, indicating that a particular set of proteins – the EV core proteome – is invariably present in *P. freudenreichii*-derived EVs, considering the four analyzed conditions. This is a robust and representative set of proteins to be used in further analysis of *P. freudenreichii* EV proteome, since it is less susceptible to purification and growth biases. The initial characterization of this core proteome showed that some proteins predicted to be extracellular or associated to the membrane were identified, although the majoritarian composition was of cytoplasmic proteins (more than 70%), which is consistent with several proteomic analysis of EVs derived from Gram-positive bacteria (Lee et al., 2009; Brown et al., 2015; Kim et al., 2015b; Briaud and Carroll, 2020).

Regarding the functional aspects of the core proteome, some of the identified proteins were previously associated to immunomodulation in *P. freudenreichii*. Those included Enolase 1 (Eno1), Aconitase (Acn), 60 kDa chaperonin 2 (GroL2), Surface-layer proteins B (SlpB) and E (SlpE), and a hypothetical protein (PFCIRM129_10785) (Deutsch et al., 2017; do Carmo et al., 2019). Importantly, we demonstrated that SlpB was partly involved in the immunomodulatory activity of SEC-purified EVs (Rodovalho et al., 2020b), as its key role in the interaction with the host has been demonstrated for the bacterial cells as well (do Carmo et al., 2017, 2019). Whether UC-purified EVs also present an immunomodulatory activity should be further investigated, but the identification of these proteins in the core proteome is a promising evidence. Moreover, proteins with functions related to peptidoglycan metabolism were identified in the core proteome, including the transferases MurG (659917354, CDP49364.1) and MurA (659917556, CDP49091.1); endopeptidases such as the secreted cell-wall peptidase of the NlpC/P60 family (659918631, CDP48034.1) and the hypothetical proteins PFCIRM129_03060 (659918230, CDP48413.1) and PFCIRM129_10650 (659917426, CDP49227.1); the transpeptidases cell division protein FtsI (659917360, CDP49370.1) and penicillin-binding protein A (659916821, CDP49790.1); and the carboxypeptidase (659916853, CDP49718.1). These identifications reinforce a hypothesis for EVs biogenesis in Gram-positive bacteria, that involves cell-wall remodeling via enzymatic action (Toyofuku et al., 2017; Briaud and Carroll, 2020). The core proteome was also enriched in metabolic enzymes and ribosomes components, which could have a role as public goods for the bacterial population, serving as metabolic and structural complements for individual bacterial cells (Rakoff-Nahoum et al., 2014; Valguarnera et al., 2018). In other environments, such as dairy matrices with multiple strains or the human gastrointestinal tract, the export of such proteins might exert a crucial role in adaptation and interspecies interactions (Rakoff-Nahoum et al., 2014; Liu et al., 2018b). That is also true for other enriched terms in the core proteome, such as protein export and quorum sensing, which also mediate bacterial interactions and comprise transporter and signal recognition proteins with important roles in adaptation.

Overall, we demonstrated that, similar to SEC, UC is also suitable for the purification of EVs from cultures of *P. freudenreichii* in UF and YEL media. Moreover, UC-purified EVs presented biophysical properties that were less variable according to growth conditions than those of SEC-purified EVs. Nonetheless, the protein content varied according to growth conditions in UC-purified EVs, and their protein content was more extensive than SEC-purified EVs. Finally, the combination of the proteomic dataset from the four studied conditions allowed the identification of 308 invariably occurring proteins. This core proteome comprises a more

representative dataset of the proteins from *P. freudenreichii*-derived EVs and probably relates to their essential roles, including immunomodulation, carbon metabolism, peptidoglycan biosynthesis, ribosome, protein export and quorum sensing. The further analysis and complementation of this core proteome are promising for the elucidation of key aspects of *P. freudenreichii*-derived EVs, including mechanisms of biogenesis, cargo sorting and interactions with the host.

Chapter 6 – General discussion and perspectives

The ubiquitous production of EVs and their role as an export system for inter-kingdom communication are being increasingly reported in the scientific literature (Gill et al., 2019; Woith et al., 2019). Here, we demonstrated that the Gram-positive probiotic *P. freudenreichii* also produces EVs with immunomodulatory role in human-bacteria communication. Our study focused on the biophysical and proteomic characterization of *P. freudenreichii*-derived EVs, as well as their functional investigation with interactomics predictions and *in vitro* immunological assays. Furthermore, we demonstrated that bacteria growth conditions and EV purification methods impact on EVs properties.

1. What are the mechanisms of EV-mediated immunomodulation?

Regarding the functional investigations, our data showed that SEC-derived EVs exerted an anti-inflammatory role towards cultured human intestinal epithelial cells, via the reduction of NF-κB activity and of IL-8 release. Moreover, this reduction was verified specifically when cells were induced to an inflammatory state by bacterial LPS. Therefore, we supposed that EVs acted on points of the NF-κB pathway that are induced specifically by LPS, and not by the other tested inductors (TNFα and IL-1β). As members of the LPS-induced NF-κB pathway, the human proteins toll-like receptor 4 (TLR4) and TIR domain-containing adapter molecule 1 (TICAM-1) were regarded as potential targets for EV-mediated immunomodulation. TLR4 is a transmembrane protein, whereas TICAM-1 is a cytoplasmic protein. Thus, it is also important to determine whether EVs act intracellularly or extracellularly, relative to the receptor cells.

EVs were reported to interact with surface proteins from the receptor cells, docking to the cell surface (French et al., 2017). Accordingly, *P. freudenreichii*-derived EVs could interact with the extracellular domain of TLR4 via EV surface components (e.g., proteins) or other cytoplasmic components after EV lysis. However, our data showed that EV surface proteolysis did not abolish their immunomodulatory roles, suggesting that the second option is more probable. Bacterial EVs were also reported to entry into eukaryotic cells via different mechanisms, including direct membrane fusion and endocytosis (O'Donoghue and Krachler, 2016; Bajic et al., 2020). Therefore, EV cargo could mediate the immunomodulation after entry into the host cell, interacting with the intracellular domain of TLR4 or with the protein TICAM-1. Another possibility is a more indirect role, in which EVs could regulate immunomodulation in other ways and the negative modulation of NF-κB pathway would be rather a consequence. For all that, further studies are necessary to elucidate the precise mechanisms of action of *P. freudenreichii*-derived EVs, including *in silico* structural approaches to analyze PPIs in higher resolution (Keskin et al., 2016) and experimental approaches to investigate if EV

integrity is required for cargo delivery (Bonsergent and Lavieu, 2019), fluorescence tracking of internalization and cargo delivery (Ofir-Birin et al., 2018), as well as inhibition of specific uptake pathways in eukaryotic cells (Bajic et al., 2020). Nonetheless, as the mechanisms of immunomodulation are not fully elucidated for *P. freudenreichii* bacterial cells, our study with *P. freuderenichii*-derived EVs opens new perspectives. Whether *P. freudenreichii* also signals through the same pathway as its EVs constitutes a basis for further studies.

2. Which EVs effectors are implicated in immunomodulation?

Regarding the bacterial effectors of EV-mediated immunomodulation, our data indicated that SlpB protein is partly involved, since EVs derived from the slpB mutant presented a less intense reduction of NF-κB activity. That is in accordance with the crucial role of SlpB in the bacterium-mediated immunomodulation (do Carmo et al., 2019). However, as EV-mediated reduction in NF-κB activity was not completely abolished without SlpB, other proteins might be involved. Our analysis that coupled interactomics predictions, quantitative proteomics and functional assays comparing UF- and YEL-derived EVs purified by SEC resulted in a small list of bacterial proteins that could interact directly with TLR4 and/or TICAM-1, thus mediating the inhibition of LPS-induced NF-κB pathway. This list includes pyruvate synthase/pyruvate-flavodoxin oxidoreductase (NifJ1), the NADH-quinone oxidoreductase chain G (NuoG), an uncharacterized protein (PFCIRM129_01355), GtfB glycosyltransferase (GtfB), a putative DNA polymerase I (PolA), and the UvrABC system protein A (UvrA3). Moreover, other proteins previously regarded as immunomodulatory in *P. freudenreichii*, such as surface layer protein E (SlpE) and chaperonin GroL2 (GroL2) (Deutsch et al., 2017), are part of the vesicular core proteome, indicating it could also be implicated in EV-mediated immunomodulation. Further studies with mutants or antibody targeting could elucidate the importance of specific proteins, as well as bioinformatics structural analysis of the predicted interactions. Moreover, although our analyses focused on proteomic investigations, it is also possible that molecules other than proteins could be implicated in EV-mediated immunomodulation. As an example, the DHNA produced by *P. freudenreichii* also inhibited the production of proinflammatory cytokines in mice (Okada et al., 2013) and could be exported by EVs. Furthermore, short chain fatty acids could also be implicated in immunomodulation (Morrison and Preston, 2016) and exported via EVs. Nucleic acids were also reported to be associated to bacterial EVs and to participate in host-bacteria interactions, particularly sRNAs with regulatory roles (Dauros-Singorenko et al., 2018; Ahmadi Badi et al., 2020; Rodriguez and Kuehn, 2020). Therefore, other approaches should be applied to fully characterize the content of *P. freudenreichii*-derived EVs, possibly unveiling the presence of nucleic acids, carbohydrates and lipids.

Regarding the cellular model used for *in vitro* assays, colon adenocarcinoma HT-29 cells were chosen, since this type of cells is constituent of the human gastrointestinal tract mucosa, modelling the host-bacteria interaction environment. However, other types of cultured intestinal epithelial cells could be equally applied for these investigations, such as human Caco-2, T84, SW480, H4 and other animal cellular models (Bourgine et al., 2012; Ponce de León-Rodríguez et al., 2019). Furthermore, the effect of *P. freudenreichii*-derived EVs in systemic inflammation could also be studied with co-culture systems including immune cells like human peripheral blood mononuclear cells (PBMC) and macrophage cell lines (THP-1, RAW 264.7) (Ponce de León-Rodríguez et al., 2019). Additionally, inflammatory biomarkers other than pro-inflammatory cytokine IL-8 could be monitored, such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17a, IFNγ, TNFα and GM-CSF. Those further experiments would allow a better glimpse of the mechanisms of immunomodulation exerted by *P. freudenreichii*-derived EVs.

3. Would EVs exert immunomodulatory roles *in vivo*?

As our functional investigation was restricted to *in vitro* approaches, it is tempting to wonder what would be the relevance of *P. freudenreichii*-derived EVs *in vivo*. Several studies demonstrated that *P. freudenreichii* cells exerted immunomodulatory roles *in vivo* (Foligné et al., 2010; do Carmo et al., 2019; Ma et al., 2020), and there is also evidence for *in vivo* immunomodulation mediated by probiotic-derived EVs (Al-Nedawi et al., 2015; Kim et al., 2018b; Yamasaki-Yashiki et al., 2019; Choi et al., 2020b), particularly the improvement of inflammatory conditions (Kang et al., 2013; Fábrega et al., 2017; Seo et al., 2018; Ashrafian et al., 2019). Moreover, bacterial EVs were successfully applied *in vivo* for vaccines, drug delivery, bioimaging and diagnosis (Gujrati et al., 2014b, 2019; Carvalho et al., 2019a, 2019b; Kim et al., 2020a). Therefore, it is possible that *P. freudenreichii*-derived EVs exert anti-inflammatory roles *in vivo* and find promising applications as well. That would be relevant for pharmacokinetic and pharmacodynamic aspects of *P. freudenreichii* probiotic effect, since EVs can reach to distant sites of the organism, relative to the gastrointestinal tract, and mediate probiotic effects in the gut-brain axis (Jang et al., 2015; Yaghoubfar et al., 2020). Moreover, the vectorization in food matrices has shown a protective effect for *P. freudenreichii* cells (Plé et al., 2015; Rabah et al., 2018b, 2018a), and that should be considered for *in vivo* administration of EVs as well, with the potential to enhance EV stability and develop enriched dairy products. Finally, EV concentration might be a key variable for *in vivo* experiments, and low-yield EV purification methods could hinder these studies. Indeed, our investigations showed important differences in EV recovery yields according to the purification method. Therefore, improved purification methods and nanotechnological strategies for EV accumulation in the desired sites should be developed.

Further studies should also consider comparisons between bacteria- and EVs-mediated probiotic effect, notably regarding their anti-inflammatory properties, and

what would be the advantages and drawbacks of using EVs for *in vivo* applications. Accordingly, further studies should be conducted to determine the *in vivo* relevance of *P. freudenreichii*-derived EVs, accounting for optimizations of administration and vectorization schemes, higher-yield purification methodologies and possible advantages over bacteria direct use.

4. What other roles could EVs play in human-bacteria interaction?

Although our study focused in the immunomodulatory role of *P. freudenreichii*-derived EVs, other potential functionalities should be addressed. Indeed, our interatomics analysis predicted interactions with human proteins involved in diverse functional modules, including metabolism, signal transduction and infectious diseases. The last one could be related to the anti-pathogenic activity of *P. freudenreichii*, previously associated to cell-free supernatants (Nair and Kollanoor Johny, 2018). Indeed, this activity could also be associated to some protein components of *P. freudenreichii*-derived EVs. Furthermore, some predicted interactions mapped to human proteins related to cancer. Although the anti-cancer activity of *P. freudenreichii* was attributed to short-chain fatty acids (Jan et al., 2002; Cousin et al., 2016), in addition to possibly contain these molecules, EVs could also contain proteins implicated in this anti-cancer activity. Interestingly, some predicted interactions mapped to the KEGG term "Cellular community – eukaryotes", which comprises focal adhesion, adherence junction, tight junction and gap junction. Therefore, it is also possible that *P. freudenreichii*-derived EVs have a role in intestinal barrier function, as evidenced for other probiotics or probiotic-derived EVs (Blackwood et al., 2017; Chelakkot et al., 2018). Additionally, some interactions mapped to neurodegenerative diseases and could offer insights of potential distal roles for *P. freudenreichii*-derived EVs, encompassing the gut-brain axis, as showed for other probiotic-derived EVs (Jang et al., 2015; Yaghoubfar et al., 2020). In spite of lacking experimental validation, these interactomics predictions offer several new approaches for investigating and understanding some aspects of the probiotic activity of *P. freudenreichii*.

In addition to *in silico* interactomics, experimental approaches could also allow the identification of novel aspects of *P. freudenreichii* EV-mediated beneficial effects. The human cellular models used for *in vitro* assays, as well as other proposed cellular models and tissues derived from *in vivo* experiments could be submitted to RNA profiling, in order to detect differentially expressed genes after EV treatment. Then, enrichment analysis would identify regulated functional modules, providing insights into a broader EV effect. For *P. freudenreichii* cells, there are evidences of the modulation of aryl hydrocarbon receptor (AhR) pathway by DHNA, with implications on metabolic detoxification and inflammation regulation (Fukumoto et al., 2014). Moreover, gut microbiota bacteria were also showed to regulate peroxisome proliferator-activated receptor γ (PPAR-γ) pathway, via the production of short-chain fatty acid butyrate, with

implications in the improvement of irritable bowel syndrome in mice (Byndloss et al., 2017; Nozu et al., 2019). Therefore, as a producer of short chain fatty acids and DHNA, it is possible that *P. freudenreichii* and its EVs may regulate other pathways in the eukaryotic cells, such as AhR and PPAR-γ pathways. Transcriptomics and proteomics profiling of the eukaryotic cells treated with EVs, as well as cellular reporter assays may allow the identification of such regulated pathways.

5. What roles could EVs play in bacteria-bacteria interaction?

Besides the host-bacteria interactions, EVs may also play a role in bacteria-bacteria interactions. That is evident in the case of intestinal bacteria communities that share EVs containing enzymes for the hydrolysis of complex polysaccharides, in such a way that specific species or strains provide molecules to serve as public goods for the community (Elhenawy et al., 2014; Rakoff-Nahoum et al., 2014; Lynch and Alegado, 2017). Since *P. freudenreichii* was identified in fecal samples of preterm breastfed infants (Colliou et al., 2017), it is possible that this species integrates the human intestinal microbiota and its EVs may provide metabolic enzymes for the community. Accordingly, a great part of the proteins identified in our proteomics approach was related to metabolic functions. Moreover, since *P. freudenreichii* is known for its bifidogenic property, mediated by DHNA and other bifidogenic factors (Mori et al., 1997; Isawa et al., 2002; Kouya et al., 2007), it is reasonable to suppose that these molecules may be exported via EVs to support the growth of bifidobacteria in the human gut. It is also possible that EVs mediate bacteria-bacteria interactions in other environments, such as dairy matrices. Since combinations of strains have been successfully used for dairy products (Plé et al., 2016; Cordeiro et al., 2018), it is possible that EV-mediated communication occur between the bacterial communities within these products, supporting the growth and fermentation processes of other bacteria. Additionally, EVs may represent a way of nutritional enrichment in such products, if they are demonstrated to contain molecules such as vitamin B12, which are produced by *P. freudenreichii* (Chamlagain et al., 2018; Piwowarek et al., 2018a). All these potential applications and functional attributions require additional experiments to characterize the diversity of EV content, and their roles and mechanisms of interactions in bacterial communities, including omics profiling and cargo tracking.

6. What environmental and technological aspects impact EVs properties?

Beyond functional investigations, our data also demonstrated that EV production and properties might be modulated by environmental conditions, specifically bacteria growth media (UF and YEL). That is in accordance with other studies, that demonstrated a great variability of EVs properties obtained from different growth conditions (Choi et

al., 2014; Lynch et al., 2019). Moreover, some studies demonstrated varying EVs properties or increased production according to bacteria growth phases (Tashiro et al., 2010b; Kim et al., 2016b; Taboada et al., 2019; Zavan et al., 2019) or stress conditions, such as nutrient shortage, antibiotics, osmotic and acid stress (Prados-Rosales et al., 2014; Chan et al., 2017; Lee et al., 2018; Gerritzen et al., 2019; Jun et al., 2019; Lynch et al., 2019). EVs biogenesis was also sometimes considered a type of stress response (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013; Schwechheimer et al., 2014). Our data reinforce these observations, since the condition with higher EV production (UF medium), also presented higher abundance of EV-associated stress proteins. Therefore, it is possible that stress factors could enhance EV production in *P. freudenreichii*, also modulating their properties. Altogether, these observations provide the opportunity to rationally optimize EVs content and improve their anti-inflammatory roles via the management of environmental conditions during bacteria growth, which could facilitate large-scale and inexpensive production of therapeutic EVs. Nevertheless, further experiments should be conducted to study how other growth variables and stressful environmental conditions relate to the properties of *P. freudenreichii*-derived EVs.

The method of choice for EV purification also impacted on the properties of *P. freudenreichii*-derived EVs, particularly the protein content. We first used SEC as a purification method, which is a size-based, high-quality, straightforward and gentle method, that preserves EVs integrity and properties (Gámez-Valero et al., 2016; Monguió-Tortajada et al., 2019a). However, UC-based methods are the most used in bacterial EV purification, particularly those incorporating ultracentrifugation rounds with density gradients, although ultracentrifugation may be time-consuming and cause damages and aggregation in EVs (Monguió-Tortajada et al., 2019a). We compared the protein content of EVs obtained in both ways. As no purification method is perfect, there might always be a tradeoff between the recovery of EVs and a certain number of contaminating proteins. Moreover, as the methods are based in different approaches (size or density separation), each one might recover different subpopulations of EVs. Therefore, it is not unexpected that some proteins were exclusive to certain conditions. Nonetheless, hundreds of proteins were conserved in EVs obtained in all the studied conditions, indicating that some proteins (the EV core proteome) might be invariably sorted into EVs. Indeed, these proteins might be involved in essential processes for EVs biogenesis and for their functions in intercellular communication. Although we identified a conservation in EV protein content across different conditions of EV obtention, is also important to account for that variability at the species level, investigating how strain variability affects EV properties. That is particularly important because the immunomodulation exerted by *P. freudenreichii* is strain-dependent (Deutsch et al., 2017), and that could also be the case of *P. freudenreichii*-derived EVs.

Other important technological aspects that should be considered for further studies on *P. freudenreichii*-derived EVs include the evaluation of their stability during

digestion throughout the gastrointestinal tract, what could be accomplished with *in vitro* digestion assays. It is equally important to characterize the stability of EVs during storage, and technological methods for conservation, such as lyophilization. Furthermore, other biophysical features should be characterized, such as rigidity, elasticity, interaction forces, surface charges and agglomeration potential, using techniques that include atomic force microscopy and dynamic light scattering. All these investigations could shed light on how EVs properties are influenced by environmental and technological parameters, and provide hints for different optimizations.

7. Conclusion

Overall, the present study comprises the first comprehensive analysis of *P. freudenreichii*-derived EVs, including their biophysical properties, protein content and immunomodulatory roles *in vitro*. It also demonstrates the importance of bacterial growth conditions and EV purification methods as analytical variables for interpreting bacterial EV properties. Furthermore, the anti-inflammatory role of *P. freudenreichii*-derived EVs and the viability of modulating their content and functions provide novel tools for the rational design of improved and safe therapeutic products. Although there are several gaps in our knowledge about *P. freudenreichii*-derived EVs, such as their effects *in vivo*, the molecules that they contain in addition to proteins, other pathways that they might modulate in eukaryotic cells, the impact of stress conditions and strain variability; still, this pioneer study has certainly initiated an exciting journey into the inner workings of EV-mediated probiotic effect of *P. freudenreichii.*

Thesis outputs

The work developed during this Ph.D. thesis resulted in the scientific products enumerated below:

1. Research articles as first author

RODOVALHO VR, LUZ BSR, RABAH H, do CARMO FLR, FOLADOR EL, NICOLAS A, JARDIN J, BRIARD-BION V, BLOTTIÈRE H, LAPAQUE N, JAN G, LE LOIR Y, de CARVALHO AZEVEDO VA, GUÉDON E. **Extracellular Vesicles Produced by the Probiotic *Propionibacterium freudenreichii* CIRM-BIA 129 Mitigate Inflammation by Modulating the NF-κB Pathway**. Front Microbiol. 2020 Jul 7;11:1544. doi: 10.3389/fmicb.2020.01544.

RODOVALHO VR, LUZ BSR, NICOLAS A, do CARMO FLR, JARDIN J, BRIARD-BION V, JAN G, LE LOIR Y, de CARVALHO AZEVEDO VA, GUEDON E. **Environmental conditions modulate the protein content and immunomodulatory activity of extracellular vesicles produced by the probiotic *Propionibacterium freudenreichii***. Appl Environ Microbiol. 2020 Dec 11:AEM.02263-20. doi: 10.1128/AEM.02263-20.

2. Research articles as co-author

TARTAGLIA NR, NICOLAS A, RODOVALHO VR, LUZ BSRD, BRIARD-BION V, KRUPOVA Z, THIERRY A, COSTE F, BUREL A, MARTIN P, JARDIN J, AZEVEDO V, LE LOIR Y, GUÉDON E. **Extracellular vesicles produced by human and animal Staphylococcus aureus strains share a highly conserved core proteome.** Sci Rep. 2020 May 21;10(1):8467. doi: 10.1038/s41598-020-64952-y. *(The contributions to this article included the sequences similarity analysis of the proteins found in EVs derived from different strains of* S. aureus*, for the core proteome composition and manuscript revision.)*

LUZ BSR, NICOLAS A, CHABELSKAYA S, RODOVALHO VR, LE LOIR Y, AZEVEDO VAC, FELDEN B, GUÉDON E. **Environmental plasticity of the RNA content of *Staphylococcus aureus* extracellular vesicles**. Front Microbiol. 2021; 12:482. doi: 10.3389/fmicb.2021.634226. *(The contributions to this article included the functional enrichment analysis of the coding sequences of nucleic acids found in* S. aureus*-derived EVs and manuscript revision.)*

3. Patent

GUEDON E.; RODOVALHO VR.; JAN G.; LE LOIR Y.; AZEVEDO VAC. **Vesicles derived from a *Propionibacterium freudenreichii* strain for the treatment of an inflammatory disease and a method for preparing thereof**. European Patent Office (EPO): 20305644.5-1132, 12/06/2020.

4. Book chapter

RODOVALHO, VR.; RODRIGUES, DLN.; JAN, G.; LE LOIR, Y.; AZEVEDO, VAC.; GUEDON, E. *Propionibacterium freudenreichii*: General Characteristics and Probiotic Traits [Online First], IntechOpen, 2021. DOI: 10.5772/intechopen.97560.

5. Conference oral presentations

RODOVALHO VR; LUZ BSR; CARMO FLR; BLOTTIERE H; LAPAQUE N; JAN G; LE LOIR Y; AZEVEDO VAC; GUEDON E. ***Propionibacterium freudenreichii* secretes extracellular vesicles with anti-inflammatory activity via NF-κB pathway modulation.** In: 2nd French Society of Extracellular Vesicles Congress, 2019, Nantes, France. Extracellular Vesicles in Health & Disease FSEV-2019 Congress - PROGRAM & ABSTRACTS, 2019.

RODOVALHO VR; LUZ BSR; CARMO FLR; JARDIN J; BRIARD-BION V; JAN G; LE LOIR Y; AZEVEDO VAC; GUEDON E. **Roles of extracellular vesicles secreted by *Propionibacterium freudenreichii*.** 2nd Associated International Laboratory Meeting - Bact-Inflam Conference, 2019, Belo Horizonte, Brazil.

6. Conference poster presentations

RODOVALHO VR; LUZ BSR; CARMO FLR; JARDIN J; BRIARD-BION V; JAN G; LE LOIR Y; AZEVEDO VAC; GUEDON E. ***Propionibacterium freudenreichii* secretes extracellular vesicles containing immunomodulatory proteins.** 2nd Associated International Laboratory Meeting - Bact-Inflam Conference, 2019, Belo Horizonte, Brazil.

RODOVALHO VR; LUZ BSR; CARMO FLR; JAN G; LE LOIR Y; AZEVEDO VAC; GUEDON E. **The extracellular vesicles produced by the probiotic *Propionibacterium freudenreichii* in different conditions share a core proteome that includes immunomodulatory proteins.** X-meeting eXperience 2020, 2020.

RODOVALHO VR. **Characteristics and roles of extracellular vesicles secreted by *Propionibacterium freudenreichii***. STLOpendays, Institut National de Recherche Agronomique (INRA). UMR UMR INRA / AgroCampus Rennes: Science et Technologie du Lait et de l'œuf (1253), 2019, Rennes, France.

Résumé étendu (Extended abstract)

1. Introduction

La production de vésicules extracellulaires (VEs) est désormais reconnue comme un phénomène omniprésent dans les trois branches du vivant et constituant une voie de sécrétion impliquée dans la communication intercellulaire (Gill et al., 2019; Woith et al., 2019). Les VEs sont des particules biologiques sphériques de taille nanométrique, composées d'une bicouche lipidique et d'une lumière interne transportant une cargaison biologique (Théry et al., 2018; Gill et al., 2019). Elles sont produites par la plupart des cellules (procaryotes, eucaryotes), principalement à partir du bourgeonnement de la membrane, et sécrétées dans le milieu environnant. Elles jouent un rôle essentiel dans la communication entre les cellules (intra- et inter-espèce) et dans la vectorisation des propriétés biologiques des cellules, de par leur capacité à transporter des molécules bioactives (protéines, acides nucléiques, lipides, enzymes, toxines, métabolites) d'une cellule productrice à une cellule receveuse. Ces médiateurs de la communication agissent par transfert de leur contenu dans les cellules cibles ou par une interaction spécifique entre des ligands présents à leur surface et des récepteurs exprimés par les cellules cibles. La production de ces vecteurs d'information a été démontrée chez de nombreuses bactéries, y compris des bactéries pathogènes, probiotiques et commensales (Bitto and Kaparakis-Liaskos, 2017; Cecil et al., 2019; Molina-Tijeras et al., 2019; Caruana and Walper, 2020). Bien que la production de VEs de bactéries ait été principalement étudiée chez des bactéries à Gram négatif et pathogènes, ces dernières années, on a constaté une augmentation, dans la littérature scientifique, des travaux sur la production de VEs par plusieurs bactéries bénéfiques, notamment les bactéries à Gram négatif *Akkermansia muciniphila*, *Bacteroides* spp., *Escherichia coli* Nissle 1917, *Lysobacter* spp., et à Gram positif *Lactobacillus* spp. et *Bifidobacterium* spp (Brown et al., 2015; Kudryakova et al., 2016; Cañas et al., 2018; Stentz et al., 2018; Ashrafian et al., 2019; Mata Forsberg et al., 2019; Molina-Tijeras et al., 2019; Nishiyama et al., 2020). Les VEs produites par ces bactéries exercent divers rôles bénéfiques, notamment des activités anti-pathogènes, immunomodulatrices, anti-obésités et antidépressives, ainsi que l'amélioration de la fonction barrière de l'épithélium intestinal et de la mobilité intestinale (López et al., 2012; Shen et al., 2012; Fábrega et al., 2017; Seo et al., 2018; Ashrafian et al., 2019; Behrouzi et al., 2020; Vargoorani et al., 2020). Les travaux sur les VEs de bactéries bénéfiques aideront à décoder la communication entre les micro-organismes et leurs hôtes et à mieux comprendre les relations qu'ils peuvent entretenir, en particulier dans le cadre des interactions entre le microbiote intestinal et les cellules humaines (Macia et al., 2019). Par conséquent, la caractérisation des propriétés des VEs dérivés de bactéries

bénéfiques présente un grand potentiel pour diverses applications cliniques et technologiques (Toyofuku et al., 2015; Liu et al., 2018b). L'une de ces bactéries bénéfiques dont l'importance est de plus en plus reconnue est *Propionibacterium freudenreichii*, une espèce probiotique laitière à Gram positif qui est utilisée depuis longtemps comme ferment dans la production de fromage de type suisse (Thierry et al., 2011; Rabah et al., 2017). Dans un contexte technologique, cette bactérie a été étudiée pour l'optimisation de la production de fromage, notamment l'affinage et la production de composés aromatiques, mais aussi pour la production de vitamine B12 et d'acides organiques (de Freitas et al., 2015; Gagnaire et al., 2015; Rabah et al., 2017; Piwowarek et al., 2018a). En outre, elle est apparue dans le panorama des probiotiques en raison de plusieurs caractéristiques bénéfiques, notamment la tolérance au stress dans le tractus gastro-intestinal, la capacité d'adhésion aux cellules hôtes, l'activité anti-pathogène, les propriétés anticancéreuses et l'immunomodulation (Jan et al., 2002; Cousin et al., 2012b; do Carmo et al., 2017, 2019; Nair and Kollanoor-Johny, 2017; Rabah et al., 2017). Ces propriétés bénéfiques ont été confirmées par des études *in vitro* et *in vivo* (Foligné et al., 2010; Colliou et al., 2017; do Carmo et al., 2019; Ge et al., 2019; Ma et al., 2020). Plusieurs approches en sciences omiques ont été utilisées pour étudier les mécanismes moléculaires impliqués dans ces processus, ce qui a permis d'identifier des acteurs moléculaires importants, tels que les protéines de surface, les acides gras à chaîne courte et les facteurs bifidogéniques (Jan et al., 2002; Okada et al., 2006; Deutsch et al., 2017; do Carmo et al., 2017). Il a été démontré que la diversité des souches est un aspect important à prendre en considération, car plusieurs de ces propriétés sont souche-dépendantes (Foligné et al., 2010; Thierry et al., 2011; Foligné et al., 2016; Deutsch et al., 2017). Plusieurs études ont réalisé un criblage multi-souches pour identifier des souches remarquables et des optimisations ont été proposées pour comprendre ou améliorer les propriétés technologiques et probiotiques dans des contextes spécifiques (Foligné et al., 2016; Plé et al., 2016; Deutsch et al., 2017). De nouvelles études devraient permettre d'approfondir les mécanismes moléculaires liés aux propriétés bénéfiques de cette espèce et de ses produits, tout en considérant la diversité phénotypique des souches et les interactions avec l'hôte et le microbiote.

2. Question de recherche

La production de VEs est désormais considérée comme un phénomène ubiquitaire, même si son identification dans des contextes particuliers ait été retardée en raison de limitations techniques ou d'idées reçues (Woith et al., 2019). Ceci a été notamment le cas des bactéries à Gram positif, dont l'épaisse paroi cellulaire de peptidoglycane semblait constituer une barrière infranchissable (Brown et al., 2015). De même, la recherche sur les VEs de bactéries bénéfiques n'en est qu'à ses débuts, si on la compare aux bactéries pathogènes (Bitto and Kaparakis-Liaskos, 2017). Néanmoins, de plus en plus de preuves suggèrent que des facteurs sécrétés sont à l'origine de

certains des effets bénéfiques associés à ces bactéries, parfois à des endroits éloignés de ceux où elles se trouvent habituellement dans le corps humain, comme le cerveau (Haas-Neill and Forsythe, 2020). Les VEs pourraient donc expliquer en partie ces observations. En effet, ces véhicules naturels d'échange intercellulaire, qui assurent la protection de leur cargaison interne, sont capables de traverser la couche de mucus dans le tractus gastro-intestinal, de migrer vers d'autres tissus et de se propager vers des sites distants (Bitto and Kaparakis-Liaskos, 2017; Molina-Tijeras et al., 2019).

De même, les VEs sont également prometteurs sur le plan technologique, avec un grand potentiel de production rentable et à grande échelle, de stabilité et de sécurité à long terme (Bitto and Kaparakis-Liaskos, 2017). Les VEs ont été utilisés en toute sécurité comme vaccins ciblés sur l'homme et sont considérés comme une alternative appropriée aux probiotiques dans le cas de personnes immunodéprimées (Bitto and Kaparakis-Liaskos, 2017; Molina-Tijeras et al., 2019). En outre, la production de VEs dérivés de bactéries bénéfiques, ainsi que leurs propriétés, ont également été optimisées pour répondre à des critères spécifiques, tels que l'amélioration du rendement, et la modulation du contenu et de l'activité (Nakayama-Imaohji et al., 2016; Pérez-Cruz et al., 2016; Hirayama and Nakao, 2020; Kuhn et al., 2020). Toutes ces caractéristiques font des VEs un système très intéressant de livraison de contenus, à la fois dans des contextes naturels et technologiques.

Avec l'amélioration des méthodes de purification et de la caractérisation omique, la tendance est à une forte augmentation des rapports scientifiques sur la prévalence des VEs et la caractérisation de leur contenu, avec un intérêt particulier pour les bactéries bénéfiques, longtemps négligées, mais très importantes. L'une de ces bactéries bénéfiques est *P. freudenreichii*, un probiotique laitier à Gram positif traditionnellement utilisé dans la production de fromage de type suisse, d'acides organiques et de vitamine B12 (Rabah et al., 2017). *P. freudenreichii* a été reconnu comme une espèce probiotique importante en raison de plusieurs caractéristiques bénéfiques, notamment l'adhésion aux cellules épithéliales humaines, l'activité anti-pathogène, la production de facteurs biogéniques qui modulent la composition du microbiote, les propriétés anticancéreuses médiées par les acides gras à chaîne courte et l'immunomodulation. Il a été montré que ces effets sont médiés par des facteurs exposés à la surface ou sécrétés (Jan et al., 2002; Okada et al., 2013; do Carmo et al., 2017), et parfois associés à des surnageants de culture sans cellules (Nair and Kollanoor Johny, 2018; Ma et al., 2020). Par ailleurs, la présence de structures ressemblant à des VEs dans le surnageant de culture de la souche JS22 de *P. freudenreichii* suggère que l'espèce puisse produire des VEs (Frohnmeyer et al., 2018).

Pour ces raisons, et comme les VEs sont de plus en plus reconnus comme un système général d'exportation, y compris pour les bactéries à Gram positif et bénéfiques (Brown et al., 2015; Molina-Tijeras et al., 2019), nous avons émis l'hypothèse que *P. freudenreichii* pourrait produire des VEs impliqués dans la médiation de ses rôles

bénéfiques, en particulier l'immunomodulation de cellules intestinales humaines. Nous avons donc formulé la question de recherche suivante :

La bactérie probiotique *P. freudenreichii* produit-elle des VEs ayant un rôle immunomodulateur dans différentes conditions ?

3. Objectifs

L'objectif général de ces travaux est de déterminer si *P. freudenreichii* produit des VEs au rôle immunomodulateur sur des cellules humaines. Pour cela, nous avons adopté pour une approche interdisciplinaire, intégrant la caractérisation biophysique des VEs, l'analyse protéomique de leur contenu, l'analyse interactomique de leurs protéines avec celles des cellules humaines, et des analyses fonctionnelles pour dévoiler les caractéristiques et les rôles des VEs dérivés de *P. freudenreichii* obtenues dans différentes conditions. Afin de répondre à cette question centrale, elle a été divisée en plusieurs objectifs spécifiques :

1. Déterminer si *P. freudenreichii* produit des VEs dans une condition standard de croissance, en utilisant la chromatographie par exclusion de taille comme méthode de purification des VEs.
2. Étudier les caractéristiques biophysiques (taille, concentration, morphologie) des VEs dérivés de *P. freudenreichii*.
3. Étudier la composition protéique des VEs dérivés de *P. freudenreichii*.
4. Appliquer des approches *in silico* pour prédire les interactions entre les protéines des VEs dérivés de *P. freudenreichii* et les protéines humaines.
5. Étudier l'activité immunomodulatrice des VEs dérivés de *P. freudenreichii* à l'égard de modèles cellulaires intestinaux humains.
6. Caractériser les VEs dérivés de *P. freudenreichii* obtenues à partir de cultures dans un milieu de croissance différent.
7. Comparer les conditions de croissance pour la modulation des propriétés des VEs dérivés de *P. freudenreichii*, notamment les paramètres biophysiques, la composition protéique, l'activité immunomodulatrice et les profils d'interaction.
8. Caractériser les propriétés des VEs dérivés de *P. freudenreichii* obtenues par une méthode de purification différente : l'ultracentrifugation avec gradient de densité.
9. Analyser la conservation de la composition en protéines des VEs dérivés de *P. freudenreichii* obtenus dans différentes conditions de croissance et par différentes méthodes de purification.

Ces objectifs ont été abordés dans trois différents chapitres (3, 4 et 5) de cette thèse, comprenant des articles de recherche publiés et des résultats supplémentaires.

4. Article de recherche 1

Certains aspects des effets probiotiques de *P. freudenreichii* ont été attribués à des facteurs exposés à la surface ou sécrétés et à des surnageants de culture sans cellules. Même si l'importance de protéines spécifiques pour l'immunomodulation exercée par l'espèce a été démontrée, il n'est pas certain que la fonctionnalité de ces protéines soit exclusivement médiée par une exposition en surface ou par des systèmes d'exportation conventionnels. En effet, il est envisageable que ces facteurs puissent être exportés par d'autres systèmes cellulaires, tels que les VEs. La présence de structures ressemblant à des VEs, récemment identifiées à partir d'images de microscopie électronique à transmission (TEM) de cette espèce, pourrait appuyer cette hypothèse. Toutefois, ces structures n'ont pas été purifiées, ni caractérisées. Nous avons donc cherché à savoir si *P. freudenreichii* produisait des VEs, et si ces dernières pouvaient être des médiateurs des propriétés probiotiques, notamment immunomodulatrices, de la bactérie.

Pour répondre à ces questions, nous avons utilisé la souche anti-inflammatoire *P. freudenreichii* CIRM-BIA129. Pour cela, elle a été cultivée en milieu ultrafiltrat de lait (UF) jusqu'au début de la phase stationnaire. Les cultures ont ensuite été centrifugées et filtrées, et les surnageants résultants ont été concentrés et appliqués à une colonne de chromatographie d'exclusion stérique (SEC) afin de purifier les VEs potentiellement présents. Les échantillons résultants ont été caractérisés par TEM et par l'analyse du suivi individuel de particules (NTA), dévoilant ainsi des VEs de taille nanométrique avec une morphologie typique sphérique et en forme de tasses. Une étude du contenu réalisée par une approche de protéomique shotgun a montré que les VEs contenaient un ensemble diversifié de protéines, dont des protéines associées aux propriétés immunomodulatrices de la bactérie, telle que SlpB. De plus, des prédictions d'interaction *in silico* ont révélé des interactions potentielles entre des protéines vésiculaires et une sous-unité du facteur de transcription humain NF-κB, qui est impliquée dans les processus inflammatoires. Par conséquent, l'activité immunomodulatrice de ces VEs a été donc évaluée dans des modèles cellulaires intestinaux adaptés. Le traitement de cellules épithéliales intestinales par les VEs de *P. freudenreichii* CIRM-BIA129 réduit la libération de la cytokine pro-inflammatoire IL-8 et l'activation du facteur de transcription NF-κB, en particulier lorsque les cellules ont été stimulées par le lipopolysaccharide (LPS) bactérien, un inducteur proinflammatoire . Cet effet est partiellement médié par la protéine SlpB, car les VEs produits par un mutant *slpB* ont un effet anti-inflammatoire moins intense.

Par conséquent, la première partie de cette étude montre que non seulement *P. freudenreichii* CIRM-BIA129 produit des VEs, mais qu'ils ont un rôle anti-inflammatoire sur des cellules épithéliales intestinales humaines, qui est lié aux protéines immunomodulatrices portées par ces VEs. Il est important de noter qu'il s'agit de la première caractérisation complète des VEs dérivés de *P. freudenreichii*, ce qui a donné lieu à un article de recherche original publié dans la revue Frontiers in Microbiology (DOI: 10.3389/fmicb.2020.01544), intitulé “Extracellular Vesicles Produced by the Probiotic *Propionibacterium freudenreichii* CIRM-BIA 129 Mitigate Inflammation by Modulating the NF-κB Pathway”.

5. Article de recherche 2

Comme démontré, *P. freudenreichii* CIRM-BIA129 cultivé en milieu UF a produit des VEs ayant une activité anti-inflammatoire envers les cellules épithéliales humaines. Les propriétés biophysiques, biochimiques et fonctionnelles de ces VEs dérivées de l'UF ont été caractérisées de manière exhaustive. Toutefois, des preuves de plus en plus nombreuses suggèrent que plusieurs facteurs, notamment le milieu de croissance, la phase de croissance, les sels, le pH et l'exposition aux antibiotiques, peuvent moduler les propriétés des VEs dérivés de bactéries. C'est la raison pour laquelle nous avons cherché, dans un second temps, à étudier l'influence du milieu de croissance la modulation des propriétés des VEs dérivés de *P. freudenreichii* CIRM-BIA129.

La souche *P. freudenreichii* CIRM-BIA129 a été cultivée en milieu UF ou YEL jusqu'au début de la phase stationnaire. Ensuite, le même protocole d'isolement des VEs a été appliqué. Celui-ci consistait en une centrifugation de la culture et une filtration pour récupérer les surnageants sans cellules, une concentration et une application dans une colonne SEC. Les échantillons résultants ont été caractérisés par des mesures de NTA, des tests d'activité immunomodulatrice, et des expériences de protéomique qualitative et quantitative. Ces analyses ont révélé des différences entre les VEs dérivées de l'UF et de l'YEL en termes d'abondance, de diamètre, d'activité anti-inflammatoire, et d'identité et d'abondance des protéines. En outre, elles ont mis en évidence la manière dont les différentes abondances des protéines associées aux VEs pourraient affecter le profil des interactions entre les protéines bactériennes et humaines, affectant ainsi les activités immunomodulatrices.

Cette étude comparative a permis de mettre en évidence l’impact des conditions de croissance de la bactérie sur les propriétés des VEs qu’elle produit, notamment via une modulation de leur protéome et de ses propriétés anti-inflammatoires. De plus, elle ouvre des perspectives sur un contrôle des propriétés des VEs par l’optimisation des conditions de croissance de la bactérie. Cette partie présente le deuxième article de

recherche original issu de cette thèse, publié dans la revue Applied and Environmental Microbiology (DOI: 10.1128/AEM.02263-20), et intitulé “Environmental conditions modulate the protein content and immunomodulatory activity of extracellular vesicles produced by the probiotic *Propionibacterium freudenreichii*”.

6. Résultats supplémentaires

Les études présentées dans les parties précédentes ont montré que *P. freudenreichii* produisait des VEs ayant un rôle anti-inflammatoire et que leurs propriétés dépendaient des conditions de croissance utilisées pour cultiver la bactérie. Par conséquent, l'optimisation des conditions de croissance représente un outil pour la modulation des propriétés des VEs, y compris leur activité anti-inflammatoire envers les cellules épithéliales humaines. Dans les études précédentes, les VEs ont été purifiés par chromatographie d'exclusion de taille (SEC), qui est une méthode de purification basée sur la taille. Néanmoins, malgré la reconnaissance croissante de l’approche SEC comme une méthode simple et de haute qualité pour la purification des VEs, les méthodes les plus utilisées pour la purification des VEs bactériennes sont toujours celles basées sur l'ultracentrifugation (UC). Par conséquent, nous avons cherché à savoir si les VEs dérivés de *P. freudenreichii* purifiés par cette méthode présenteraient des propriétés similaires, notamment en ce qui concerne la composition protéique.

P. freudenreichii CIRM-BIA129 a été cultivé en milieu UF et YEL jusqu'au début de la phase stationnaire, et les cultures ont été centrifugées, filtrées et le surnageant concentré. La purification des VEs a été effectuée par une série d'étapes d'ultracentrifugation, dont une avec un gradient de densité de saccharose. La caractérisation microscopique et la caractérisation de la taille ont confirmé la purification de VEs par cette approche. La protéomique shotgun a permis l'identification d'un ensemble diversifié de protéines, dépendant également des conditions de croissance des bactéries. Une analyse comparative de la composition protéique des VEs dérivés d'UC et de SEC, isolés à partir de cultures d'UF et de YEL, a montré que, bien que certaines protéines soient spécifiques à chaque condition, 308 étaient partagées entre toutes les conditions. Ce protéome central était enrichi en protéines liées au métabolisme de l'énergie et du carbone, à la structure ribosomique et à la biogenèse, au quorum sensing, à l'exportation de protéines et à la biosynthèse de peptidoglycane. Ces résultats offrent donc quelques indices des fonctions conservées dans les VEs dérivés de *P. freudenreichii*, éliminant les biais des conditions de croissance et de purification.

7. Discussion

La production de VEs et leur rôle en tant que système d'exportation pour la communication intercellulaire intra- et inter-espèce sont de plus en plus rapportés dans la littérature scientifique (Gill et al., 2019; Woith et al., 2019). Dans cette étude, nous avons démontré que le probiotique à Gram positif *P. freudenreichii* produit également des VEs ayant un rôle immunomodulateur dans la communication entre certaines cellules de l'homme et la bactérie. Notre étude s'est concentrée sur la caractérisation biophysique et protéomique des VEs dérivées de *P. freudenreichii*, ainsi que sur leur étude fonctionnelle à l'aide de prédictions interactomiques et d'essais immunologiques *in vitro*. En outre, nous avons démontré que les conditions de croissance des bactéries et les méthodes de purification des VEs ont un impact sur les propriétés de ces dernières.

En ce qui concerne les investigations fonctionnelles, nos données ont montré que les VEs purifiés par SEC ont un rôle anti-inflammatoire sur les cellules épithéliales intestinales humaines cultivées, via la réduction de l'activité du facteur de transcription NF-κB et de la libération d'IL-8. Cet effet anti-inflammatoire des VEs est observé spécifiquement lorsque les cellules sont dans un état inflammatoire induit par le LPS bactérien. Par conséquent, nous avons supposé que les VEs agissent sur des points de la voie de signalisation NF-κB qui sont induits spécifiquement par le LPS, et non par les autres inducteurs que nous avons pu tester (TNFα et IL-1β). En tant que membres de la voie NF-κB induite par le LPS, les protéines humaines toll-like receptor 4 (TLR4) et TIR domain-containing adapter molecule 1 (TICAM-1) sont des cibles potentielles à tester de l'immunomodulation médiée par les VEs de *P. freudenreichii* CIRM-BIA129. TLR4 est une protéine transmembranaire, tandis que TICAM-1 est une protéine cytoplasmique. Ainsi, il semble important de déterminer si les VEs agissent de manière intracellulaire ou extracellulaire, par rapport aux cellules réceptrices.

Il a été rapporté que les VEs interagissent avec des protéines de surface des cellules réceptrices, se fixant à la surface de la cellule (French et al., 2017). Par conséquent, les VEs dérivés de *P. freudenreichii* pourraient interagir avec le domaine extracellulaire de TLR4 via des composants de surface des VEs (par exemple, les protéines) ou d'autres composants cytoplasmiques après leur lyse. Cependant, nos données ont montré que la protéolyse de la surface des VEs n'abolissait pas leur rôle immunomodulateur, ce qui suggère que la seconde option soit plus probable. Il a également été rapporté que les VEs bactériens pouvaient entrer dans les cellules eucaryotes par différents mécanismes, notamment la fusion directe des membranes ou par endocytose (O'Donoghue and Krachler, 2016; Bajic et al., 2020). Par conséquent, le contenu des VEs pourrait servir de médiateur à l'immunomodulation après leur entrée dans la cellule hôte, en interagissant avec le domaine intracellulaire de TLR4 ou avec la protéine TICAM-1. Une autre possibilité envisageable serait un rôle plus indirect, dans lequel les VEs pourraient réguler l'immunomodulation via une autre voie de signalisation et la modulation négative de la voie NF-κB serait plutôt une conséquence. Pour autant, d'autres études sont nécessaires pour élucider les mécanismes d'action précis des VEs

dérivés de *P. freudenreichii*, y compris des approches structurelles *in silico* pour analyser les interactions proteíne-proteíne à plus haute résolution (Keskin et al., 2016) et des approches expérimentales pour déterminer si l'intégrité des VEs est nécessaire pour la livraison des biomolécules qu'elles transportent (Bonsergent and Lavieu, 2019), le suivi par fluorescence de l'internalisation et de la libération des biomolécules (Ofir-Birin et al., 2018), ainsi que l'inhibition de voies d'assimilation spécifiques par les cellules eucaryotes (Bajic et al., 2020). Néanmoins, comme les mécanismes de l'immunomodulation ne sont pas entièrement élucidés pour les cellules bactériennes de *P. freudenreichii*, notre étude sur les VEs dérivés de *P. freuderenichii* ouvre de nouvelles perspectives. En particulier, déterminer si l'activité anti-inflammatoire de la bactérie *P. freudenreichii* cible la même voie de signalisation, NF-κB, que les VEs qu'elle produit, constitue une base pour d'autres études.

En ce qui concerne les effecteurs bactériens de l'immunomodulation médiée par les VEs, nos données indiquent que la protéine SlpB est partiellement impliquée, puisque les VEs dérivés du mutant *slpB* présentent une réduction moins intense de l'activité régulatrice de NF-κB que les VEs produits par la souche sauvage. Ce résultat est en partie en adéquation avec le rôle crucial de SlpB dans l'immunomodulation exercée par la bactérie (do Carmo et al., 2019). Cependant, comme la réduction de l'activité de NF-κB par les VEs n'est pas complètement abolie en absence de SlpB, d'autres protéines pourraient être impliquées. Notre analyse qui a couplé les prédictions de l'interactomique, la protéomique quantitative et les essais fonctionnels comparant les VEs dérivés de l'UF et de l'YEL purifiés par SEC a abouti à une petite liste de protéines bactériennes candidates qui pourraient interagir directement avec TLR4 et/ou TICAM-1, et ainsi déclencher l'inhibition de la voie NF-κB induite par LPS. Cette liste comprend le pyruvate synthase/pyruvate-flavodoxine oxydoréductase (NifJ1), la chaîne G de la NADH-quinone oxydoréductase (NuoG), une protéine non caractérisée (PFCIRM129_01355), la GtfB glycosyltransférase (GtfB), une ADN polymérase I putative (PolA), et la protéine A du système UvrABC (UvrA3). En outre, d'autres protéines auparavant considérées comme immunomodulatrices chez *P. freudenreichii*, telles que la protéine de la couche superficielle E (SlpE) et la chaperonine GroL2 (GroL2) (Deutsch et al., 2017), font partie du protéome central vésiculaire, ce qui indique qu'elles pourraient également être impliquées dans l'immunomodulation induite par les VEs. D'autres études à partir de mutants pour ces protéines ou des anticorps pourraient élucider l'importance de protéines spécifiques, ainsi que l'analyse structurale bioinformatique des interactions prédites. De plus, bien que nos analyses se soient concentrées sur les recherches protéomiques, il est également possible que des molécules autres que des protéines puissent être impliquées dans l'immunomodulation médiée par les VEs. Par exemple, le DHNA produit par *P. freudenreichii* a également la capacité à inhiber la production de cytokines pro-inflammatoires chez la souris (Okada et al., 2013) et pourrait être exporté par les VEs. En outre, les acides gras à chaîne courte pourraient également être impliqués dans l'immunomodulation (Morrison and Preston,

2016) et exporté à l'intérieur des VEs. Il a été récemment montré que des acides nucléiques associés aux VEs bactériens participaient aux interactions hôte-bactérie, en particulier de petits ARNs régulateurs (Dauros-Singorenko et al., 2018; Ahmadi Badi et al., 2020; Rodriguez and Kuehn, 2020). Par conséquent, d'autres approches devraient être mises en œuvre pour caractériser de manière exhaustive, au-delà de sa cargaison protéique, le contenu des VEs dérivés de *P. freudenreichii*, en révélant éventuellement la présence d'acides nucléiques, de glucides et de lipides.

En ce qui concerne le modèle cellulaire utilisé pour les essais *in vitro*, les cellules d'adénocarcinome HT-29 du colon ont été choisies, car ce type de cellules est constitutif de la muqueuse du tractus gastro-intestinal humain, modélisant l'environnement d'interaction hôte-bactérie. Toutefois, d'autres types de cellules épithéliales intestinales cultivées pourraient également être utilisées pour ces recherches, comme les modèles cellulaires Caco-2, T84, SW480, H4 et d'autres modèles cellulaires animaux (Bourgine et al., 2012; Ponce de León-Rodríguez et al., 2019). En outre, l'effet des VEs dérivés de *P. freudenreichii* dans l'inflammation systémique pourrait également être étudié avec des systèmes de co-culture comprenant des cellules immunitaires comme les cellules mononucléaires du sang périphérique humain (PBMC) et les lignées de cellules macrophages (THP-1, RAW 264.7) (Ponce de León-Rodríguez et al., 2019). En outre, des biomarqueurs inflammatoires autres que la cytokine pro-inflammatoire IL-8 pourraient être évalués, tels que IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17a, IFNγ, TNFα et GM-CSF. Ces nouvelles expériences permettraient de mieux comprendre les mécanismes d'immunomodulation exercés par les VEs dérivées de *P. freudenreichii*.

Notre étude fonctionnelle s'étant limitée aux approches *in vitro*, il est tentant de se demander quelle serait la pertinence des VEs dérivés de *P. freudenreichii in vivo*. Plusieurs études ont démontré que les cellules de *P. freudenreichii* exerçaient des rôles immunomodulateurs *in vivo* (Foligné et al., 2010; do Carmo et al., 2019; Ma et al., 2020). Par ailleurs, il existe également des preuves de l'immunomodulation *in vivo* médiée par des VEs dérivées de probiotiques (Al-Nedawi et al., 2015; Kim et al., 2018b; Yamasaki-Yashiki et al., 2019; Choi et al., 2020b), en particulier l'amélioration des conditions inflammatoires (Kang et al., 2013; Fábrega et al., 2017; Seo et al., 2018; Ashrafian et al., 2019). Par conséquent, il est possible que les VEs dérivés de *P. freudenreichii* exercent également des rôles anti-inflammatoires *in vivo*. Cela serait pertinent pour les aspects pharmacocinétiques et pharmacodynamiques de l'effet probiotique de *P. freudenreichii*, ainsi que pour sa portée sur des sites éloignés de l'organisme, par rapport au tractus gastro-intestinal, comme les effets probiotiques sur l'axe intestin-cerveau (Jang et al., 2015; Yaghoubfar et al., 2020). De plus, la vectorisation dans les matrices alimentaires de la bactérie a montré un effet protecteur pour les cellules de *P. freudenreichii* (Plé et al., 2015; Rabah et al., 2018b, 2018a). Des expériences similaires pourraient être envisagées pour l'administration *in vivo* des VEs, notamment pour améliorer leur stabilité et développer des produits laitiers enrichis. Enfin, la concentration en VEs pourrait être un facteur clé pour les expériences *in vivo*, et les méthodes de purification

des VEs à faible rendement pourraient entraver ce type d'études. En effet, nos investigations ont montré des différences importantes dans les rendements de récupération des VEs selon la méthode de purification. Il convient donc de mettre au point des méthodes de purification améliorées et des stratégies nanotechnologiques pour l'accumulation des VEs dans les sites souhaités. En conséquence, des études supplémentaires devraient être menées pour déterminer la pertinence *in vivo* des VEs dérivés de *P. freudenreichii*, en tenant compte des optimisations des schémas d'administration et de vectorisation, ainsi que des méthodes de purification de plus haut rendement.

Bien que notre étude se soit concentrée sur le rôle immunomodulateur des VEs dérivés de *P. freudenreichii*, d'autres fonctionnalités potentielles devraient être abordées. En effet, notre analyse interatomique a prédit des interactions avec des protéines humaines impliquées dans divers modules fonctionnels, notamment le métabolisme, la transduction de signaux et les maladies infectieuses. Cette dernière pourrait être liée à l'activité anti-pathogène de *P. freudenreichii*, précédemment associée à des surnageants sans cellules (Nair and Kollanoor Johny, 2018). En effet, cette activité pourrait également être associée à certains composants protéiques des VEs dérivés de *P. freudenreichii*. En outre, certaines interactions prédites ont été associées à des protéines humaines liées au cancer. Bien que l'activité anticancéreuse de *P. freudenreichii* ait été attribuée aux acides gras à chaîne courte (Jan et al., 2002; Cousin et al., 2016), en plus de contenir éventuellement ces molécules, les VEs pourraient également contenir des protéines impliquées dans cette activité anticancéreuse. Il est intéressant de noter que certaines interactions prédites ont été associées au terme KEGG "Communauté cellulaire - eucaryotes", qui comprend l'adhésion focale, la jonction par adhérence, la jonction serrée et la jonction par fente. Par conséquent, il est également possible que les VEs dérivés de *P. freudenreichii* jouent un rôle dans la fonction barrière de l'épithélium intestinale, comme l'ont montré d'autres probiotiques ou VEs dérivés de probiotiques (Blackwood et al., 2017; Chelakkot et al., 2018). En outre, certaines interactions ont été identifiées pour les maladies neurodégénératives et pourraient permettre de mieux comprendre les rôles distales potentiels des VEs dérivés de *P. freudenreichii*, qui englobent l'axe intestin-cerveau, comme cela a été démontré pour d'autres VEs dérivés de probiotiques (Jang et al., 2015; Yaghoubfar et al., 2020). Malgré l'absence de validation expérimentale, ces prédictions de l'interactomique offrent plusieurs nouvelles approches pour étudier et comprendre certains aspects de l'activité probiotique de *P. freudenreichii*.

Au-delà de l'interactomique *in silico*, des approches expérimentales pourraient également permettre d'identifier de nouveaux aspects des effets bénéfiques induits par les VEs de *P. freudenreichii*. Les modèles cellulaires humains utilisés pour les essais *in vitro*, ainsi que d'autres modèles cellulaires proposés et des tissus issus d'expériences *in vivo* pourraient être soumis à un profilage de l'ARN, afin de détecter les gènes exprimés de manière différentielle après un traitement aux VEs. Ensuite, l'analyse de

l'enrichissement permettrait d'identifier les modules fonctionnels régulés, ce qui donnerait un aperçu de l'effet plus large des VEs. Pour les cellules de *P. freudenreichii*, il existe des preuves de la modulation de la voie du récepteur d'hydrocarbure arylique (AhR) par DHNA, avec des implications sur la détoxification métabolique et la régulation de l'inflammation (Fukumoto et al., 2014). En outre, il a également été démontré que les bactéries du microbiote intestinal régulent la voie du récepteur activé par les prolifératéurs de peroxysomes γ (PPAR-γ), via la production de butyrate, avec des implications dans l'amélioration du syndrome du côlon irritable chez la souris (Byndloss et al., 2017; Nozu et al., 2019). Par conséquent, en tant que producteur d'acides gras à chaîne courte et de DHNA, il est possible que *P. freudenreichii* et ses VEs puissent réguler d'autres voies des cellules eucaryotes, comme les voies AhR et PPAR-γ. Le profilage transcriptomique et protéomique des cellules eucaryotes traitées aux VEs, ainsi que des tests avec des systèmes cellulaires rapporteurs pourraient permettre l'identification de telles voies régulées.

Au-delà des interactions hôte-bactérie, les VEs peuvent également jouer un rôle dans les interactions bactéries-bactéries. Cela est évident dans le cas des communautés de bactéries intestinales qui partagent des VEs contenant des enzymes pour l'hydrolyse de polysaccharides complexes, de telle sorte que des espèces ou souches spécifiques fournissent des molécules utiles à d'autres espèces de la communauté (Elhenawy et al., 2014; Rakoff-Nahoum et al., 2014; Lynch and Alegado, 2017). Comme *P. freudenreichii* a été identifié dans des échantillons de matières fécales de nourrissons prématurés allaités (Colliou et al., 2017), il est possible que cette espèce fasse partie du microbiote intestinal humain et que ses VEs puissent fournir des enzymes métaboliques à la communauté. Il est à noter qu'une grande partie des protéines des VEs identifiées dans notre approche protéomique correspondent à des fonctions métaboliques. De plus, puisque *P. freudenreichii* est connu pour ses propriétés bifidogènes, médiées par le DHNA et d'autres facteurs bifidogéniques (Mori et al., 1997; Isawa et al., 2002; Kouya et al., 2007), il est raisonnable de supposer que ces molécules puissent être exportées via des VEs pour soutenir la croissance des bifidobactéries dans l'intestin humain. Il est également possible que les VEs servent de médiateurs pour les interactions entre bactéries dans d'autres environnements, tels que les matrices laitières. Étant donné que des combinaisons de souches ont été utilisées avec succès pour les produits laitiers (Plé et al., 2016; Cordeiro et al., 2018), il est possible qu'une communication par les VEs se produise au sein de ses communautés dans ces produits, soutenant la croissance et les processus de fermentation d'autres bactéries. En outre, les VEs peuvent représenter un moyen d'enrichissement nutritionnel dans ces produits, s'il est démontré qu'ils contiennent des molécules telles que la vitamine B12, qui sont produites par *P. freudenreichii* (Chamlagain et al., 2018; Piwowarek et al., 2018a). Toutes ces applications potentielles et attributions fonctionnelles nécessitent des expériences supplémentaires pour caractériser la diversité du contenu des VEs, ainsi que leurs rôles et mécanismes

d'interaction dans les communautés bactériennes, notamment via des profilage omique et le suivi des cargaisons biologiques.

Au-delà des investigations fonctionnelles, nos données ont également démontré que la production et les propriétés des VEs pouvaient être modulées par les conditions environnementales, en particulier les milieux de croissance des bactéries (UF et YEL). Ceci est en accord avec d'autres études, qui ont démontré une grande variabilité des propriétés des VEs obtenus à partir de différentes conditions de croissance (Choi et al., 2014; Lynch et al., 2019). En outre, certaines études ont démontré que les propriétés des VEs variaient ou que leur production augmentait en fonction des phases de croissance des bactéries (Tashiro et al., 2010b; Kim et al., 2016b; Taboada et al., 2019; Zavan et al., 2019) ou des conditions de stress, telles que la carence en nutriments, les antibiotiques, le stress osmotique et acide (Prados-Rosales et al., 2014; Chan et al., 2017; Lee et al., 2018; Gerritzen et al., 2019; Jun et al., 2019; Lynch et al., 2019). La biogenèse des VEs étant aussi parfois considérée comme une réponse cellulaire face à un stress (McBroom and Kuehn, 2007; Schwechheimer and Kuehn, 2013; Schwechheimer et al., 2014). Nos données renforcent ces observations, puisque la condition avec une production plus élevée des VEs (milieu UF), présentait également une plus grande abondance de protéines de stress associées aux VEs. Par conséquent, il est possible que les facteurs de stress puissent augmenter la production des VEs chez *P. freudenreichii*, en modulant également leurs propriétés. Dans l'ensemble, ces observations offrent la possibilité d'optimiser rationnellement le contenu des VEs et d'améliorer leurs rôles anti-inflammatoires par la gestion des conditions environnementales pendant la croissance des bactéries, ce qui pourrait faciliter la production à grande échelle et peu coûteuse de VEs thérapeutiques. Néanmoins, d'autres expériences devraient être menées pour étudier comment d'autres variables de croissance et des conditions environnementales stressantes sont liées aux propriétés des VEs dérivées de *P. freudenreichii*.

La méthode de choix pour la purification des VEs a également eu un impact sur les propriétés des VEs dérivés de *P. freudenreichii*, en particulier la composition protéique. Nous avons d'abord utilisé la SEC comme méthode de purification, qui est une méthode basée sur la taille, de haute qualité, simple et délicate, qui préserve l'intégrité et les propriétés des VEs (Gámez-Valero et al., 2016; Monguió-Tortajada et al., 2019a). Cependant, les méthodes basées sur l'UC sont les plus utilisées pour la purification des VEs de bactéries, en particulier celles incorporant des cycles d'ultracentrifugation avec des gradients de densité, même si l'ultracentrifugation peut être longue et causer des dommages aux VEs et engendrer des phénomènes d'agrégation (Monguió-Tortajada et al., 2019a). Nous avons comparé la composition protéique des VEs obtenus par ces deux approches. Comme aucune méthode de purification n'est parfaite, un compromis entre la récupération de grande quantité de VEs et la présence de protéines contaminantes doit être trouvé. De plus, comme les méthodes sont basées sur des approches différentes (séparation par taille ou par densité), chacune d'entre elles pourrait

permettre de recueillir des sous-populations différentes de VEs. Il n'est donc pas surprenant que certaines protéines aient été exclusives à certaines conditions. Néanmoins, des centaines de protéines ont été conservées dans les VEs obtenus dans toutes les conditions étudiées, ce qui indique que certaines protéines (le protéome central des VEs) pourraient être invariablement exportées dans des VEs. En effet, ces protéines pourraient être impliquées dans des processus essentiels pour la biogenèse des VEs et pour leurs fonctions dans la communication intercellulaire. Bien que nous ayons identifié une conservation du contenu en protéines des VEs dans différentes conditions d'obtention des VEs, il est également important de tenir compte de cette variabilité au niveau des espèces, en étudiant comment la variabilité des souches affecte les propriétés des VEs. Cela est particulièrement important parce que l'immunomodulation exercée par *P. freudenreichii* dépend de la souche (Deutsch et al., 2017), et cela pourrait également être le cas des VEs dérivés de *P. freudenreichii*.

8. Conclusion

La présente étude constitue la première analyse complète des VEs dérivés de *P. freudenreichii*, y compris leurs propriétés biophysiques, leur contenu protéique et leurs rôles immunomodulateurs *in vitro*. Elle démontre également l'importance des conditions de croissance bactérienne et des méthodes de purification des VEs comme variables analytiques pour l'interprétation des propriétés des VEs de bactéries. En outre, le rôle anti-inflammatoire des VEs dérivés de *P. freudenreichii* et la modulation de leur contenu et de leurs fonctions fournissent de nouveaux outils pour la conception raisonnée de produits thérapeutiques améliorés et sûrs. Bien que nos connaissances sur les VEs dérivés de *P. freudenreichii* présentent plusieurs lacunes, telles que leurs effets *in vivo*, les molécules qu'ils contiennent en plus des protéines, les autres voies qu'ils pourraient moduler dans les cellules eucaryotes, l'impact des conditions de stress et la variabilité des souches, cette étude pionnière a certainement lancé un voyage passionnant dans les rouages internes de l'effet probiotique de *P. freudenreichii* médié par les VEs.

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Annexes

This section contains supplementary information for this thesis. For research articles, supplementary images are provided, whereas longer supplementary tables are available online, with their electronic addresses provided.

All Python code used in this work is available online at:
https://github.com/vrrodovalho/evs-proteomics

Supplementary files for Research article 1 (Chapter 3) can be found online at:
https://www.frontiersin.org/articles/10.3389/fmicb.2020.01544/full#supplementary-material

Supplementary files for Research article 2 (Chapter 4) can be found online at:
https://journals.asm.org/doi/suppl/10.1128/AEM.02263-20/suppl_file/aem.02263-20-s0002.xlsx and
https://journals.asm.org/doi/suppl/10.1128/AEM.02263-20/suppl_file/aem.02263-20-s0001.pdf

Moreover, this annexes section includes the following subsections:

1. Proteins and their characteristics
2. Supplementary figures for Research article 1 (Chapter 3)
3. Supplementary figure for Research article 2 (Chapter 4)
4. Research articles as co-author

1. Proteins and their characteristics

Table S1. Proteins found in *P. freudenreichii*-derived EVs and their main characteristics.

	IDENTIFICATION					FEATURES			CONDITIONS			
#	**ID**	**GI**	**ACCESSION**	**LOCUS TAG**	**DESCRIPTION**	**COG**	**LOCALIZATION**	**LIPO-PROTEIN**	**SEC_UF**	**SEC_YEL**	**UC_UF**	**UC_YEL**
1	PFCIRM129_07415	659918774	CDP47792.1	PFCIRM129_07415	Cell envelope-related transcriptional attenuator	K	Cytoplasmic	Other	1	1	1	1
2	PFCIRM129_00620	659917060	CDP49575.1	PFCIRM129_00620	Hypothetical secreted protein	S	Cytoplasmic	TM	1	1	1	1
3	PFCIRM129_11635	659917259	CDP49392.1	PFCIRM129_11635	Hypothetical protein PFCIRM129_11635	J	Cytoplasmic	Other	1	1	1	1
4	OpuCA	659918860	CDP47878.1	PFCIRM129_07855	ATP-binding protein opuCA of Glycine betaine/carnitine/choline ABC transporter	E	Cytoplasmic	Other	1	1	1	1
5	Hsp202	659918241	CDP48424.1	PFCIRM129_03120	Heat shock protein 20 2 (20 kDa chaperone 2)	O	Cytoplasmic	Other	1	1	1	1
6	GroEL1	659918856	CDP47874.1	PFCIRM129_07835	60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1) (Heat shock protein 60 1)	O	Cytoplasmic	Other	1	1	1	1
7	PFCIRM129_07430	659918777	CDP47795.1	PFCIRM129_07430	Peptidase S51 family protein, dipeptidase E	E	Membrane	Other	1	1	1	1

8	GuaB1	65991 8855	CDP478 73.1	PFCIRM129 _07830	Inosine-5-monophosphate dehydrogenase (IMP dehydrogenase) (IMPDH) (IMPD) / GMP reductase)	F	Cytoplasmic	Other	1	1	1	1
9	PFCIRM12 9_01350	65991 6956	CDP496 49.1	PFCIRM129 _01350	Putative uncharacterized protein	S	Cytoplasmic	Other	1	1	1	1
10	PFCIRM12 9_07810	65991 8851	CDP478 69.1	PFCIRM129 _07810	Hypothetical protein PFCIRM129_07810	S	Extracellular	Other	1	1	1	1
11	Ask	65991 8550	CDP481 37.1	PFCIRM129 _08960	Aspartokinase (Aspartate kinase)	E	Cytoplasmic	Other	1	1	1	1
12	CysE	65991 8848	CDP478 66.1	PFCIRM129 _07795	Serine acetyltransferase	E	Cytoplasmic	Other	1	1	1	1
13	PFCIRM12 9_10155	65991 7468	CDP491 35.1	PFCIRM129 _10155	ABC transporter ATP-binding protein	P	Cytoplasmic	Other	1	1	1	1
14	Pyk1	65991 7343	CDP493 53.1	PFCIRM129 _11435	Pyruvate kinase 1	G	Cytoplasmic	Other	1	1	1	1
15	PFCIRM12 9_02975	65991 8323	CDP483 96.1	PFCIRM129 _02975	Hypothetical protein PFCIRM129_02975	S	Cytoplasmic	Other	1	1	1	1
16	SodA	65991 8893	CDP479 11.1	PFCIRM129 _08020	Iron/Manganese superoxide dismutase (Superoxide dismutase [Mn/Fe]) (SODM)	P	Cytoplasmic	Other	1	1	1	1
17	PFCIRM12 9_03805	65991 8118	CDP485 53.1	PFCIRM129 _03805	Hypothetical protein PFCIRM129_03805		Extracellular	TM	1	1	1	1
18	PhoU	65991 7486	CDP491 53.1	PFCIRM129 _10265	Putative phosphate transport system protein	P	Cytoplasmic	Other	1	1	1	1

19	PFCIRM129_09300	65991 8623	CDP481 25.1	PFCIRM129 _09300	FAD-dependent pyridine nucleotide-disulphide oxidoreductase:4Fe-4S ferredoxin, iron-sulfur binding:Aromatic-ring hydroxylase	C	Cytoplasmic	Other	1	1	1	1
20	PFCIRM129_07990	65991 8887	CDP479 05.1	PFCIRM129 _07990	Hypothetical protein PFCIRM129_07990		Extracellular	Lipo	1	1	1	1
21	PspA	65991 7270	CDP494 03.1	PFCIRM129 _11690	Phage shock protein A	KT	Cytoplasmic	Other	1	1	1	1
22	HtrA1	65991 7710	CDP490 24.1	PFCIRM129 _06325	Trypsin-like serine protease	O	Cytoplasmic	Other	1	1	1	1
23	GroL2	65991 7458	CDP491 25.1	PFCIRM129 _10100	60 kDa chaperonin 2 (Protein Cpn60 2) (groEL protein 2) (Heat shock protein 60 2)	O	Cytoplasmic	Other	1	1	1	1
24	GuaB3	65991 7266	CDP493 99.1	PFCIRM129 _11670	Inosine-5-monophosphate dehydrogenase (IMP dehydrogenase) (IMPDH) (IMPD) / GMP reductase	F	Cytoplasmic	Other	1	1	1	1
25	TyrB	65991 8246	CDP484 29.1	PFCIRM129 _03145	Aspartate transaminase (Aminotransferase)	E	Cytoplasmic	Other	1	1	1	1
26	PFCIRM129_00555	65991 7047	CDP495 62.1	PFCIRM129 _00555	sugar transporter	EGP	Membrane	TM	1	1	1	1
27	RplM	65991 8879	CDP478 97.1	PFCIRM129 _07950	50S ribosomal protein L13	J	Cytoplasmic	Other	1	1	1	1

28	Gcp	65991 8867	CDP478 85.1	PFCIRM129 _07890	Putative O-sialoglycoprotein endopeptidase	O	Cytoplasmic	Other	1	1	1	1
29	PFCIRM12 9_03845	65991 8126	CDP485 61.1	PFCIRM129 _03845	polar amino acid ABC transporter, binding protein component	ET	Membrane	Lipo	1	1	1	1
30	PFCIRM12 9_03855	65991 8128	CDP485 63.1	PFCIRM129 _03855	polar amino acid ABC transporter, inner membrane component	E	Membrane	Other	1	1	1	1
31	PFCIRM12 9_03860	65991 8129	CDP485 64.1	PFCIRM129 _03860	polar amino acid ABC transporter, ATP binding component	E	Cytoplasmic	Other	1	1	1	1
32	TrpD	65991 7264	CDP493 97.1	PFCIRM129 _11660	Anthranilate phosphoribosyltransferase	E	Cytoplasmic	Other	1	1	1	1
33	PpgK	65991 7053	CDP495 68.1	PFCIRM129 _00585	Polyphosphate glucokinase	GK	Cytoplasmic	Other	1	1	1	1
34	YfmC	65991 7054	CDP495 69.1	PFCIRM129 _00590	ABC transporter, binding lipoprotein	P	Membrane	Lipo	1	1	1	1
35	AceE	65991 8245	CDP484 28.1	PFCIRM129 _03140	pyruvate dehydrogenase E1 component (2-oxo-acid dehydrogenase E1 subunit, homodimeric type)	C	Cytoplasmic	Other	1	1	1	1
36	PFCIRM12 9_09290	65991 8621	CDP481 23.1	PFCIRM129 _09290	Dihydroorotate dehydrogenase	F	Cytoplasmic	Sec	1	1	1	1
37	CydA	65991 7025	CDP495 92.1	PFCIRM129 _01005	Cytochrome d ubiquinol oxidase subunit I	C	Membrane	Other	1	1	1	1

38	PFCIRM129_03850	659918127	CDP48562.1	PFCIRM129_03850	polar amino acid ABC transporter, binding protein	ET	Membrane	Lipo	1	1	1	1
39	NifJ1	659918622	CDP48124.1	PFCIRM129_09295	Pyruvate synthase/Pyruvate-flavodoxin oxidoreductase	C	Cytoplasmic	Other	1	1	1	1
40	RbsB	659918841	CDP47859.1	PFCIRM129_07760	binding protein of ribose ABC transporter	G	Membrane	Lipo	1	1	1	1
41	PFCIRM129_04165	659918020	CDP48619.1	PFCIRM129_04165	Hypothetical protein PFCIRM129_04165	D	Cytoplasmic	Other	1	1	1	1
42	ArgG	659917010	CDP49614.1	PFCIRM129_01135	Argininosuccinate synthase	E	Cytoplasmic	Other	1	1	1	1
43	CpsA1	659917011	CDP49615.1	PFCIRM129_01140	Cell envelope-related function transcriptional attenuator common domain	K	Cytoplasmic	TM	1	1	1	1
44	CarA	659918004	CDP48603.1	PFCIRM129_04085	Carbamoyl-phosphate synthase small chain	F	Cytoplasmic	Other	1	1	1	1
45	TetR7	659917671	CDP48985.1	PFCIRM129_06130	Regulatory protein, TetR	K	Cytoplasmic	Other	1	1	1	1
46	PFCIRM129_07610	659918812	CDP47830.1	PFCIRM129_07610	Hypothetical secreted protein	S	Extracellular	Lipo	1	1	1	1
47	SecF	659918023	CDP48622.1	PFCIRM129_04180	Protein-export membrane protein secF	U	Membrane	TM	1	1	1	1
48	AroB	659918008	CDP48607.1	PFCIRM129_04105	3-dehydroquinate synthase / shikimate kinase,bifunctionnal enzyme	E	Cytoplasmic	Other	1	1	1	1

49	Hpf	659918223	CDP48406.1	PFCIRM129_03025	ribosomal S30AE, sigma 54 modulation protein	J	Cytoplasmic	Other	1	1	1	1
50	MltG	659918010	CDP48609.1	PFCIRM129_04115	Aminodeoxychorismate lyase	S	Membrane	Other	1	1	1	1
51	SecD	659918024	CDP48623.1	PFCIRM129_04185	Protein-export membrane protein secD	U	Membrane	TM	1	1	1	1
52	SecA	659918222	CDP48405.1	PFCIRM129_03020	Preprotein translocase SecA subunit	U	Cytoplasmic	Other	1	1	1	1
53	AlaS	659918012	CDP48611.1	PFCIRM129_04125	Alanyl-tRNA synthetase (Alanine--tRNA ligase) (AlaRS)	J	Cytoplasmic	Other	1	1	1	1
54	PFCIRM129_09060	659918569	CDP48156.1	PFCIRM129_09060	Hypothetical secreted protein	L	Extracellular	Sec	1	1	1	1
55	AccA	659918794	CDP47812.1	PFCIRM129_07520	Acetyl-CoA carboxylase	I	Cytoplasmic	Other	1	1	1	1
56	AspS	659918015	CDP48614.1	PFCIRM129_04140	Aspartyl-tRNA synthetase (Aspartate--tRNA ligase) (AspRS)	J	Cytoplasmic	Other	1	1	1	1
57	MurG	659917354	CDP49364.1	PFCIRM129_11490	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (Undecaprenyl-PP-MurNAc-pentapeptide-UDPGlcNAc GlcNAc transferase)	M	Cytoplasmic	Other	1	1	1	1

58	PFCIRM129_07545	65991879	CDP47817.1	PFCIRM129_07545	zinc-binding dehydrogenase	E	Cytoplasmic	Other	1	1	1	1
59	HisS	65991801	CDP48617.1	PFCIRM129_04155	Histidyl-tRNA synthetase (Histidine--tRNA ligase) (HisRS)	J	Cytoplasmic	Other	1	1	1	1
60	NifJ2	65991879	CDP47815.1	PFCIRM129_07535	Pyruvate:ferredoxin (Flavodoxin) oxidoreductase	C	Cytoplasmic	Other	1	1	1	1
61	Eno1	65991766	CDP48974.1	PFCIRM129_06070	Enolase 1	G	Cytoplasmic	Other	1	1	1	1
62	PFCIRM129_01130	65991700	CDP49613.1	PFCIRM129_01130	NADPH:quinone reductase and related Zn-dependent oxidoreductases	C	Cytoplasmic	Other	1	1	1	1
63	CarB	65991800	CDP48602.1	PFCIRM129_04080	Carbamoyl-phosphate synthase large chain (Carbamoyl-phosphate synthetase ammonia chain)	F	Cytoplasmic	Other	1	1	1	1
64	YihN	65991715	CDP49458.1	PFCIRM129_12035	Membrane protein, Transporter, MFS superfamily	G	Membrane	TM	1	1	1	1
65	FtsE	65991823	CDP48415.1	PFCIRM129_03070	ABC transporter, ATP-binding protein	D	Cytoplasmic	Other	1	1	1	1
66	SppA	65991798	CDP48588.1	PFCIRM129_04010	SppA, Periplasmic serine proteases	OU	Membrane	Other	1	1	1	1
67	Pep	65991823	CDP48413.1	PFCIRM129_03060	Hypothetical protein PFCIRM129_03060	M	Extracellular	TM	1	1	1	1

68	Blmh	659917992	CDP48591.1	PFCIRM129_04025	Aminopeptidase C (Bleomycin hydrolase)	E	Cytoplasmic	Other	1	1	1	1
69	FtsZ	659917351	CDP49361.1	PFCIRM129_11475	Cell division protein FtsZ	D	Cytoplasmic	Other	1	1	1	1
70	YkgG	659917472	CDP49139.1	PFCIRM129_10175	Hypothetical protein PFCIRM129_10175	S	Cytoplasmic	Other	1	1	1	1
71	LutB	659917473	CDP49140.1	PFCIRM129_10180	iron-sulfur protein	C	Cytoplasmic	Other	1	1	1	1
72	PFCIRM129_01055	659917035	CDP49602.1	PFCIRM129_01055	ATP binding protein of ABC transporter	V	Cytoplasmic	Other	1	1	1	1
73	PFCIRM129_01060	659917036	CDP49603.1	PFCIRM129_01060	ABC transporter permease	Q	Membrane	Sec	1	1	1	1
74	LutA	659917474	CDP49141.1	PFCIRM129_10185	Oxidoreductase	C	Cytoplasmic	Other	1	1	1	1
75	MetK	659917997	CDP48596.1	PFCIRM129_04050	S-adenosylmethionine synthetase (Methionine adenosyltransferase) (AdoMet synthetase) (MAT)	H	Cytoplasmic	Other	1	1	1	1
76	AroE	659917039	CDP49606.1	PFCIRM129_01075	Shikimate 5-dehydrogenase	E	Cytoplasmic	Other	1	1	1	1
77	RsmI	659917675	CDP48989.1	PFCIRM129_06150	Methylase	H	Cytoplasmic	Other	1	1	1	1
78	MraW	659917362	CDP49372.1	PFCIRM129_11530	S-adenosyl-L-methionine-dependent methyltransferase mraW	M	Cytoplasmic	Other	1	1	1	1
79	LldP	659917475	CDP49142.1	PFCIRM129_10190	L-lactate permease	C	Membrane	Other	1	1	1	1

80	FtsI	65991 7360	CDP493 70.1	PFCIRM129 _11520	Cell division protein FtsI (penicillin-binding protein 2) (Peptidoglycan glycosyltransferase)	M	Extracellular	TM	1	1	1	1
81	PFCIRM12 9_04075	65991 8002	CDP486 01.1	PFCIRM129 _04075	Dihydroorotate dehydrogenase 2		Cytoplasmic	Other	1	1	1	1
82	PFCIRM12 9_09500	65991 8520	CDP481 68.1	PFCIRM129 _09500	Protein of unknown function		Extracellular	Sec	1	1	1	1
83	TyrS	65991 9103	CDP475 54.1	PFCIRM129 _00005	Tyrosyl-tRNA synthetase (Tyrosine--tRNA ligase) (TyrRS)	J	Cytoplasmic	Other	1	1	1	1
84	PFCIRM12 9_11720	65991 7238	CDP494 07.1	PFCIRM129 _11720	Hypothetical protein PFCIRM129_11720	S	Cytoplasmic	TM	1	1	1	1
85	NuoC	65991 8731	CDP480 05.1	PFCIRM129 _08515	NADH-quinone oxidoreductase chain C (NADH dehydrogenase I, chain C)	C	Cytoplasmic	Other	1	1	1	1
86	NuoD	65991 8730	CDP480 04.1	PFCIRM129 _08510	NADH-quinone oxidoreductase chain D (NADH dehydrogenase I, chain D)	C	Cytoplasmic	Other	1	1	1	1
87	NuoF	65991 8728	CDP480 02.1	PFCIRM129 _08500	NADH-quinone oxidoreductase chain F (NADH dehydrogenase I, chain F) (NDH-1, chain F)	C	Cytoplasmic	Other	1	1	1	1

88	NuoG	659918727	CDP48001.1	PFCIRM129_08495	NADH-quinone oxidoreductase chain G (NADH dehydrogenase I, chain G)	C	Cytoplasmic	Other	1	1	1	1
89	NuoI	659918725	CDP47999.1	PFCIRM129_08485	NADH-quinone oxidoreductase subunit I (NADH dehydrogenase I subunit I) (NDH-1 subunit I)	C	Cytoplasmic	Other	1	1	1	1
90	InlA	659917120	CDP49497.1	PFCIRM129_12235	internaline A		Extracellular	Sec	1	1	1	1
91	IdsA	659918719	CDP47993.1	PFCIRM129_08455	Heptaprenyl diphosphate synthase component II	H	Cytoplasmic	Other	1	1	1	1
92	RtcB	659917123	CDP49500.1	PFCIRM129_12250	Hypothetical protein PFCIRM129_12250	S	Cytoplasmic	Other	1	1	1	1
93	PFCIRM129_03560	659918163	CDP48508.1	PFCIRM129_03560	ErfK/YbiS/YcfS/YnhG precursor	S	Extracellular	Other	1	1	1	1
94	PFCIRM129_08935	659918681	CDP48084.1	PFCIRM129_08935	FAD linked oxidase domain protein	C	Cytoplasmic	Other	1	1	1	1
95	Mrc	659917127	CDP49504.1	PFCIRM129_12270	penicillin-binding protein (peptidoglycan glycosyltransferase)	M	Extracellular	TM	1	1	1	1
96	GlgC	659917204	CDP49430.1	PFCIRM129_11835	Glucose-1-phosphate adenylyltransferase (ADP-glucose synthase) (ADP-glucose pyrophosphorylase) (ADPGlc PPase)	G	Cytoplasmic	Other	1	1	1	1

97	PFCIRM129_03580	659918167	CDP48512.1	PFCIRM129_03580	Hypothetical transmembrane protein	M	Membrane	Other	1	1	1	1
98	XthA	659918711	CDP47985.1	PFCIRM129_08415	Exodeoxyribonuclease III/exonuclease III	L	Cytoplasmic	Other	1	1	1	1
99	SdhC1	659918268	CDP48451.1	PFCIRM129_03255	Succinate dehydrogenase subunit C	C	Membrane	TM	1	1	1	1
100	DapA	659917202	CDP49428.1	PFCIRM129_11825	Dihydrodipicolinate synthase	E	Cytoplasmic	Other	1	1	1	1
101	NuoB	659918732	CDP48006.1	PFCIRM129_08520	NADH-quinone oxidoreductase chain B	C	Cytoplasmic	Other	1	1	1	1
102	Pf279	659918679	CDP48082.1	PFCIRM129_08925	Carboxylic ester hydrolase	S	Membrane	Sec	1	1	1	1
103	MenJ	659918735	CDP48009.1	PFCIRM129_08535	electron transfer oxidoreductase	C	Cytoplasmic	Other	1	1	1	1
104	SkfE	659918738	CDP48012.1	PFCIRM129_08550	ABC transporter ATP-binding protein	V	Cytoplasmic	Other	1	1	1	1
105	BraC	659917558	CDP49093.1	PFCIRM129_09775	Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein [Precursor] (LIVAT-BP)	E	Extracellular	Lipo	1	1	1	1
106	Lpd	659917557	CDP49092.1	PFCIRM129_09770	Dihydrolipoyl dehydrogenase (E3 component of alpha keto acid dehydrogenase complexes) (Dihydrolipoamide dehydrogenase)	C	Cytoplasmic	Other	1	1	1	1

107	MurA	65991 7556	CDP490 91.1	PFCIRM129 _09765	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (Enoylpyruvate transferase)	M	Cytoplasmic	Other	1	1	1	1
108	CbiM	65991 8653	CDP480 56.1	PFCIRM129 _08795	Cobalt transport protein CbiM	P	Membrane	TM	1	1	1	1
109	CbiN	65991 8654	CDP480 57.1	PFCIRM129 _08800	Cobalt transport protein CbiN	P	Membrane	TM	1	1	1	1
110	Dpm	65991 7597	CDP490 86.1	PFCIRM129 _09740	dolichyl-phosphate beta-D-mannosyltransferase	S	Cytoplasmic	Other	1	1	1	1
111	GlpT	65991 7186	CDP494 52.1	PFCIRM129 _11990	glycerol-3-phosphate transporter	G	Membrane	TM	1	1	1	1
112	PFCIRM12 9_12095	65991 7162	CDP494 70.1	PFCIRM129 _12095	dehydrogenase/reductase	IQ	Cytoplasmic	Other	1	1	1	1
113	PFCIRM12 9_08715	65991 8637	CDP480 40.1	PFCIRM129 _08715	Hypothetical protein PFCIRM129_08715	KLT	Extracellular	Other	1	1	1	1
114	PFCIRM12 9_11950	65991 7195	CDP494 49.1	PFCIRM129 _11950	Hypothetical protein PFCIRM129_11950	H	Cytoplasmic	Other	1	1	1	1
115	SdhA	65991 8267	CDP484 50.1	PFCIRM129 _03250	Succinate dehydrogenase, subunit A	C	Cytoplasmic	Sec	1	1	1	1
116	PFCIRM12 9_09710	65991 7591	CDP490 80.1	PFCIRM129 _09710	transporter	P	Membrane	Lipo	1	1	1	1
117	Vpr	65991 7193	CDP494 47.1	PFCIRM129 _11940	Peptidase Subtilases	O	Extracellular	Other	1	1	1	1
118	PFCIRM12 9_11930	65991 7191	CDP494 45.1	PFCIRM129 _11930	Hypothetical protein PFCIRM129_11930	S	Cytoplasmic	Sec	1	1	1	1
119	FumC	65991 7216	CDP494 42.1	PFCIRM129 _11895	Fumarate hydratase, class-II	C	Cytoplasmic	Other	1	1	1	1

120	PFCIRM129_11875	659917212	CDP49438.1	PFCIRM129_11875	Hypothetical secreted protein	M	Extracellular	Lipo	1	1	1	1
121	DapD	659917211	CDP49437.1	PFCIRM129_11870	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	E	Cytoplasmic	Other	1	1	1	1
122	ZnuA	659918274	CDP48457.1	PFCIRM129_03285	ABC-transporter metal-binding lipoprotein	P	Membrane	Lipo	1	1	1	1
123	SlpA	659917603	CDP49065.1	PFCIRM129_09350	Surface layer protein A (S-layer protein A)	O	Extracellular	Sec	1	1	1	1
124	PFCIRM129_08670	659918631	CDP48034.1	PFCIRM129_08670	cell-wall peptidases, NlpC/P60 family secreted protein	M	Extracellular	Sec	1	1	1	1
125	CypA	659917133	CDP49510.1	PFCIRM129_12305	Cytochrome P450 (Heme-thiolate monooxygenase)	C	Cytoplasmic	Other	1	1	1	1
126	BkdB	659918587	CDP48089.1	PFCIRM129_09100	dihydrolipoyllysine-residue (2-methylpropanoyl)transferase. Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex (E2) (Dihydrolipoamide branched chain transacylase)	C	Cytoplasmic	Other	1	1	1	1

127	BkdA2	659918588	CDP48090.1	PFCIRM129_09105	2-oxoisovalerate dehydrogenase subunit beta (Branched-chain alpha-keto acid dehydrogenase E1 component beta chain) (BCKDH E1-beta) Pyruvate dehydrogenase E1 component subunit beta	C	Cytoplasmic	Other	1	1	1	1
128	HtrA2	659917538	CDP49118.1	PFCIRM129_09910	secreted serine protease, trypsin-like serine proteases	O	Extracellular	Other	1	1	1	1
129	GtfB	659918191	CDP48536.1	PFCIRM129_03710	Glycosyltransferase	S	Cytoplasmic	Other	1	1	1	1
130	RpsH	659918922	CDP47940.1	PFCIRM129_08165	30S ribosomal protein S8	J	Cytoplasmic	Other	1	1	1	1
131	RplF	659918921	CDP47939.1	PFCIRM129_08160	50S ribosomal protein L6	J	Cytoplasmic	Other	1	1	1	1
132	PFCIRM129_09915	659917539	CDP49119.1	PFCIRM129_09915	Hypothetical protein PFCIRM129_09915		Membrane	Lipo	1	1	1	1
133	RpsE	659918919	CDP47937.1	PFCIRM129_08150	30S ribosomal protein S5	J	Cytoplasmic	Other	1	1	1	1
134	Cys2	659917082	CDP49535.1	PFCIRM129_00390	cysteine synthase 2	E	Cytoplasmic	Other	1	1	1	1
135	MoeA1	659917722	CDP49036.1	PFCIRM129_06395	Molybdenum cofactor synthesis domain	H	Cytoplasmic	Other	1	1	1	1

136	PFCIRM129_00410	659917086	CDP49539.1	PFCIRM129_00410	glycine betaine/choline Binding protein of ABC transporter	M	Cytoplasmic	Lipo	1	1	1	1
137	BopA	659918913	CDP47931.1	PFCIRM129_08120	solute binding protein of the ABC transport system	E	Extracellular	Lipo	1	1	1	1
138	RplE	659918925	CDP47943.1	PFCIRM129_08180	50S ribosomal protein L5	J	Cytoplasmic	Other	1	1	1	1
139	OppD	659918912	CDP47930.1	PFCIRM129_08115	ABC2 protein of oligopeptide ABC transporter (OPN:undef)	P	Cytoplasmic	Other	1	1	1	1
140	PFCIRM129_11735	659917229	CDP49410.1	PFCIRM129_11735	Hypothetical protein PFCIRM129_11735		Extracellular	Lipo	1	1	1	1
141	AckA	659918198	CDP48543.1	PFCIRM129_03745	Acetate kinase	C	Cytoplasmic	Other	1	1	1	1
142	CstA	659917091	CDP49544.1	PFCIRM129_00435	Carbon starvation protein	T	Membrane	TM	1	1	1	1
143	Pta	659918199	CDP48544.1	PFCIRM129_03750	Phosphate acetyltransferase	C	Cytoplasmic	Other	1	1	1	1
144	GlnA1	659917240	CDP49409.1	PFCIRM129_11730	Glutamine synthetase	E	Cytoplasmic	Other	1	1	1	1
145	CodB	659916960	CDP49653.1	PFCIRM129_01370	Permease for cytosine/purines, uracil, thiamine,allantoin	F	Membrane	TM	1	1	1	1
146	RpsK	659918900	CDP47918.1	PFCIRM129_08055	30S ribosomal protein S11	J	Cytoplasmic	Other	1	1	1	1
147	RpsD	659918899	CDP47917.1	PFCIRM129_08050	30S ribosomal protein S4	J	Cytoplasmic	Other	1	1	1	1

148	RpoA	65991 8898	CDP479 16.1	PFCIRM129 _08045	DNA-directed RNA polymerase alpha chain (RNAP alpha subunit) (Transcriptase alpha chain) (RNA polymerase subunit alpha)	K	Cytoplasmic	Other	1	1	1	1
149	PFCIRM12 9_00865	65991 8386	CDP483 06.1	PFCIRM129 _00865	Hypothetical protein PFCIRM129_00865	S	Cytoplasmic	TM	1	1	1	1
150	PFCIRM12 9_08025	65991 8894	CDP479 12.1	PFCIRM129 _08025	Resuscitation-promoting factor RpfB	M	Membrane	Sec	1	1	1	1
151	Gpt	65991 7078	CDP495 31.1	PFCIRM129 _00370	Putative purine phosphoribosyltransferase	F	Cytoplasmic	Other	1	1	1	1
152	PFCIRM12 9_03205	65991 8258	CDP484 41.1	PFCIRM129 _03205	ATPases MoxR family	S	Cytoplasmic	Other	1	1	1	1
153	PFCIRM12 9_08360	65991 8703	CDP479 77.1	PFCIRM129 _08360	Hypothetical protein PFCIRM129_08360		Membrane	Other	1	1	1	1
154	BkdA1	65991 8589	CDP480 91.1	PFCIRM129 _09110	2-oxoisovalerate dehydrogenase subunit alpha (Branched-chain alpha-keto acid dehydrogenase E1 component alpha chain) (BCKDH E1-alpha)	C	Cytoplasmic	Other	1	1	1	1
155	SecE	65991 8701	CDP479 75.1	PFCIRM129 _08345	SecE/Sec61-gamma subunit of protein translocation complex	U	Cytoplasmic	Other	1	1	1	1
156	RplA	65991 8698	CDP479 72.1	PFCIRM129 _08330	50S ribosomal protein L1	J	Cytoplasmic	Other	1	1	1	1

157	RmlA	659918176	CDP48521.1	PFCIRM129_03625	Glucose-1-phosphate thymidylyltransferase		Cytoplasmic	Other	1	1	1	1
158	PFCIRM129_09870	659917577	CDP49112.1	PFCIRM129_09870	Hypothetical secreted protein		Cytoplasmic	Other	1	1	1	1
159	RplJ	659918695	CDP47969.1	PFCIRM129_08315	50S ribosomal protein L10	J	Cytoplasmic	Other	1	1	1	1
160	Gtf	659918179	CDP48524.1	PFCIRM129_03640	Glycosyl transferase, family 2	M	Cytoplasmic	Other	1	1	1	1
161	RpoB	659918692	CDP47966.1	PFCIRM129_08300	DNA-directed RNA polymerase beta chain (RNAP beta subunit) (Transcriptase beta chain) (RNA polymerase subunit beta)	K	Cytoplasmic	Other	1	1	1	1
162	RpoC	659918691	CDP47965.1	PFCIRM129_08295	DNA-directed RNA polymerase beta chain (RNAP beta subunit) (Transcriptase beta chain) (RNA polymerase beta subunit)	K	Cytoplasmic	Other	1	1	1	1
163	RplN	659918927	CDP47945.1	PFCIRM129_08190	50S ribosomal protein L14	J	Cytoplasmic	Other	1	1	1	1
164	FusA	659918688	CDP47962.1	PFCIRM129_08280	Elongation factor G (EF-G)	J	Cytoplasmic	Other	1	1	1	1
165	SlpD	659917219	CDP49418.1	PFCIRM129_11775	Surface protein D with SLH domain	O	Extracellular	Sec	1	1	1	1
166	RpsJ	659918938	CDP47956.1	PFCIRM129_08245	30S ribosomal protein S10	J	Cytoplasmic	Other	1	1	1	1

167	RplC	659918937	CDP47955.1	PFCIRM129_08240	50S ribosomal protein L3	J	Cytoplasmic	Other	1	1	1	1
168	RplD	659918936	CDP47954.1	PFCIRM129_08235	50S ribosomal protein L4	J	Cytoplasmic	Other	1	1	1	1
169	RplB	659918934	CDP47952.1	PFCIRM129_08225	50S ribosomal protein L2	J	Cytoplasmic	Other	1	1	1	1
170	RfbB	659918186	CDP48531.1	PFCIRM129_03675	DTDP-glucose 4,6-dehydratase	M	Cytoplasmic	Other	1	1	1	1
171	RplV	659918932	CDP47950.1	PFCIRM129_08215	50S ribosomal protein L22	J	Cytoplasmic	Other	1	1	1	1
172	RpsC	659918931	CDP47949.1	PFCIRM129_08210	30S ribosomal protein S3	J	Cytoplasmic	Other	1	1	1	1
173	PFCIRM129_11765	659917235	CDP49416.1	PFCIRM129_11765	Hypothetical protein PFCIRM129_11765		Extracellular	TM	1	1	1	1
174	GtfA	659918188	CDP48533.1	PFCIRM129_03695	Glycosyltransferase, family 2	S	Cytoplasmic	Other	1	1	1	1
175	RpsG	659918689	CDP47963.1	PFCIRM129_08285	30S ribosomal protein S7	J	Cytoplasmic	Other	1	1	1	1
176	PFCIRM129_06000	659917647	CDP48961.1	PFCIRM129_06000	Hypothetical protein PFCIRM129_06000		Extracellular	Lipo	1	1	1	1
177	PFCIRM129_07390	659918769	CDP47787.1	PFCIRM129_07390	Hypothetical protein PFCIRM129_07390		Membrane	TM	1	1	1	1
178	IspG	659917376	CDP49278.1	PFCIRM129_11045	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase)	I	Cytoplasmic	Other	1	1	1	1

179	PFCIRM129_01960	659916902	CDP49767.1	PFCIRM129_01960	inositol-1-phosphate synthase	I	Cytoplasmic	Other	1	1	1	1
180	ProS	659917374	CDP49276.1	PFCIRM129_11035	Prolyl-tRNA synthetase (Proline--tRNA ligase) (ProRS)	J	Cytoplasmic	Other	1	1	1	1
181	NorB	659918446	CDP48254.1	PFCIRM129_09425	Nitric-oxide reductase subunit B (nitric-oxide reductase)	P	Membrane	Other	1	1	1	1
182	Phk	659917936	CDP48739.1	PFCIRM129_04815	Phosphoketolase pyrophosphate	G	Cytoplasmic	Other	1	1	1	1
183	DeoR2	659917938	CDP48741.1	PFCIRM129_04825	DeoR transcriptional regulator	K	Cytoplasmic	Other	1	1	1	1
184	GlpC	659917939	CDP48742.1	PFCIRM129_04830	Anaerobic glycerol-3-phosphate dehydrogenase subunit C	C	Cytoplasmic	Other	1	1	1	1
185	GlpB	659917940	CDP48743.1	PFCIRM129_04835	Anaerobic glycerol-3-phosphate dehydrogenase subunit B	E	Cytoplasmic	Other	1	1	1	1
186	GlpA	659917941	CDP48744.1	PFCIRM129_04840	Anaerobic glycerol-3-phosphate dehydrogenase subunit A	C	Cytoplasmic	Other	1	1	1	1
187	HrdB	659917970	CDP48733.1	PFCIRM129_04775	RNA polymerase principal sigma factor HrdB	K	Cytoplasmic	Sec	1	1	1	1
188	Upp	659916834	CDP49778.1	PFCIRM129_02025	Uracil phosphoribosyltransferase (UMP pyrophosphorylase) (UPRTase)	F	Cytoplasmic	Other	1	1	1	1

189	InfB	659917370	CDP49272.1	PFCIRM129_11015	Translation initiation factor IF-2	J	Cytoplasmic	Other	1	1	1	1
190	PFCIRM129_02060	659916840	CDP49784.1	PFCIRM129_02060	Hypothetical protein PFCIRM129_02060	S	Membrane	TM	1	1	1	1
191	FeoB	659916842	CDP49786.1	PFCIRM129_02070	Ferrous iron uptake protein B 9.A.8.1.x	P	Membrane	Other	1	1	1	1
192	GlpK	659917366	CDP49268.1	PFCIRM129_10995	Glycerol kinase (ATP:glycerol 3-phosphotransferase) (Glycerokinase) (GK)	C	Cytoplasmic	Other	1	1	1	1
193	GalK2	659916844	CDP49788.1	PFCIRM129_02080	Galactokinase	G	Cytoplasmic	Other	1	1	1	1
194	PknB	659916820	CDP49789.1	PFCIRM129_02090	Serine/threonine protein kinase	KLT	Membrane	Other	1	1	1	1
195	PbpA	659916821	CDP49790.1	PFCIRM129_02095	Penicillin-binding protein	M	Extracellular	Sec	1	1	1	1
196	FtsW2	659916822	CDP49791.1	PFCIRM129_02100	Cell division protein	D	Membrane	TM	1	1	1	1
197	NrdJ	659917943	CDP48746.1	PFCIRM129_04850	Vitamin B12-dependent ribonucleotide reductase (Ribonucleoside-diphosphate reductase NrdJ)	F	Cytoplasmic	Other	1	1	1	1
198	RlmN	659917378	CDP49280.1	PFCIRM129_11055	Hypothetical protein PFCIRM129_11055	J	Cytoplasmic	Other	1	1	1	1
199	PstS	659916895	CDP49760.1	PFCIRM129_01925	binding protein of phosphate ABC transporter (BP:MOI:undef:phosphate)	P	Membrane	Lipo	1	1	1	1

200	PanE	65991 7966	CDP487 29.1	PFCIRM129 _04755	2-dehydropantoate 2-reductase	H	Cytoplasmic	Other	1	1	1	1
201	IolT3	65991 7518	CDP491 85.1	PFCIRM129 _10425	iolT3 (myo-inositol transporter iolT3)	EGP	Membrane	TM	1	1	1	1
202	Dxs	65991 8105	CDP487 04.1	PFCIRM129 _04620	Deoxyxylulose-5-phosphate synthase	H	Cytoplasmic	Other	1	1	1	1
203	HsdM1	65991 7285	CDP492 95.1	PFCIRM129 _11140	Type I restriction-modification system DNA methylase	V	Cytoplasmic	Other	1	1	1	1
204	Acn	65991 8109	CDP487 08.1	PFCIRM129 _04640	Aconitase, Aconitate hydratase	C	Cytoplasmic	Other	1	1	1	1
205	Mrr	65991 7284	CDP492 94.1	PFCIRM129 _11135	restriction system protein	L	Cytoplasmic	Other	1	1	1	1
206	PFCIRM12 9_06845	65991 8987	CDP476 88.1	PFCIRM129 _06845	Ribonuclease, Rne/Rng family	J	Cytoplasmic	Other	1	1	1	1
207	Gst	65991 7947	CDP487 10.1	PFCIRM129 _04660	glutathione S-transferase	O	Cytoplasmic	Other	1	1	1	1
208	YdfJ	65991 7797	CDP488 95.1	PFCIRM129 _05660	drug exporters of the RND superfamily	P	Membrane	TM	1	1	1	1
209	HsdS-2	65991 7283	CDP492 93.1	PFCIRM129 _11130	Putative uncharacterized protein	V	Cytoplasmic	Other	1	1	1	1
210	OdhA	65991 8335	CDP483 52.1	PFCIRM129 _02730	2-oxoglutarate dehydrogenase, E1 and E2 components	C	Cytoplasmic	Other	1	1	1	1
211	Asd	65991 6880	CDP497 45.1	PFCIRM129 _01850	Aspartate-semialdehyde dehydrogenase (Semialdehyde dehydrogenase)	E	Cytoplasmic	Other	1	1	1	1

212	FepC2	659917790	CDP48888.1	PFCIRM129_05625	Iron transport system substrate-binding protein ABC transporter	P	Extracellular	Lipo	1	1	1	1
213	LemA	659917526	CDP49193.1	PFCIRM129_10465	transmembrane protein	S	Extracellular	TM	1	1	1	1
214	PFCIRM129_05605	659917786	CDP48884.1	PFCIRM129_05605	Hypothetical protein PFCIRM129_05605	NU	Membrane	TM	1	1	1	1
215	PFCIRM129_10935	659918483	CDP48243.1	PFCIRM129_10935	Protein of unknown function		Cytoplasmic	Other	1	1	1	1
216	Gpm1	659917784	CDP48882.1	PFCIRM129_05595	phosphoglycerate mutase/fructose-2,6-bisphosphatase	G	Cytoplasmic	Other	1	1	1	1
217	MalE	659917783	CDP48881.1	PFCIRM129_05590	binding protein of maltose ABC transporter	G	Membrane	Lipo	1	1	1	1
218	Tsf	659917382	CDP49284.1	PFCIRM129_11075	Elongation factor Ts (EF-Ts)	J	Cytoplasmic	Other	1	1	1	1
219	FhaB	659916824	CDP49793.1	PFCIRM129_02110	Forkhead-associated protein	T	Cytoplasmic	TM	1	1	1	1
220	FhaA	659916825	CDP49794.1	PFCIRM129_02115	Forkhead-associated protein	T	Cytoplasmic	Other	1	1	1	1
221	PFCIRM129_00780	659918369	CDP48289.1	PFCIRM129_00780	Hypothetical protein PFCIRM129_00780		Cytoplasmic	Other	1	1	1	1
222	TrpB2	659919133	CDP47584.1	PFCIRM129_00160	Tryptophan synthase beta subunit	E	Cytoplasmic	Other	1	1	1	1
223	SlgT	659918370	CDP48290.1	PFCIRM129_00785	Sodium/glucose cotransporter (Na(+)/glucose symporter) 2.A.21.3.2	E	Membrane	TM	1	1	1	1

224	PFCIRM129_05105	659917879	CDP48792.1	PFCIRM129_05105	Hypothetical protein PFCIRM129_05105	M	Cytoplasmic	Other	1	1	1	1
225	PFCIRM129_02410	659916788	CDP49839.1	PFCIRM129_02410	Sulfate-binding protein precursor	P	Membrane	Lipo	1	1	1	1
226	PFCIRM129_10740	659917406	CDP49243.1	PFCIRM129_10740	Hypothetical protein PFCIRM129_10740	M	Extracellular	Other	1	1	1	1
227	DeoR3	659917856	CDP48826.1	PFCIRM129_05295	Putative regulatory protein, DeoR family	K	Cytoplasmic	Other	1	1	1	1
228	PntA	659916762	CDP49845.1	PFCIRM129_02445	NAD(P)(+) transhydrogenase (AB-specific)	C	Cytoplasmic	Other	1	1	1	1
229	PntB	659916763	CDP49846.1	PFCIRM129_02450	NADH dehydrogenase	C	Membrane	TM	1	1	1	1
230	RplT	659919116	CDP47567.1	PFCIRM129_00070	50S ribosomal protein L20	J	Cytoplasmic	Other	1	1	1	1
231	AtpG	659917888	CDP48801.1	PFCIRM129_05150	ATP synthase gamma chain (ATP synthase F1 sector gamma subunit)	C	Cytoplasmic	Other	1	1	1	1
232	PFCIRM129_10650	659917426	CDP49227.1	PFCIRM129_10650	Hypothetical protein PFCIRM129_10650	M	Extracellular	Other	1	1	1	1
233	PFCIRM129_05250	659917861	CDP48817.1	PFCIRM129_05250	2-dehydropantoate 2-reductase	H	Cytoplasmic	Other	1	1	1	1
234	PFCIRM129_05245	659917860	CDP48816.1	PFCIRM129_05245	Transcriptional regulator, RpiR family	K	Cytoplasmic	Other	1	1	1	1
235	PFCIRM129_10690	659917396	CDP49233.1	PFCIRM129_10690	Putative uncharacterized protein		Cytoplasmic	TM	1	1	1	1

236	PurC	659917409	CDP49246.1	PFCIRM129_10755	Phosphoribosylaminoimidazole-succinocarboxamidesynthase	F	Cytoplasmic	Other	1	1	1	1
237	PFCIRM129_06895	659918997	CDP47698.1	PFCIRM129_06895	thiredoxine like membrane protein	O	Cytoplasmic	Sec	1	1	1	1
238	LepB	659919139	CDP47590.1	PFCIRM129_00190	Signal peptidase I	U	Membrane	TM	1	1	1	1
239	SdhB3	659919141	CDP47592.1	PFCIRM129_00200	Succinate dehydrogenase	C	Cytoplasmic	Other	1	1	1	1
240	PFCIRM129_02195	659916818	CDP49799.1	PFCIRM129_02195	TRNA processing ribonuclease BN	S	Cytoplasmic	Other	1	1	1	1
241	SlpE	659917805	CDP48858.1	PFCIRM129_05460	Surface protein with SLH domain	O	Extracellular	Sec	1	1	1	1
242	PnpA	659917386	CDP49265.1	PFCIRM129_10980	Polyribonucleotide nucleotidyltransferase (Polynucleotide phosphorylase) (PNPase) (Guanosine pentaphosphate synthetase)	J	Cytoplasmic	Other	1	1	1	1
243	RecA	659917912	CDP48758.1	PFCIRM129_04920	RecA (Recombinase A)	L	Cytoplasmic	Other	1	1	1	1
244	PFCIRM129_10970	659917384	CDP49263.1	PFCIRM129_10970	Hypothetical protein PFCIRM129_10970	S	Membrane	Sec	1	1	1	1
245	PFCIRM129_02590	659919086	CDP47617.1	PFCIRM129_02590	Hypothetical transmembrane protein		Cytoplasmic	Other	1	1	1	1

246	PonA	65991 7443	CDP492 12.1	PFCIRM129 _10570	Penicillin-binding protein (Transglycosylase/transpeptid ase)	M	Extracellular	TM	1	1	1	1
247	SlpB	65991 8413	CDP482 73.1	PFCIRM129 _00700	Surface layer protein B (S-layer protein B)	O	Extracellular	Sec	1	1	1	1
248	PFCIRM12 9_00705	65991 8414	CDP482 74.1	PFCIRM129 _00705	Surface protein of unknown function		Membrane	Sec	1	1	1	1
249	PFCIRM12 9_00955	65991 8404	CDP483 24.1	PFCIRM129 _00955	transferase	J	Cytoplasmic	Other	1	1	1	1
250	MoxR	65991 8419	CDP482 79.1	PFCIRM129 _00730	Protein of unknown function	S	Cytoplasmic	Other	1	1	1	1
251	IclR	65991 7928	CDP487 74.1	PFCIRM129 _05000	IclR transcriptional regulator	K	Cytoplasmic	Other	1	1	1	1
252	PFCIRM12 9_05365	65991 7831	CDP488 40.1	PFCIRM129 _05365	Hypothetical protein PFCIRM129_05365		Extracellular	Lipo	1	1	1	1
253	FtsY	65991 9151	CDP476 02.1	PFCIRM129 _00250	Signal recognition particle receptor	U	Cytoplasmic	TM	1	1	1	1
254	Ffh	65991 9150	CDP476 01.1	PFCIRM129 _00245	GTP binding signal recognition particle protein	U	Cytoplasmic	Other	1	1	1	1
255	PFCIRM12 9_10785	65991 7415	CDP492 52.1	PFCIRM129 _10785	Hypothetical protein PFCIRM129_10785		Membrane	Lipo	1	1	1	1
256	PFCIRM12 9_10780	65991 7414	CDP492 51.1	PFCIRM129 _10780	Hypothetical protein PFCIRM129_10780		Extracellular	Lipo	1	1	1	1
257	SdhC2	65991 9143	CDP475 94.1	PFCIRM129 _00210	Succinate dehydrogenase cytochrome B-558 subunit	S	Membrane	TM	1	1	1	1
258	SdhA3	65991 9142	CDP475 93.1	PFCIRM129 _00205	Succinate dehydrogenase flavoprotein subunit	C	Cytoplasmic	Other	1	1	1	1

259	RplS	659919140	CDP47591.1	PFCIRM129_00195	50S ribosomal protein L19	J	Cytoplasmic	Other	1	1	1	1
260	InhA	659917290	CDP49300.1	PFCIRM129_11165	Enoyl-[acyl-carrier-protein] reductase (NADH)	I	Cytoplasmic	Other	1	1	1	1
261	Rho	659918327	CDP48344.1	PFCIRM129_02685	Transcription termination factor Rho	K	Cytoplasmic	Other	1	1	1	1
262	IolA	659917517	CDP49184.1	PFCIRM129_10420	iolA (Myo-inositol catabolism IolA protein) (Methylmalonic acid semialdehyde dehydrogenase)	C	Cytoplasmic	Other	1	1	1	1
263	PFCIRM129_09985	659918492	CDP48202.1	PFCIRM129_09985	Putative peptidyl-prolyl cis-trans isomerase,FKBP-type (Precursor)	O	Membrane	Sec	1	1	1	1
264	PFCIRM129_05935	659917764	CDP48948.1	PFCIRM129_05935	Hypothetical membrane protein	S	Cytoplasmic	Other	1	1	1	1
265	HemA	659917504	CDP49171.1	PFCIRM129_10355	Glutamyl-tRNA reductase	H	Cytoplasmic	Other	1	1	1	1
266	GsiB	659918299	CDP48372.1	PFCIRM129_02850	binding protein of oligopeptide ABC transporter (OPN : undef : Oligopeptides)	E	Extracellular	Lipo	1	1	1	1
267	PFCIRM129_07085	659919031	CDP47732.1	PFCIRM129_07085	ABC2 protein of ABC transporter (ART:REG)	S	Cytoplasmic	Other	1	1	1	1
268	AsnB	659917505	CDP49172.1	PFCIRM129_10360	Asparagine synthase (Glutamine-hydrolyzing)	E	Cytoplasmic	Other	1	1	1	1
269	PFCIRM129_07105	659919035	CDP47736.1	PFCIRM129_07105	ABC transporter	S	Cytoplasmic	Other	1	1	1	1

270	PFCIRM129_04325	659918052	CDP48651.1	PFCIRM129_04325	ABC transporter substrate-binding protein	S	Membrane	Lipo	1	1	1	1
271	MutA	659919057	CDP47758.1	PFCIRM129_07235	Methylmalonyl-CoA mutase small subunit (Methylmalonyl-CoA mutase beta subunit) (MCB-beta)	I	Cytoplasmic	Other	1	1	1	1
272	PdxT	659917307	CDP49317.1	PFCIRM129_11250	Glutamine amidotransferase subunit pdxT (Glutamine amidotransferase glutaminase subunit pdxT)	H	Cytoplasmic	Other	1	1	1	1
273	PyrG	659918500	CDP48210.1	PFCIRM129_10025	CTP synthase (UTP--ammonia ligase) (CTP synthetase)	F	Cytoplasmic	TM	1	1	1	1
274	Cat	659916973	CDP49666.1	PFCIRM129_01440	Coenzyme A transferase (Putative succinyl-CoA or butyryl-CoA:coenzyme A transferase)	C	Cytoplasmic	Other	1	1	1	1
275	PFCIRM129_07050	659919024	CDP47725.1	PFCIRM129_07050	oxidoreductase	C	Cytoplasmic	Other	1	1	1	1
276	UvrA3	659917331	CDP49341.1	PFCIRM129_11370	UvrABC system protein A (UvrA protein) (Excinuclease ABC subunit A)	L	Cytoplasmic	Other	1	1	1	1
277	CycA1	659916913	CDP49708.1	PFCIRM129_01665	D-serine/D-alanine/glycine transporter	E	Membrane	TM	1	1	1	1

278	PFCIRM129_02820	659918293	CDP48366.1	PFCIRM129_02820	D-3-phosphoglycerate dehydrogenase / erythronate 4-phosphate dehydrogenase	EH	Cytoplasmic	Other	1	1	1	1
279	CbiP	659918073	CDP48672.1	PFCIRM129_04440	Cobyric acid synthase CbiP/CobQ	H	Cytoplasmic	Other	1	1	1	1
280	CbiE	659919054	CDP47755.1	PFCIRM129_07220	precorrin methylase (precorrin-3B C17-methyltransferase) CbiE/G/H fusion protein	H	Cytoplasmic	Other	1	1	1	1
281	CbiX	659919053	CDP47754.1	PFCIRM129_07215	CysG/CbiX	H	Cytoplasmic	Other	1	1	1	1
282	PFCIRM129_02895	659918307	CDP48380.1	PFCIRM129_02895	Secreted protease with a PDZ domain	T	Membrane	Sec	1	1	1	1
283	MutB	659919058	CDP47759.1	PFCIRM129_07240	Methylmalonyl-CoA mutase large subunit (Methylmalonyl-CoA mutase alpha subunit) (MCM-alpha) (MUTB-(R)-2-Methyl-3-oxopropanoyl-CoA CoA-carbonylmutase)	I	Cytoplasmic	Other	1	1	1	1
284	XseA	659917756	CDP48940.1	PFCIRM129_05895	Exodeoxyribonuclease VII large subunit	L	Cytoplasmic	Sec	1	1	1	1
285	MetQ	659916945	CDP49684.1	PFCIRM129_01530	ABC-type transport systems, periplasmic component	P	Membrane	TM	1	1	1	1
286	PFCIRM129_09980	659918491	CDP48201.1	PFCIRM129_09980	peptidyl-prolyl cis-trans isomerase	O	Extracellular	Lipo	1	1	1	1

287	PFCIRM129_02885	659918305	CDP48378.1	PFCIRM129_02885	Hypothetical secreted and membrane protein	T	Membrane	TM	1	1	1	1
288	ThyA	659919062	CDP47763.1	PFCIRM129_07260	Thymidylate synthase	F	Cytoplasmic	Other	1	1	1	1
289	YqfL	659916937	CDP49676.1	PFCIRM129_01490	Hypothetical protein PFCIRM129_01490	S	Cytoplasmic	Other	1	1	1	1
290	PFCIRM129_07170	659919045	CDP47746.1	PFCIRM129_07170	Hypothetical protein PFCIRM129_07170	S	Cytoplasmic	Other	1	1	1	1
291	PFCIRM129_07150	659919042	CDP47743.1	PFCIRM129_07150	UDP-N-acetylmuramyl tripeptide synthase (Mur ligase)	M	Cytoplasmic	Other	1	1	1	1
292	RsmC	659916980	CDP49673.1	PFCIRM129_01475	rRNA (guanine-N2-)-methyltransferase	J	Cytoplasmic	Other	1	1	1	1
293	WhiA	659917322	CDP49332.1	PFCIRM129_11325	Hypothetical protein PFCIRM129_11325	K	Cytoplasmic	Other	1	1	1	1
294	MmdA	659917492	CDP49159.1	PFCIRM129_10295	Methylmalonyl-CoA carboxytransferase 12S subunit (Transcarboxylase 12S subunit). 610 bp	I	Cytoplasmic	Other	1	1	1	1
295	NudC	659918317	CDP48390.1	PFCIRM129_02945	NUDIX hydrolase	L	Cytoplasmic	Other	1	1	1	1
296	Dld	659917724	CDP48908.1	PFCIRM129_05730	D-lactate dehydrogenase	C	Cytoplasmic	Other	1	1	1	1
297	SufC	659917299	CDP49309.1	PFCIRM129_11210	ABC-type transport system involved in Fe-S cluster assembly, ATPase component, SufC	O	Cytoplasmic	Other	1	1	1	1

298	PFCIRM129_02785	659918346	CDP48363.1	PFCIRM129_02785	Hypothetical protein PFCIRM129_02785	S	Extracellular	Sec	1	1	1	1
299	Tal2	659917511	CDP49178.1	PFCIRM129_10390	Transaldolase 2	H	Cytoplasmic	Other	1	1	1	1
300	SufD	659917301	CDP49311.1	PFCIRM129_11220	FeS assembly protein SufD	O	Cytoplasmic	Other	1	1	1	1
301	Hpt	659916856	CDP49721.1	PFCIRM129_01730	hypoxanthine phosphoribosyltransferase	F	Cytoplasmic	Other	1	1	1	1
302	PFCIRM129_07345	659918760	CDP47778.1	PFCIRM129_07345	Hypothetical protein PFCIRM129_07345	S	Extracellular	Other	1	1	1	1
303	SufB	659917302	CDP49312.1	PFCIRM129_11225	FeS assembly protein SufB	O	Cytoplasmic	Other	1	1	1	1
304	Mao	659916915	CDP49710.1	PFCIRM129_01675	flavin-containing amine oxidase	E	Cytoplasmic	Other	1	1	1	1
305	TrpC	659918046	CDP48645.1	PFCIRM129_04295	Indole-3-glycerol phosphate synthase (TrpC)	E	Cytoplasmic	Other	1	1	1	1
306	RpsA	659917338	CDP49348.1	PFCIRM129_11405	30S ribosomal protein S1	J	Cytoplasmic	Other	1	1	1	1
307	Dac	659916853	CDP49718.1	PFCIRM129_01715	carboxypeptidase (serine-type D-Ala-D-Ala carboxypeptidase) (D-alanyl-D-alanine-carboxypeptidase)	M	Membrane	TM	1	1	1	1
308	PFCIRM129_07365	659918764	CDP47782.1	PFCIRM129_07365	Hypothetical protein PFCIRM129_07365	K	Cytoplasmic	Other	1	1	1	1
309	YajC	659918025	CDP48624.1	PFCIRM129_04190	Preprotein translocase subunit YajC	U	Cytoplasmic	TM	1	1	1	0

310	Pf456	65991 6823	CDP497 92.1	PFCIRM129 _02105	Hypothetical protein PFCIRM129_02105	T	Cytoplasmic	Other	1	1	1	0
311	Gpi	65991 7425	CDP492 26.1	PFCIRM129 _10645	Glucose-6-phosphate isomerase (GPI) (Phosphoglucose isomerase) (PGI) (Phosphohexose isomerase) (PHI)	G	Cytoplasmic	Other	1	1	1	0
312	RplQ	65991 8897	CDP479 15.1	PFCIRM129 _08040	50S ribosomal protein L17	J	Cytoplasmic	Other	1	1	0	1
313	PolA	65991 7341	CDP493 51.1	PFCIRM129 _11420	Putative DNA polymerase I	L	Cytoplasmic	Other	1	1	0	0
314	AcpP	65991 8250	CDP484 33.1	PFCIRM129 _03165	Acyl carrier protein (ACP)	IQ	Cytoplasmic	Other	1	1	0	0
315	BluB	65991 8866	CDP478 84.1	PFCIRM129 _07885	Phosphoribosyltransferase/nitroreductase (fusion gene) (Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase)	CH	Cytoplasmic	Other	1	1	0	0
316	CydB	65991 7024	CDP495 91.1	PFCIRM129 _01000	Cytochrome d ubiquinol oxidase, subunit II	C	Membrane	TM	1	1	0	0
317	GlyA	65991 7480	CDP491 47.1	PFCIRM129 _10235	Glycine hydroxymethyltransferase precursor	E	Cytoplasmic	Other	1	1	0	0
318	Mdh	65991 8819	CDP478 37.1	PFCIRM129 _07645	Malate dehydrogenase	C	Cytoplasmic	Other	1	1	0	0

319	PFCIRM129_11455	659917347	CDP49357.1	PFCIRM129_11455	Hypothetical protein PFCIRM129_11455	D	Cytoplasmic	Other	1	1	0	0
320	FtsX	659918231	CDP48414.1	PFCIRM129_03065	Cell division protein	D	Membrane	TM	1	1	0	0
321	NuoE	659918729	CDP48003.1	PFCIRM129_08505	NADH-quinone oxidoreductase chain E	C	Cytoplasmic	Other	1	1	0	0
322	NuoH	659918726	CDP48000.1	PFCIRM129_08490	NADH-quinone oxidoreductase subunit H (NADH dehydrogenase I subunit H)	C	Membrane	TM	1	1	0	0
323	NuoJ	659918724	CDP47998.1	PFCIRM129_08480	NADH-quinone oxidoreductase chain J (NADH dehydrogenase I, chain J)	C	Membrane	Other	1	1	0	0
324	Ald	659918161	CDP48506.1	PFCIRM129_03550	Alanine dehydrogenase	E	Cytoplasmic	Other	1	1	0	0
325	NuoM	659918721	CDP47995.1	PFCIRM129_08465	NADH dehydrogenase I chain M	C	Membrane	TM	1	1	0	0
326	CycA2	659918162	CDP48507.1	PFCIRM129_03555	D-serine/D-alanine/glycine transporter	E	Membrane	TM	1	1	0	0
327	AhpC	659918709	CDP47983.1	PFCIRM129_08400	Peroxiredoxin/Alkyl hydroperoxide reductase subunit C /Thioredoxin peroxidase/Alkyl hydroperoxide reductase protein C22/General stress protein 22	O	Cytoplasmic	Other	1	1	0	0

328	ProV	65991 7164	CDP494 72.1	PFCIRM129 _12105	ABC-type choline/glycine betaine transport,ATP-binding protein	E	Cytoplasmic	TM	1	1	0	0
329	Tal1	65991 8750	CDP480 24.1	PFCIRM129 _08610	Transaldolase 1	G	Cytoplasmic	Other	1	1	0	0
330	PFCIRM12 9_08605	65991 8749	CDP480 23.1	PFCIRM129 _08605	Hypothetical protein PFCIRM129_08605		Cytoplasmic	TM	1	1	0	0
331	PFCIRM12 9_09455	65991 7600	CDP490 67.1	PFCIRM129 _09455	Restriction endonuclease PvuRts1 I		Cytoplasmic	Other	1	1	0	0
332	PFCIRM12 9_03530	65991 8157	CDP485 02.1	PFCIRM129 _03530	transporter	EGP	Membrane	Other	1	1	0	0
333	PFCIRM12 9_00395	65991 7083	CDP495 36.1	PFCIRM129 _00395	Membrane protease subunits, stomatin/prohibitin homologs (Membrane protease subunit, stomatin/prohibitin homolog)	O	Cytoplasmic	TM	1	1	0	0
334	OppB	65991 8910	CDP479 28.1	PFCIRM129 _08105	IM protein of oligopeptide ABC transporter (OPN:undef)	EP	Membrane	TM	1	1	0	0
335	SecY	65991 8909	CDP479 27.1	PFCIRM129 _08100	Preprotein translocase SecY subunit	U	Membrane	TM	1	1	0	0
336	SdhB	65991 8266	CDP484 49.1	PFCIRM129 _03245	Succinate dehydrogenase, subunit B	C	Cytoplasmic	Other	1	1	0	0
337	PFCIRM12 9_11795	65991 7223	CDP494 22.1	PFCIRM129 _11795	Hypothetical protein PFCIRM129_11795		Membrane	Other	1	1	0	0

338	UgpQ2	65991 7149	CDP494 57.1	PFCIRM129 _12030	Glycerophosphoryl diester phosphodiesterase	C	Cytoplasmic	Other	1	1	0	0
339	PFCIRM12 9_02050	65991 6838	CDP497 82.1	PFCIRM129 _02050	Hypothetical protein PFCIRM129_02050		Cytoplasmic	Sec	1	1	0	0
340	Fba2	65991 7766	CDP489 02.1	PFCIRM129 _05695	Fructose-bisphosphate aldolase class I	G	Cytoplasmic	Other	1	1	0	0
341	Fba1	65991 7522	CDP491 89.1	PFCIRM129 _10445	Fructose-bisphosphate aldolase class II	G	Cytoplasmic	Other	1	1	0	0
342	Gpm2	65991 7487	CDP491 54.1	PFCIRM129 _10270	phosphoglycerate mutase	G	Cytoplasmic	Other	1	1	0	0
343	PFCIRM12 9_05480	65991 7809	CDP488 62.1	PFCIRM129 _05480	ABC transporter	Q	Membrane	TM	1	1	0	0
344	Pgm1	65991 7875	CDP487 88.1	PFCIRM129 _05085	Phosphoglucomutase	G	Cytoplasmic	Other	1	1	0	0
345	Epi	65991 7877	CDP487 90.1	PFCIRM129 _05095	Methylmalonyl-CoA epimerase	E	Cytoplasmic	Other	1	1	0	0
346	FtsH	65991 9123	CDP475 74.1	PFCIRM129 _00105	FtsH	O	Cytoplasmic	Other	1	1	0	0
347	AtpH	65991 7890	CDP488 03.1	PFCIRM129 _05160	ATP synthase delta chain	C	Cytoplasmic	Other	1	1	0	0
348	AtpF	65991 7891	CDP488 04.1	PFCIRM129 _05165	ATP synthase B chain (F0F1 ATP synthase subunit B)	C	Cytoplasmic	Other	1	1	0	0
349	PFCIRM12 9_00335	65991 8434	CDP482 66.1	PFCIRM129 _00335	Protein of unknown function	V	Cytoplasmic	Other	1	1	0	0
350	GlgP	65991 7868	CDP487 81.1	PFCIRM129 _05050	Alpha-glucan phosphorylase	G	Cytoplasmic	Other	1	1	0	0

351	DkgA	65991 6978	CDP496 71.1	PFCIRM129 _01465	Putative aldo/keto reductase (oxidoreductase)	S	Cytoplasmic	Other	1	1	0	0
352	PFCIRM12 9_05940	65991 7635	CDP489 49.1	PFCIRM129 _05940	Hypothetical protein PFCIRM129_05940		Cytoplasmic	Other	1	1	0	0
353	Tpi1	65991 7315	CDP493 25.1	PFCIRM129 _11290	triosephosphate isomerase 1	G	Cytoplasmic	Other	1	1	0	0
354	Pfp	65991 8060	CDP486 59.1	PFCIRM129 _04365	pyrophosphate phosphofructokinase	G	Cytoplasmic	Sec	1	1	0	0
355	MetN	65991 6946	CDP496 85.1	PFCIRM129 _01535	Methionine import ATP-binding protein metN	P	Cytoplasmic	Other	1	1	0	0
356	Pgk	65991 7316	CDP493 26.1	PFCIRM129 _11295	Phosphoglycerate kinase	G	Cytoplasmic	Other	1	1	0	0
357	PepN	65991 9018	CDP477 19.1	PFCIRM129 _07020	Aminopeptidase N, Lysyl aminopeptidase	E	Cytoplasmic	Other	1	1	0	0
358	Gab	65991 7339	CDP493 49.1	PFCIRM129 _11410	Aldehyde dehydrogenase (Succinate-semialdehyde dehydrogenase) (NAD-dependent aldehyde dehydrogenase)	C	Cytoplasmic	Other	1	1	0	0
359	Rnj	65991 9164	CDP476 15.1	PFCIRM129 _00315	Beta-lactamase-like:RNA-metabolising metallo-beta-lactamase	S	Cytoplasmic	Other	1	0	1	1
360	DhbC	65991 8736	CDP480 10.1	PFCIRM129 _08540	Menaquinone-specific isochorismate synthase	HQ	Cytoplasmic	Other	1	0	1	1
361	SerS1	65991 6997	CDP496 24.1	PFCIRM129 _01200	Seryl-tRNA synthetase	J	Cytoplasmic	Other	1	0	1	1

362	GlmU	65991 7669	CDP489 83.1	PFCIRM129 _06115	UDP-N-acetylglucosamine pyrophosphorylase	M	Cytoplasmic	Other	1	0	1	1
363	Hom	65991 8329	CDP483 46.1	PFCIRM129 _02695	Homoserine dehydrogenase	E	Cytoplasmic	Other	1	0	1	1
364	OpcA	65991 7311	CDP493 21.1	PFCIRM129 _11270	glucose 6-phosphate dehydrogenase effector OpcA	G	Cytoplasmic	TM	1	0	1	1
365	HelD	65991 7247	CDP493 80.1	PFCIRM129 _11575	Helicase protein	L	Cytoplasmic	Other	1	0	1	1
366	CysN	65991 6784	CDP498 35.1	PFCIRM129 _02390	Sulfate adenylyltransferase, large subunit/ ATP-sulfurylase, subunit 1 (ATP:sulfate adenylyltransferase)	P	Cytoplasmic	Other	1	0	1	1
367	IolE1	65991 7508	CDP491 75.1	PFCIRM129 _10375	IolE (Myo-inositol catabolism IolAEprotein) (Inosose dehydratase) (2-keto-myo-inositol dehydratase)	G	Cytoplasmic	Other	1	0	1	1
368	DnaN	65991 7808	CDP488 61.1	PFCIRM129 _05475	DNA polymerase III, beta chain	L	Cytoplasmic	Other	1	0	1	1
369	MetF	65991 7248	CDP493 81.1	PFCIRM129 _11580	5,10-methylenetetrahydrofolate reductase	E	Cytoplasmic	Other	1	0	1	1
370	AroH	65991 7253	CDP493 86.1	PFCIRM129 _11605	Phospho-2-dehydro-3-deoxyheptonate aldolase	E	Cytoplasmic	Other	1	0	1	1
371	LacZ	65991 7113	CDP495 26.1	PFCIRM129 _12415	Beta-galactosidase (Lactase) LacZ	G	Cytoplasmic	Other	1	0	1	1

372	IolC	65991 7513	CDP491 80.1	PFCIRM129 _10400	iolC (Myo-inositol catabolism iolC protein)	G	Cytoplasmic	Other	1	0	1	1
373	ThrS	65991 8097	CDP486 96.1	PFCIRM129 _04560	Threonyl-tRNA synthetase (Threonine--tRNA ligase) (ThrRS)	J	Cytoplasmic	Other	1	0	1	1
374	Nfo	65991 8119	CDP485 54.1	PFCIRM129 _03810	endonuclease IV (deoxyribonuclease IV (phage-T4-induced))	L	Cytoplasmic	Other	1	0	1	1
375	HepA	65991 8116	CDP485 51.1	PFCIRM129 _03795	Helicase (SNF2-related protein)	L	Cytoplasmic	Other	1	0	1	1
376	GlgX	65991 9098	CDP476 29.1	PFCIRM129 _02665	Glycogen debranching enzyme GlgX	G	Cytoplasmic	Other	1	0	1	1
377	PurQ	65991 7407	CDP492 44.1	PFCIRM129 _10745	Phosphoribosylformylglycinamidine synthase 1 (Phosphoribosylformylglycinamidine synthase I) (FGAM synthase I)	F	Cytoplasmic	Other	1	0	1	1
378	NirA2	65991 6787	CDP498 38.1	PFCIRM129 _02405	Sulfite reductase [ferredoxin]	C	Cytoplasmic	Other	1	0	1	1
379	UvrB	65991 7335	CDP493 45.1	PFCIRM129 _11390	UvrABC system protein B (Protein uvrB) (Excinuclease ABC subunit B)	L	Cytoplasmic	Other	1	0	1	1
380	GyrB2	65991 8140	CDP484 85.1	PFCIRM129 _03435	DNA gyrase subunit B	L	Cytoplasmic	Other	1	0	1	1
381	PFCIRM12 9_01355	65991 6957	CDP496 50.1	PFCIRM129 _01355	Putative uncharacterized protein	L	Cytoplasmic	Other	1	0	1	1

382	DapL	65991 7213	CDP494 39.1	PFCIRM129 _11880	aminotransferase	E	Cytoplasmic	Other	1	0	1	1
383	PurF	65991 8636	CDP480 39.1	PFCIRM129 _08710	Amidophosphoribosyltransfer ase	F	Cytoplasmic	Other	1	0	1	1
384	PFCIRM12 9_01505	65991 6940	CDP496 79.1	PFCIRM129 _01505	Hypothetical protein PFCIRM129_01505	T	Cytoplasmic	Other	1	0	0	0
385	Tkt	65991 7825	CDP488 34.1	PFCIRM129 _05335	Transketolase	G	Cytoplasmic	Other	1	0	0	0
386	Icd	65991 8818	CDP478 36.1	PFCIRM129 _07640	Putative isocitrate/isopropylmalate dehydrogenase	C	Cytoplasmic	Other	1	0	0	0
387	TopA	65991 8405	CDP483 25.1	PFCIRM129 _00960	DNA topoisomerase I	L	Cytoplasmic	Other	1	0	0	0
388	Gnd2	65991 9019	CDP477 20.1	PFCIRM129 _07025	6-phosphogluconate dehydrogenase,decarboxylatin g	G	Cytoplasmic	Other	1	0	0	0
389	SufS	65991 7298	CDP493 08.1	PFCIRM129 _11205	Cysteine desulphurases, SufS	E	Cytoplasmic	Other	1	0	0	0
390	FabF	65991 8251	CDP484 34.1	PFCIRM129 _03170	3-oxoacyl-[acyl-carrier-protein] synthase (Beta-ketoacyl-ACP synthase)	I	Cytoplasmic	Other	1	0	0	0
391	PFCIRM12 9_01765	65991 6863	CDP497 28.1	PFCIRM129 _01765	Lsr2-like protein	S	Cytoplasmic	Other	0	1	0	0
392	PFCIRM12 9_10305	65991 7494	CDP491 61.1	PFCIRM129 _10305	Methylmalonyl-CoA carboxytransferase 5S subunit. (transcarboxylase 5S) 505 bp	C	Cytoplasmic	Other	0	0	1	1

393	PFCIRM129_10275	659917488	CDP49155.1	PFCIRM129_10275	Regulatory protein	K	Cytoplasmic	Other	0	0	1	1
394	CE1826	659917450	CDP49219.1	PFCIRM129_10605	PTS enzyme I	G	Cytoplasmic	Sec	0	0	1	1
395	PFCIRM129_09820	659917567	CDP49102.1	PFCIRM129_09820	Putative uncharacterized protein	S	Membrane	TM	0	0	1	1
396	Bccp	659917490	CDP49157.1	PFCIRM129_10285	Methylmalonyl-CoA carboxytransferase, 1.3S subunit (Transcarboxylase, 1.3S subunit). 123bp	C	Cytoplasmic	Other	0	0	1	1
397	PFCIRM129_10630	659917455	CDP49224.1	PFCIRM129_10630	Hypothetical membrane protein	O	Membrane	Other	0	0	1	1
398	PFCIRM129_10165	659917470	CDP49137.1	PFCIRM129_10165	Hypothetical protein PFCIRM129_10165	T	Cytoplasmic	Other	0	0	1	1
399	SrtA	659917528	CDP49195.1	PFCIRM129_10475	Sortase family protein	M	Membrane	Sec	0	0	1	1
400	SdaA	659917467	CDP49134.1	PFCIRM129_10150	L-serine dehydratase (L-serine ammonia-lyase)	E	Cytoplasmic	Other	0	0	1	1
401	DedD	659917525	CDP49192.1	PFCIRM129_10460	Conserved membrane protein (DedA family)	I	Membrane	TM	0	0	1	1
402	SpoU3	659917524	CDP49191.1	PFCIRM129_10455	TRNA/rRNA methyltransferase	J	Cytoplasmic	Other	0	0	1	1
403	PFCIRM129_10395	659917512	CDP49179.1	PFCIRM129_10395	GntR-family protein transcriptional regulator	K	Cytoplasmic	Other	0	0	1	1
404	PFCIRM129_10435	659917520	CDP49187.1	PFCIRM129_10435	Zinc-containing alcohol dehydrogenase superfamily	C	Cytoplasmic	Other	0	0	1	1

405	IolD	65991 7516	CDP491 83.1	PFCIRM129 _10415	iolD (Myo-inositol catabolism iolD protein) (acetolactate synthase protein) (pyruvate:pyruvate acetaldehydetransferase (decarboxylating))	E	Cytoplasmic	Other	0	0	1	1
406	PFCIRM12 9_10490	65991 7531	CDP491 98.1	PFCIRM129 _10490	Oligopeptide transporter, OPT family protein	S	Membrane	TM	0	0	1	1
407	PFCIRM12 9_10545	65991 7439	CDP492 08.1	PFCIRM129 _10545	multicopper oxidase		Extracellular	Lipo	0	0	1	1
408	HemY	65991 7500	CDP491 67.1	PFCIRM129 _10335	Protoporphyrinogen oxidase (PPO) HemY	H	Cytoplasmic	Other	0	0	1	1
409	PFCIRM12 9_10340	65991 7501	CDP491 68.1	PFCIRM129 _10340	Fe-S oxidoreductase	C	Cytoplasmic	Other	0	0	1	1
410	AldB	65991 7434	CDP492 03.1	PFCIRM129 _10520	Lactaldehyde dehydrogenase	C	Cytoplasmic	Other	0	0	1	1
411	IspD	65991 7482	CDP491 49.1	PFCIRM129 _10245	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (4-diphosphocytidyl-2C-methyl-D-erythritol synthase) (MEP cytidylyltransferase) (MCT)	I	Cytoplasmic	Other	0	0	1	1
412	PFCIRM12 9_05700	65991 7767	CDP489 03.1	PFCIRM129 _05700	Hypothetical protein PFCIRM129_05700		Membrane	TM	0	0	1	1
413	GltA1	65991 7802	CDP489 00.1	PFCIRM129 _05685	Citrate synthase	C	Cytoplasmic	Other	0	0	1	1

414	YghZ	659917801	CDP48899.1	PFCIRM129_05680	Oxidoreductase	C	Cytoplasmic	Other	0	0	1	1
415	UppS	659917759	CDP48943.1	PFCIRM129_05910	Undecaprenyl pyrophosphate synthase (di-trans,poly-cis-decaprenylcistransferase)	I	Cytoplasmic	Other	0	0	1	1
416	ApeB	659917752	CDP48936.1	PFCIRM129_05875	Aminopeptidase	E	Cytoplasmic	Other	0	0	1	1
417	PFCIRM129_05845	659917746	CDP48930.1	PFCIRM129_05845	Abortive infection bacteriophage resistance protein Abi	V	Cytoplasmic	Other	0	0	1	1
418	PcnB	659917788	CDP48886.1	PFCIRM129_05615	tRNA nucleotidyltransferase PcnB	J	Cytoplasmic	Other	0	0	1	1
419	PrsA	659917835	CDP48844.1	PFCIRM129_05385	ribose-phosphate pyrophosphokinase (RPPK) (Phosphoribosyl pyrophosphate synthetase) (P-Rib-PP synthetase) (PRPP synthetase)	EF	Cytoplasmic	Other	0	0	1	1
420	LutR	659917853	CDP48823.1	PFCIRM129_05280	Transcriptional Regulator, GntR family protein	K	Cytoplasmic	Other	0	0	1	1
421	GidB	659917775	CDP48873.1	PFCIRM129_05550	Methyltransferase gidB (Glucose-inhibited division protein B)	J	Cytoplasmic	Other	0	0	1	1
422	OxaA	659917774	CDP48872.1	PFCIRM129_05540	Conserved membrane protein	U	Membrane	TM	0	0	1	1

423	DnaA	65991 7815	CDP488 68.1	PFCIRM129 _05510	Chromosomal replication initiator protein	L	Cytoplasmic	Other	0	0	1	1
424	RecF	65991 7807	CDP488 60.1	PFCIRM129 _05470	DNA replication and repair protein recF	L	Cytoplasmic	Other	0	0	1	1
425	DtxR	65991 7626	CDP490 49.1	PFCIRM129 _06475	Iron-dependent repressor	K	Cytoplasmic	Other	0	0	1	1
426	PFCIRM12 9_06350	65991 7715	CDP490 29.1	PFCIRM129 _06350	Hypothetical protein PFCIRM129_06350	T	Cytoplasmic	Other	0	0	1	1
427	PFCIRM12 9_06340	65991 7713	CDP490 27.1	PFCIRM129 _06340	Two-component system response regulator	T	Cytoplasmic	Other	0	0	1	1
428	Lnt	65991 7595	CDP490 84.1	PFCIRM129 _09730	Hypothetical protein PFCIRM129_09730	M	Membrane	TM	0	0	1	1
429	MetW	65991 7593	CDP490 82.1	PFCIRM129 _09720	Methionine biosynthesis protein MetW	Q	Cytoplasmic	Other	0	0	1	1
430	Pf3022	65991 7592	CDP490 81.1	PFCIRM129 _09715	Homoserine O-acetyltransferase	E	Cytoplasmic	Other	0	0	1	1
431	RedLA2	65991 7581	CDP490 70.1	PFCIRM129 _09650	Putative uncharacterized protein	IQ	Cytoplasmic	Other	0	0	1	1
432	ActII-1	65991 7580	CDP490 69.1	PFCIRM129 _09645	Putative uncharacterized protein	K	Cytoplasmic	TM	0	0	1	1
433	PFCIRM12 9_09640	65991 7579	CDP490 68.1	PFCIRM129 _09640	Prolipoprotein diacylglyceryl transferase		Membrane	TM	0	0	1	1
434	Prs	65991 7668	CDP489 82.1	PFCIRM129 _06110	Ribose-phosphate pyrophosphokinase	F	Cytoplasmic	Other	0	0	1	1
435	Mfd	65991 7663	CDP489 77.1	PFCIRM129 _06085	Transcription-repair coupling factor	L	Cytoplasmic	Other	0	0	1	1
436	PFCIRM12 9_06040	65991 7655	CDP489 69.1	PFCIRM129 _06040	tetR-family transcriptional regulator	K	Cytoplasmic	Other	0	0	1	1

437	Mpg	65991 7646	CDP489 60.1	PFCIRM129 _05995	3-methyladenine DNA glycosylase	L	Cytoplasmic	Other	0	0	1	1
438	PFCIRM12 9_05980	65991 7643	CDP489 57.1	PFCIRM129 _05980	Hypothetical protein PFCIRM129_05980		Membrane	TM	0	0	1	1
439	DnaJ1	65991 7706	CDP490 20.1	PFCIRM129 _06305	Chaperone protein dnaJ 1 (DnaJ1 protein) (Heat shock protein 40 1)	O	Cytoplasmic	Other	0	0	1	1
440	HspR1	65991 7705	CDP490 19.1	PFCIRM129 _06300	Heat shock protein transcriptional repressor HspR1 (Hspr1 protein)	K	Cytoplasmic	Other	0	0	1	1
441	UhpT	65991 6962	CDP496 55.1	PFCIRM129 _01380	Major facilitator superfamily MFS_1	G	Membrane	TM	0	0	1	1
442	AatB	65991 6987	CDP496 41.1	PFCIRM129 _01300	Leucyl/phenylalanyl-tRNA-protein transferase	O	Cytoplasmic	Other	0	0	1	1
443	FixC	65991 7063	CDP495 78.1	PFCIRM129 _00635	Electron transfer flavoprotein-quinone oxidoreductase (FixC protein)	C	Cytoplasmic	Other	0	0	1	1
444	PFCIRM12 9_01220	65991 7001	CDP496 28.1	PFCIRM129 _01220	Hypothetical protein PFCIRM129_01220	EH	Cytoplasmic	Other	0	0	1	1
445	PFCIRM12 9_01115	65991 7019	CDP496 11.1	PFCIRM129 _01115	Cell envelope-related transcriptional attenuator	K	Extracellular	Other	0	0	1	1
446	UgpQ1	65991 7040	CDP496 07.1	PFCIRM129 _01080	Glycerophosphoryl diester phosphodiesterase	C	Cytoplasmic	Other	0	0	1	1
447	PFCIRM12 9_01155	65991 7014	CDP496 18.1	PFCIRM129 _01155	extracellular protein without function		Membrane	Sec	0	0	1	1
448	PFCIRM12 9_02270	65991 6805	CDP498 13.1	PFCIRM129 _02270	Major facilitator super family MSF1	S	Membrane	TM	0	0	1	1

449	CysD	659916785	CDP49836.1	PFCIRM129_02395	Sulfate adenylyltransferase subunit 2 (Sulfate adenylate transferase) (SAT) (ATP-sulfurylase small subunit) (Mitomycin biosynthesis protein V)	EH	Cytoplasmic	TM	0	0	1	1
450	PFCIRM129_02380	659916782	CDP49833.1	PFCIRM129_02380	Methylase	H	Cytoplasmic	Other	0	0	1	1
451	DisA	659916874	CDP49739.1	PFCIRM129_01820	DNA integrity scanning protein disA	L	Cytoplasmic	Other	0	0	1	1
452	FolE	659916858	CDP49723.1	PFCIRM129_01740	GTP cyclohydrolase I (GTP-CH-I)	H	Cytoplasmic	Other	0	0	1	1
453	PFCIRM129_01720	659916854	CDP49719.1	PFCIRM129_01720	Hypothetical protein PFCIRM129_01720	S	Cytoplasmic	Other	0	0	1	1
454	PFCIRM129_01710	659916852	CDP49717.1	PFCIRM129_01710	Inorganic pyrophosphatase		Cytoplasmic	Other	0	0	1	1
455	TypA	659916830	CDP49774.1	PFCIRM129_01995	GTP-binding protein typA/BipA (Tyrosine phosphorylated protein A)	T	Cytoplasmic	Other	0	0	1	1
456	PFCIRM129_01880	659916886	CDP49751.1	PFCIRM129_01880	secreted glycosyl hydrolase	G	Membrane	Lipo	0	0	1	1
457	Fbp	659916884	CDP49749.1	PFCIRM129_01870	phosphoglycerate mutase/Fructose-2,6-bisphosphatase	G	Cytoplasmic	Other	0	0	1	1
458	MshD	659916898	CDP49763.1	PFCIRM129_01940	GCN5-related N-acetyltransferase	K	Cytoplasmic	Other	0	0	1	1

459	PFCIRM129_00505	659917105	CDP49558.1	PFCIRM129_00505	Transcriptional regulator (Glycerol uptake operon antiterminator regulatory protein)	K	Cytoplasmic	Other	0	0	1	1
460	YhbJ	659917324	CDP49334.1	PFCIRM129_11335	ATPase	S	Cytoplasmic	Other	0	0	1	1
461	Gap	659917317	CDP49327.1	PFCIRM129_11300	Glyceraldehyde-3-phosphate dehydrogenase / erythrose 4 phosphate dehydrogenase	G	Cytoplasmic	Other	0	0	1	1
462	Zwf	659917310	CDP49320.1	PFCIRM129_11265	Glucose-6-phosphate 1-dehydrogenase	G	Cytoplasmic	Other	0	0	1	1
463	PFCIRM129_11470	659917350	CDP49360.1	PFCIRM129_11470	Hypothetical protein PFCIRM129_11470	S	Cytoplasmic	Other	0	0	1	1
464	FtsQ	659917352	CDP49362.1	PFCIRM129_11480	Cell division protein FtsQ	D	Membrane	TM	0	0	1	1
465	PFCIRM129_11620	659917256	CDP49389.1	PFCIRM129_11620	Peptidase M23B family / metalloendopeptidase	D	Extracellular	Sec	0	0	1	1
466	PlsC3	659917255	CDP49388.1	PFCIRM129_11615	Acyltransferase PlsC	I	Cytoplasmic	Other	0	0	1	1
467	PFCIRM129_11550	659917242	CDP49375.1	PFCIRM129_11550	Hypothetical membrane protein	S	Cytoplasmic	TM	0	0	1	1

468	MurE	659917359	CDP49369.1	PFCIRM129_11515	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (UDP-N-acetylmuramyl-tripeptide synthetase) (Meso-diaminopimelate-adding enzyme) (UDP-MurNAc-tripeptide synthetase)	M	Cytoplasmic	Other	0	0	1	1
469	MurF	659917358	CDP49368.1	PFCIRM129_11510	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase (UDP-MurNAc-pentapeptide synthetase) (D-alanyl-D-alanine-adding enzyme)	M	Cytoplasmic	Other	0	0	1	1
470	FtsW1	659917355	CDP49365.1	PFCIRM129_11495	Cell division protein FtsW	D	Membrane	TM	0	0	1	1
471	EcsA	659917305	CDP49315.1	PFCIRM129_11240	ABC transporter, ATP-binding protein	V	Cytoplasmic	Other	0	0	1	1
472	GlpF	659917367	CDP49269.1	PFCIRM129_11000	Glycerol uptake facilitator protein	G	Membrane	TM	0	0	1	1
473	RibF	659917388	CDP49267.1	PFCIRM129_10990	Riboflavin biosynthesis protein (Riboflavin kinase) FAD synthetase	H	Cytoplasmic	Other	0	0	1	1
474	PFCIRM129_10715	659917401	CDP49238.1	PFCIRM129_10715	Secreted protein of unknown function		Extracellular	Lipo	0	0	1	1

475	PFCIRM129_11190	659917295	CDP49305.1	PFCIRM129_11190	ABC2 protein of ABC transporter (ART:REG)	S	Cytoplasmic	Other	0	0	1	1
476	MyrA	659917293	CDP49303.1	PFCIRM129_11180	resistance protein	Q	Cytoplasmic	Other	0	0	1	1
477	FabG	659917291	CDP49301.1	PFCIRM129_11170	3-oxoacyl-[acyl-carrier protein] reductase	IQ	Cytoplasmic	Other	0	0	1	1
478	PPA1530	659917288	CDP49298.1	PFCIRM129_11155	ABC transporter ATP-binding protein	P	Cytoplasmic	Other	0	0	1	1
479	PyrH	659917381	CDP49283.1	PFCIRM129_11070	Uridylate kinase PyrH	F	Cytoplasmic	Other	0	0	1	1
480	PFCIRM129_11040	659917375	CDP49277.1	PFCIRM129_11040	Acetyltransferase family protein	S	Cytoplasmic	Other	0	0	1	1
481	PFCIRM129_11030	659917373	CDP49275.1	PFCIRM129_11030	Hypothetical protein PFCIRM129_11030		Membrane	Sec	0	0	1	1
482	PFCIRM129_11695	659917271	CDP49404.1	PFCIRM129_11695	Hypothetical protein PFCIRM129_11695	S	Cytoplasmic	Other	0	0	1	1
483	PFCIRM129_12315	659917135	CDP49512.1	PFCIRM129_12315	Hypothetical protein PFCIRM129_12315		Extracellular	Other	0	0	1	1
484	PFCIRM129_12310	659917134	CDP49511.1	PFCIRM129_12310	Aldo/keto reductases, related to diketogulonate reductase	K	Cytoplasmic	Other	0	0	1	1
485	PigV	659917125	CDP49502.1	PFCIRM129_12260	GPI mannosyltransferase 2	S	Membrane	TM	0	0	1	1
486	DadA1	659917098	CDP49551.1	PFCIRM129_00470	DadA, Glycine/D-amino acid oxidases	C	Cytoplasmic	Other	0	0	1	1
487	GalE1	659917111	CDP49524.1	PFCIRM129_12405	UDP-glucose 4-epimerase	M	Cytoplasmic	Other	0	0	1	1

488	RplI	659917179	CDP49487.1	PFCIRM129_12185	50S ribosomal protein L9	J	Cytoplasmic	Other	0	0	1	1
489	PFCIRM129_11920	659917198	CDP49444.1	PFCIRM129_11920	secreted transglycosydase	M	Extracellular	Sec	0	0	1	1
490	PF47	659917227	CDP49426.1	PFCIRM129_11815	Protein mrp homolog (ATP-binding protein)	D	Cytoplasmic	Other	0	0	1	1
491	PFCIRM129_11755	659917233	CDP49414.1	PFCIRM129_11755	Hypothetical protein PFCIRM129_11755	I	Cytoplasmic	Other	0	0	1	1
492	GlnE	659917232	CDP49413.1	PFCIRM129_11750	Glutamate-ammonia-ligase adenylyltransferase ([Glutamate--ammonia-ligase] adenylyltransferase) (Glutamine-synthetase adenylyltransferase) (ATase)	H	Cytoplasmic	Other	0	0	1	1
493	MgtE	659917225	CDP49424.1	PFCIRM129_11805	magnesium (Mg2+) transporter	P	Cytoplasmic	Other	0	0	1	1
494	LacI4	659917154	CDP49462.1	PFCIRM129_12055	Transcriptional regulator, LacI family (HTH-type transcriptional regulator degA)	K	Cytoplasmic	Other	0	0	1	1
495	LipB	659918697	CDP47971.1	PFCIRM129_08325	Lipoyltransferase (Lipoyl-[acyl-carrier-protein]-protein-N-lipoyltransferase) (Lipoate-protein ligase B)	H	Cytoplasmic	Other	0	0	1	1

496	RpsL	659918690	CDP47964.1	PFCIRM129_08290	30S ribosomal protein S12	J	Cytoplasmic	Other	0	0	1	1
497	Tuf	659918687	CDP47961.1	PFCIRM129_08275	Elongation factor Tu	J	Cytoplasmic	Other	0	0	1	1
498	RplP	659918930	CDP47948.1	PFCIRM129_08205	50S ribosomal protein L16	J	Cytoplasmic	Other	0	0	1	1
499	LipA	659918696	CDP47970.1	PFCIRM129_08320	Lipoic acid synthetase	H	Cytoplasmic	Other	0	0	1	1
500	ArsR2	659918756	CDP48030.1	PFCIRM129_08640	Transcriptional regulator, ArsR family	K	Cytoplasmic	Other	0	0	1	1
501	PFCIRM129_08620	659918752	CDP48026.1	PFCIRM129_08620	Zinc-containing alcohol dehydrogenase superfamily	C	Cytoplasmic	Other	0	0	1	1
502	UbiE	659918739	CDP48013.1	PFCIRM129_08555	Menaquinone biosynthesis methyltransferase ubiE	H	Cytoplasmic	Other	0	0	1	1
503	RpsN1	659918923	CDP47941.1	PFCIRM129_08170	30S ribosomal protein S14 type Z	J	Cytoplasmic	Other	0	0	1	1
504	RplR	659918920	CDP47938.1	PFCIRM129_08155	Ribosomal protein L18	J	Cytoplasmic	Other	0	0	1	1
505	PFCIRM129_07910	659918871	CDP47889.1	PFCIRM129_07910	Hypothetical protein PFCIRM129_07910	L	Cytoplasmic	Other	0	0	1	1
506	TsaB	659918869	CDP47887.1	PFCIRM129_07900	Peptidase, family M22	O	Cytoplasmic	Other	0	0	1	1
507	PepC	659918865	CDP47883.1	PFCIRM129_07880	Aminopeptidase C, Bleomycin hydrolase	E	Cytoplasmic	Other	0	0	1	1
508	PFCIRM129_07845	659918858	CDP47876.1	PFCIRM129_07845	binding protein of choline ABC transporter	M	Membrane	TM	0	0	1	1

509	GuaB2	65991 8854	CDP478 72.1	PFCIRM129 _07825	Inosine-5-monophosphate dehydrogenase (IMP dehydrogenase) (IMPDH) (IMPD) / GMP reductase	F	Cytoplasmic	Other	0	0	1	1
510	Cys1	65991 8847	CDP478 65.1	PFCIRM129 _07790	Cysteine synthase 1	E	Cytoplasmic	Other	0	0	1	1
511	LacI1	65991 8842	CDP478 60.1	PFCIRM129 _07765	arabinose operon repressor	K	Cytoplasmic	Other	0	0	1	1
512	RplO	65991 8917	CDP479 35.1	PFCIRM129 _08140	50S ribosomal protein L15	J	Cytoplasmic	Other	0	0	1	1
513	RpsM	65991 8901	CDP479 19.1	PFCIRM129 _08060	30S ribosomal protein S13	J	Cytoplasmic	Other	0	0	1	1
514	CoaA	65991 8876	CDP478 94.1	PFCIRM129 _07935	Pantothenate kinase (Pantothenic acid kinase)	H	Cytoplasmic	Other	0	0	1	1
515	PFCIRM12 9_08990	65991 8556	CDP481 43.1	PFCIRM129 _08990	Hypothetical protein PFCIRM129_08990	L	Extracellular	Other	0	0	1	1
516	PFCIRM12 9_08825	65991 8659	CDP480 62.1	PFCIRM129 _08825	Hypothetical transmembrane protein	S	Membrane	TM	0	0	1	1
517	PurL	65991 8644	CDP480 47.1	PFCIRM129 _08750	Phosphoribosylformylglycinam idine synthase II (FGAM synthase II)	F	Cytoplasmic	Other	0	0	1	1
518	SbcC	65991 8677	CDP480 80.1	PFCIRM129 _08915	ATP-dependent dsDNA exonuclease SbcC	L	Cytoplasmic	Other	0	0	1	1
519	Fic	65991 8608	CDP481 10.1	PFCIRM129 _09225	Fic protein	S	Cytoplasmic	Other	0	0	1	1
520	PFCIRM12 9_09175	65991 8600	CDP481 02.1	PFCIRM129 _09175	NAD-dependent epimerase/dehydratase	GM	Cytoplasmic	Other	0	0	1	1

521	PFCIRM129_09095	65991 8586	CDP480 88.1	PFCIRM129 _09095	Hypothetical protein PFCIRM129_09095	L	Cytoplasmic	Other	0	0	1	1
522	PFCIRM129_07725	65991 8835	CDP478 53.1	PFCIRM129 _07725	NADPH:quinone reductase related Zn-dependent oxidoreductase	C	Cytoplasmic	Other	0	0	1	1
523	PFCIRM129_06675	65991 8954	CDP476 55.1	PFCIRM129 _06675	metallophosphoesterase	S	Cytoplasmic	Other	0	0	1	1
524	Tgt	65991 8953	CDP476 54.1	PFCIRM129 _06670	Queuine tRNA-ribosyltransferase	J	Cytoplasmic	Other	0	0	1	1
525	Era	65991 8943	CDP476 44.1	PFCIRM129 _06620	GTP-binding protein	S	Cytoplasmic	Other	0	0	1	1
526	RplU	65991 8986	CDP476 87.1	PFCIRM129 _06840	50S ribosomal protein L21	J	Cytoplasmic	Other	0	0	1	1
527	Obg	65991 8984	CDP476 85.1	PFCIRM129 _06830	GTPase	S	Cytoplasmic	Other	0	0	1	1
528	ProB	65991 8983	CDP476 84.1	PFCIRM129 _06825	Glutamate 5-kinase (Gamma-glutamyl kinase) (GK)	E	Cytoplasmic	Other	0	0	1	1
529	PyrC	65991 8970	CDP476 71.1	PFCIRM129 _06755	Dihydroorotase multifunctional complex type (dihydroorotase)	F	Cytoplasmic	Other	0	0	1	1
530	HolA	65991 8965	CDP476 66.1	PFCIRM129 _06730	DNA polymerase III, delta subunit	L	Cytoplasmic	Other	0	0	1	1
531	PFCIRM129_00140	65991 9129	CDP475 80.1	PFCIRM129 _00140	Hypothetical protein PFCIRM129_00140		Extracellular	Lipo	0	0	1	1
532	PFCIRM129_00135	65991 9128	CDP475 79.1	PFCIRM129 _00135	Hypothetical protein PFCIRM129_00135		Cytoplasmic	Other	0	0	1	1

533	PFCIRM129_00115	659919125	CDP47576.1	PFCIRM129_00115	oxidoreductase	C	Cytoplasmic	Other	0	0	1	1
534	PFCIRM129_00090	659919120	CDP47571.1	PFCIRM129_00090	Hypothetical protein PFCIRM129_00090		Membrane	TM	0	0	1	1
535	Got	659919111	CDP47562.1	PFCIRM129_00045	Aspartate aminotransferase	E	Cytoplasmic	Other	0	0	1	1
536	ArgB	659919108	CDP47559.1	PFCIRM129_00030	Acetylglutamate kinase	E	Cytoplasmic	Other	0	0	1	1
537	RpmB	659919163	CDP47614.1	PFCIRM129_00310	Ribosomal protein L28	J	Cytoplasmic	Other	0	0	1	1
538	PspC	659918834	CDP47852.1	PFCIRM129_07720	Possible stress-response transcriptional regulator protein PspC	KT	Cytoplasmic	TM	0	0	1	1
539	PFCIRM129_06890	659918996	CDP47697.1	PFCIRM129_06890	Hypothetical membrane protein	O	Cytoplasmic	TM	0	0	1	1
540	PFCIRM129_06905	659918999	CDP47700.1	PFCIRM129_06905	hydrolase, haloacid dehalogenase-like hydrolase	S	Cytoplasmic	Other	0	0	1	1
541	MutM2	659918793	CDP47811.1	PFCIRM129_07515	Formamidopyrimidine-DNA glycosylase (DNA-formamidopyrimidine glycosylase)	L	Cytoplasmic	Other	0	0	1	1
542	Maf	659918789	CDP47807.1	PFCIRM129_07495	Maf-like protein	D	Cytoplasmic	Other	0	0	1	1
543	PFCIRM129_07435	659918778	CDP47796.1	PFCIRM129_07435	transcriptional regulator TetR family	K	Cytoplasmic	Other	0	0	1	1

544	PurK	65991 8776	CDP477 94.1	PFCIRM129 _07425	Phosphoribosylaminoimidazol e carboxylase ATPase subunit	F	Cytoplasmic	Other	0	0	1	1
545	PyrD	65991 8796	CDP478 14.1	PFCIRM129 _07530	Dihydroorotate dehydrogenase	F	Cytoplasmic	Other	0	0	1	1
546	Cls	65991 8826	CDP478 44.1	PFCIRM129 _07680	Phospholipase D/Transphosphatidylase	I	Cytoplasmic	Other	0	0	1	1
547	PFCIRM12 9_07040	65991 9022	CDP477 23.1	PFCIRM129 _07040	long-chain fatty-acid CoA ligase (AMP-binding enzyme) (long-chain-fatty-acid---CoA ligase)	I	Cytoplasmic	Other	0	0	1	1
548	ThiD2	65991 9020	CDP477 21.1	PFCIRM129 _07030	hydroxymethylpyrimidine/pho sphomethylpyrimidinekinase	H	Cytoplasmic	Sec	0	0	1	1
549	MurI	65991 9065	CDP477 66.1	PFCIRM129 _07275	Glutamate racemase	M	Cytoplasmic	Other	0	0	1	1
550	Rph	65991 9064	CDP477 65.1	PFCIRM129 _07270	Ribonuclease PH (RNase PH) (tRNA nucleotidyltransferase)	J	Cytoplasmic	Other	0	0	1	1
551	UvrA2	65991 9040	CDP477 41.1	PFCIRM129 _07140	UvrA, Excinuclease ATPase subunit	L	Cytoplasmic	Other	0	0	1	1
552	MetH	65991 7990	CDP485 89.1	PFCIRM129 _04015	methionine synthase (5-methyltetrahydrofolate:L-homocysteine S-methyltransferase)	E	Cytoplasmic	Other	0	0	1	1
553	EngA	65991 7982	CDP485 81.1	PFCIRM129 _03975	GTP binding protein	S	Cytoplasmic	Other	0	0	1	1

554	RsmB	65991 7994	CDP485 93.1	PFCIRM129 _04035	Sun, tRNA and rRNA cytosine-C5-methylases	JK	Cytoplasmic	Other	0	0	1	1
555	RelA	65991 8021	CDP486 20.1	PFCIRM129 _04170	GTP pyrophosphokinase	KT	Cytoplasmic	Other	0	0	1	1
556	MetN2	65991 8054	CDP486 53.1	PFCIRM129 _04335	ABC protein of ABC transporter	S	Cytoplasmic	Other	0	0	1	1
557	PFCIRM12 9_04310	65991 8049	CDP486 48.1	PFCIRM129 _04310	Hypothetical protein PFCIRM129_04310	C	Cytoplasmic	Other	0	0	1	1
558	TrpA	65991 8044	CDP486 43.1	PFCIRM129 _04285	Tryptophan synthase alpha chain (TrpA)	E	Cytoplasmic	Other	0	0	1	1
559	Lgt	65991 8043	CDP486 42.1	PFCIRM129 _04280	Prolipoprotein diacylglyceryl transferase	M	Membrane	Other	0	0	1	1
560	GltB	65991 8042	CDP486 41.1	PFCIRM129 _04275	Glutamate synthase large subunit (Ferredoxin)	E	Cytoplasmic	Other	0	0	1	1
561	DnaE1	65991 8039	CDP486 38.1	PFCIRM129 _04260	DNA polymerase III alpha subunit	L	Cytoplasmic	Other	0	0	1	1
562	PFCIRM12 9_04215	65991 8030	CDP486 29.1	PFCIRM129 _04215	GCN5-related N-acetyltransferase	K	Cytoplasmic	Other	0	0	1	1
563	PFCIRM12 9_03650	65991 8181	CDP485 26.1	PFCIRM129 _03650	Protein of unknown function	M	Cytoplasmic	Other	0	0	1	1
564	CpsK	65991 8180	CDP485 25.1	PFCIRM129 _03645	Putative uncharacterized protein	V	Cytoplasmic	Other	0	0	1	1
565	PFCIRM12 9_03620	65991 8175	CDP485 20.1	PFCIRM129 _03620	Hypothetical transmembrane protein	S	Membrane	Sec	0	0	1	1
566	WcqF	65991 8171	CDP485 16.1	PFCIRM129 _03600	Putative uncharacterized protein	M	Cytoplasmic	Other	0	0	1	1
567	NadE	65991 8131	CDP485 66.1	PFCIRM129 _03870	Glutamine-dependent NAD(+) synthetase	H	Cytoplasmic	Other	0	0	1	1

568	PFCIRM129_03865	659918130	CDP48565.1	PFCIRM129_03865	Transcriptional regulator PadR family	K	Cytoplasmic	Other	0	0	1	1
569	PFCIRM129_03840	659918125	CDP48560.1	PFCIRM129_03840	polar amino acid ABC transporter, binding protein	ET	Extracellular	Lipo	0	0	1	1
570	PPA2234	659918120	CDP48555.1	PFCIRM129_03815	Putative long-chain-fatty-acid--CoA ligase/synthetase	I	Cytoplasmic	Other	0	0	1	1
571	Slh2	659918117	CDP48552.1	PFCIRM129_03800	S-layer protein precursor	O	Extracellular	Sec	0	0	1	1
572	PFCIRM129_03740	659918197	CDP48542.1	PFCIRM129_03740	dehydrogenase	S	Cytoplasmic	Other	0	0	1	1
573	PFCIRM129_03730	659918195	CDP48540.1	PFCIRM129_03730	Hypothetical protein PFCIRM129_03730	S	Membrane	TM	0	0	1	1
574	GtfD	659918193	CDP48538.1	PFCIRM129_03720	Glycosyl transferase, family 2	M	Cytoplasmic	Other	0	0	1	1
575	GtfC	659918192	CDP48537.1	PFCIRM129_03715	Glycosyltransferase, family 2	M	Cytoplasmic	Other	0	0	1	1
576	MiaB2	659917919	CDP48765.1	PFCIRM129_04955	2-methylthioadenine synthetase MiaB protein	J	Cytoplasmic	Other	0	0	1	1
577	MiaB1	659917908	CDP48754.1	PFCIRM129_04900	2-methylthioadenine synthetase MiaB protein	J	Cytoplasmic	Other	0	0	1	1
578	HrpA2	659917935	CDP48738.1	PFCIRM129_04810	ATP-dependent helicase HrpA	L	Cytoplasmic	Other	0	0	1	1
579	HrdD2	659917971	CDP48734.1	PFCIRM129_04780	RNA polymerase principal sigma factor HrdD	K	Cytoplasmic	Other	0	0	1	1
580	PlsC1	659917925	CDP48771.1	PFCIRM129_04985	1-acylglycerol-3-phosphate O-acyltransferase	I	Cytoplasmic	Other	0	0	1	1

581	AtpA	659917889	CDP48802.1	PFCIRM129_05155	ATP synthase subunit alpha (ATPase subunit alpha) (ATP synthase F1 sector subunit alpha)	C	Cytoplasmic	Other	0	0	1	1
582	AtpD	659917887	CDP48800.1	PFCIRM129_05145	ATP synthase subunit beta (ATPase subunit beta) (ATP synthase F1 sector subunit beta)	C	Cytoplasmic	Other	0	0	1	1
583	LplA	659917884	CDP48797.1	PFCIRM129_05130	Lipoate-protein ligase A	H	Cytoplasmic	Other	0	0	1	1
584	LeuA2	659917931	CDP48777.1	PFCIRM129_05025	2-isopropylmalate synthase	E	Cytoplasmic	Other	0	0	1	1
585	Ldh2	659917969	CDP48732.1	PFCIRM129_04770	L-lactate dehydrogenase	C	Cytoplasmic	Other	0	0	1	1
586	GyrB1	659917967	CDP48730.1	PFCIRM129_04760	DNA gyrase subunit B	L	Cytoplasmic	Other	0	0	1	1
587	CobU	659918076	CDP48675.1	PFCIRM129_04455	CobU Bifunctional cobalamin biosynthesis pyrophosphate enzyme	H	Cytoplasmic	Other	0	0	1	1
588	CbiA	659918075	CDP48674.1	PFCIRM129_04450	Cobyrinic acid A,C-diamide synthase, CbiA	H	Cytoplasmic	Other	0	0	1	1
589	CbiB	659918072	CDP48671.1	PFCIRM129_04435	Cobalamin biosynthesis protein	H	Cytoplasmic	Sec	0	0	1	1
590	DnaG1	659918063	CDP48662.1	PFCIRM129_04385	DNA primase	L	Cytoplasmic	Other	0	0	1	1
591	PimA	659918093	CDP48692.1	PFCIRM129_04540	Phosphatidylinositol alpha-mannosyltransferase (glycosyltransferase)	M	Cytoplasmic	Other	0	0	1	1

592	PFCIRM129_04720	659917959	CDP48722.1	PFCIRM129_04720	Putative uncharacterized protein	S	Cytoplasmic	Sec	0	0	1	1
593	PFCIRM129_04710	659917957	CDP48720.1	PFCIRM129_04710	Nucleic acid binding, OB-fold,tRNA/helicase-type	L	Cytoplasmic	Other	0	0	1	1
594	PFCIRM129_04595	659918100	CDP48699.1	PFCIRM129_04595	Hypothetical protein PFCIRM129_04595	S	Cytoplasmic	Other	0	0	1	1
595	PFCIRM129_03455	659918144	CDP48489.1	PFCIRM129_03455	Hypothetical protein PFCIRM129_03455	C	Cytoplasmic	Other	0	0	1	1
596	PFCIRM129_00735	659918420	CDP48280.1	PFCIRM129_00735	Putative uncharacterized protein	S	Cytoplasmic	Other	0	0	1	1
597	PFCIRM129_00950	659918403	CDP48323.1	PFCIRM129_00950	helicase	L	Cytoplasmic	Other	0	0	1	1
598	NtcA2	659918437	CDP48260.1	PFCIRM129_12370	Protein of unknown function	K	Cytoplasmic	Other	0	0	1	1
599	NagD	659918508	CDP48218.1	PFCIRM129_10065	Phosphatase	G	Cytoplasmic	Other	0	0	1	1
600	PpnK	659918502	CDP48212.1	PFCIRM129_10035	Probable inorganic polyphosphate/ATP-NAD kinase (Poly(P)/ATP NAD kinase)	G	Cytoplasmic	Other	0	0	1	1
601	RecN	659918501	CDP48211.1	PFCIRM129_10030	DNA repair protein	L	Cytoplasmic	Other	0	0	1	1
602	PFCIRM129_09965	659918488	CDP48198.1	PFCIRM129_09965	Regulatory protein	K	Cytoplasmic	Other	0	0	1	1
603	PFCIRM129_10920	659918480	CDP48240.1	PFCIRM129_10920	AAA ATPase, central region	O	Cytoplasmic	Other	0	0	1	1
604	PksA	659918473	CDP48233.1	PFCIRM129_10885	Putative uncharacterized protein	K	Cytoplasmic	Other	0	0	1	1

605	PFCIRM129_10855	65991 8467	CDP482 27.1	PFCIRM129 _10855	Putative uncharacterized protein	C	Cytoplasmic	Other	0	0	1	1
606	PFCIRM129_03200	65991 8257	CDP484 40.1	PFCIRM129 _03200	Hypothetical protein PFCIRM129_03200	S	Cytoplasmic	Other	0	0	1	1
607	FabD	65991 8248	CDP484 31.1	PFCIRM129 _03155	Carboxylic ester hydrolase	I	Cytoplasmic	Other	0	0	1	1
608	Fnk	65991 8235	CDP484 18.1	PFCIRM129 _03090	fructosamine kinase	G	Cytoplasmic	Other	0	0	1	1
609	PFCIRM129_03055	65991 8229	CDP484 12.1	PFCIRM129 _03055	Hypothetical membrane protein	S	Membrane	Sec	0	0	1	1
610	GyrA	65991 8141	CDP484 86.1	PFCIRM129 _03440	DNA gyrase subunit A	L	Cytoplasmic	Other	0	0	1	1
611	HisF	65991 8210	CDP484 80.1	PFCIRM129 _03405	Imidazole glycerol phosphate synthase subunit HisF	E	Cytoplasmic	Other	0	0	1	1
612	PFCIRM129_03400	65991 8209	CDP484 79.1	PFCIRM129 _03400	Aldo/keto reductase	C	Cytoplasmic	Other	0	0	1	1
613	GlyQS	65991 8273	CDP484 56.1	PFCIRM129 _03280	Glycyl-tRNA synthetase (Glycine--tRNA ligase) (GlyRS)	J	Cytoplasmic	Other	0	0	1	1
614	PFCIRM129_02740	65991 8337	CDP483 54.1	PFCIRM129 _02740	Sensory transduction histidine kinase	T	Cytoplasmic	Other	0	0	1	1
615	PFCIRM129_03045	65991 8227	CDP484 10.1	PFCIRM129 _03045	Hypothetical transmembrane protein	S	Cytoplasmic	Other	0	0	1	1
616	PFCIRM129_02955	65991 8319	CDP483 92.1	PFCIRM129 _02955	ATP-dependent DNA helicase	L	Cytoplasmic	Other	0	0	1	1
617	UrvD	65991 8318	CDP483 91.1	PFCIRM129 _02950	UvrD/REP helicase / ATP-dependent DNA helicase	L	Cytoplasmic	Other	0	0	1	1

618	PFCIRM129_02880	65991 8304	CDP483 77.1	PFCIRM129 _02880	Zn dependant peptidase	S	Cytoplasmic	Other	0	0	1	1
619	CysS2	65991 7477	CDP491 44.1	PFCIRM129 _10200	Cysteinyl-tRNA synthetase (Cysteine--tRNA ligase)	J	Cytoplasmic	Other	0	0	1	0
620	PFCIRM129_10660	65991 7428	CDP492 29.1	PFCIRM129 _10660	DeaD/DeaH box helicase	L	Cytoplasmic	Other	0	0	1	0
621	HemC	65991 7503	CDP491 70.1	PFCIRM129 _10350	Porphobilinogen deaminase (PBG) (Hydroxymethylbilane synthase) (HMBS) (Pre-uroporphyrinogen synthase)	H	Cytoplasmic	Other	0	0	1	0
622	Mca	65991 7762	CDP489 46.1	PFCIRM129 _05925	LMBE-related protein	S	Cytoplasmic	Other	0	0	1	0
623	YchF	65991 7751	CDP489 35.1	PFCIRM129 _05870	GTPase YchF	J	Cytoplasmic	Other	0	0	1	0
624	UvrA4	65991 7827	CDP488 36.1	PFCIRM129 _05345	Excinuclease ATPase subunit UvrA	L	Cytoplasmic	Other	0	0	1	0
625	TrxC	65991 7718	CDP490 32.1	PFCIRM129 _06365	Putative uncharacterized protein		Cytoplasmic	Other	0	0	1	0
626	TatD	65991 7674	CDP489 88.1	PFCIRM129 _06145	DNase	L	Cytoplasmic	Other	0	0	1	0
627	PFCIRM129_01830	65991 6876	CDP497 41.1	PFCIRM129 _01830	Hypothetical protein PFCIRM129_01830	S	Cytoplasmic	Other	0	0	1	0
628	Sms	65991 6875	CDP497 40.1	PFCIRM129 _01825	DNA repair protein	O	Cytoplasmic	Other	0	0	1	0

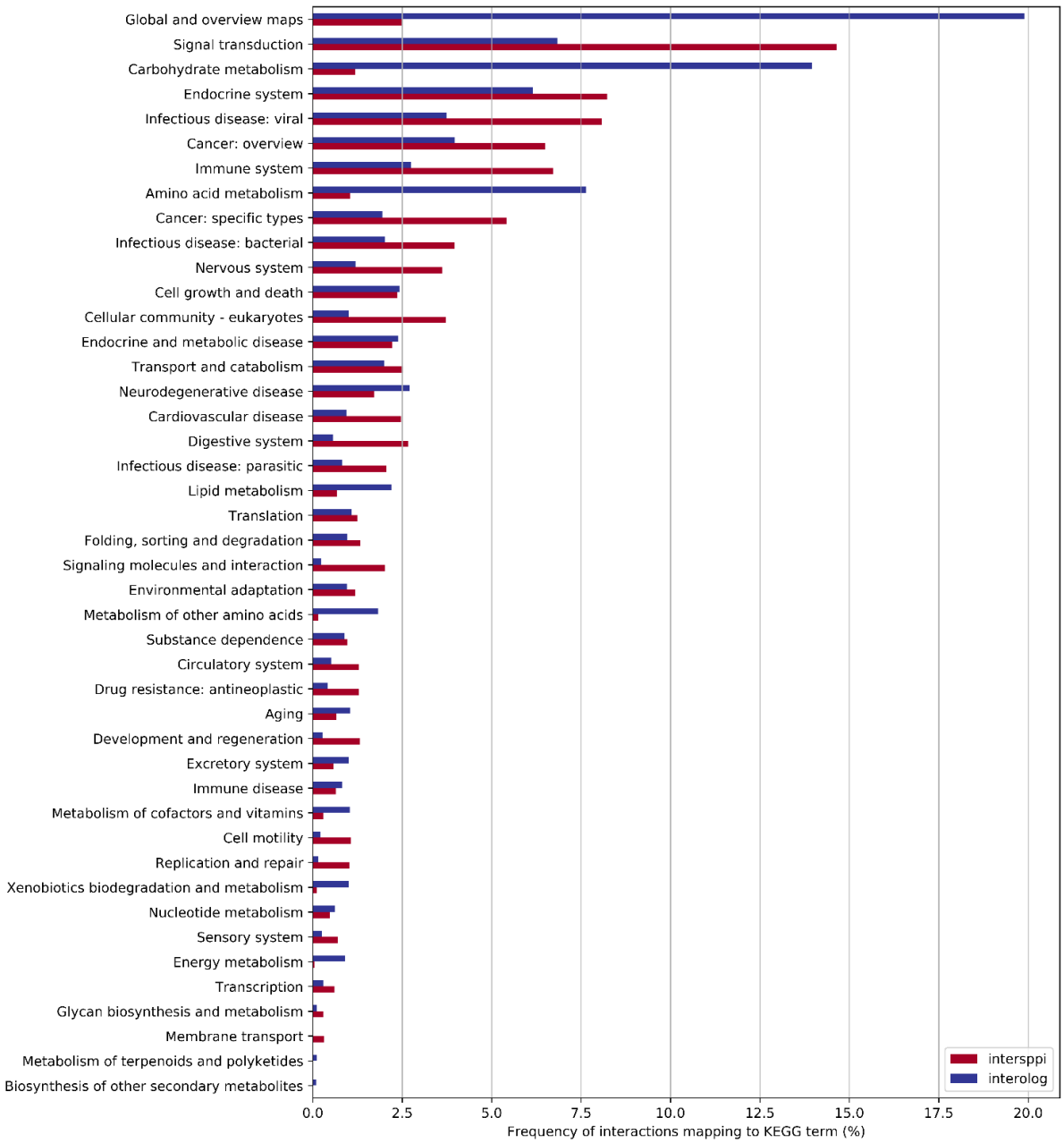
629	RluA	65991 7344	CDP493 54.1	PFCIRM129 _11440	Pseudouridylate synthase, Uncharacterized RNA pseudouridine synthase	J	Cytoplasmic	Other	0	0	1	0
630	MurD	65991 7356	CDP493 66.1	PFCIRM129 _11500	UDP-N-acetylmuramoylalanine--D-glutamate ligase (UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase) (D-glutamic acid-adding enzyme)	M	Cytoplasmic	Other	0	0	1	0
631	ThiE2	65991 7099	CDP495 52.1	PFCIRM129 _00475	thiamine-phosphate pyrophosphorylase	H	Cytoplasmic	Other	0	0	1	0
632	GltX	65991 8684	CDP479 58.1	PFCIRM129 _08260	Glutamyl-tRNA synthetase (Glutamate--tRNA ligase) (GluRS)	J	Cytoplasmic	Other	0	0	1	0
633	RplW	65991 8935	CDP479 53.1	PFCIRM129 _08230	50S ribosomal protein L23	J	Cytoplasmic	Other	0	0	1	0
634	PFCIRM12 9_08560	65991 8740	CDP480 14.1	PFCIRM129 _08560	nuclease (RecB family)	V	Cytoplasmic	Other	0	0	1	0
635	Map	65991 8907	CDP479 25.1	PFCIRM129 _08090	Methionine aminopeptidase (MAP) (Peptidase M)	J	Cytoplasmic	Other	0	0	1	0
636	CobA	65991 8657	CDP480 60.1	PFCIRM129 _08815	CobA Uroporphyrinogen III methyltransferase	H	Cytoplasmic	Other	0	0	1	0
637	PFCIRM12 9_06870	65991 8992	CDP476 93.1	PFCIRM129 _06870	Fe-S oxidoreductases family 2	C	Cytoplasmic	Other	0	0	1	0
638	CysS1	65991 9124	CDP475 75.1	PFCIRM129 _00110	Cysteine--tRNA ligase	J	Cytoplasmic	Other	0	0	1	0

639	CbiL	659919056	CDP47757.1	PFCIRM129_07230	CbiL Precorrin-2 C20-methyltransferase	H	Cytoplasmic	Other	0	0	1	0
640	Orn	659919038	CDP47739.1	PFCIRM129_07125	Oligoribonuclease	L	Cytoplasmic	Other	0	0	1	0
641	AldC	659918017	CDP48616.1	PFCIRM129_04150	Alpha-acetolactate decarboxylase	Q	Cytoplasmic	Other	0	0	1	0
642	GlnS	659918050	CDP48649.1	PFCIRM129_04315	Glutaminyl-tRNA synthetase	J	Cytoplasmic	Other	0	0	1	0
643	YwlC	659918058	CDP48657.1	PFCIRM129_04355	Sua5_yciO_yrdC	J	Cytoplasmic	Other	0	0	1	0
644	NucS	659917880	CDP48793.1	PFCIRM129_05110	Nuclease of the RecB family	L	Cytoplasmic	Other	0	0	1	0
645	PFCIRM129_00720	659918417	CDP48277.1	PFCIRM129_00720	ABC transporter, ATP-binding protein	P	Cytoplasmic	Other	0	0	1	0
646	DnaB	659918380	CDP48300.1	PFCIRM129_00835	Replicative DNA helicase	L	Cytoplasmic	Other	0	0	1	0
647	Soj	659918497	CDP48207.1	PFCIRM129_10010	ATPase involved in cell division		Cytoplasmic	Other	0	0	1	0
648	RluB	659918493	CDP48203.1	PFCIRM129_09990	Pseudouridine synthase	J	Cytoplasmic	Other	0	0	1	0
649	IlvC	659918214	CDP48484.1	PFCIRM129_03425	Ketol-acid reductoisomerase (Acetohydroxy-acid isomeroreductase) (Alpha-keto-beta-hydroxylacil reductoisomerase)	EH	Cytoplasmic	Other	0	0	1	0
650	PFCIRM129_02985	659918325	CDP48398.1	PFCIRM129_02985	DEAD/DEAH box helicase domain protein	L	Cytoplasmic	Other	0	0	1	0

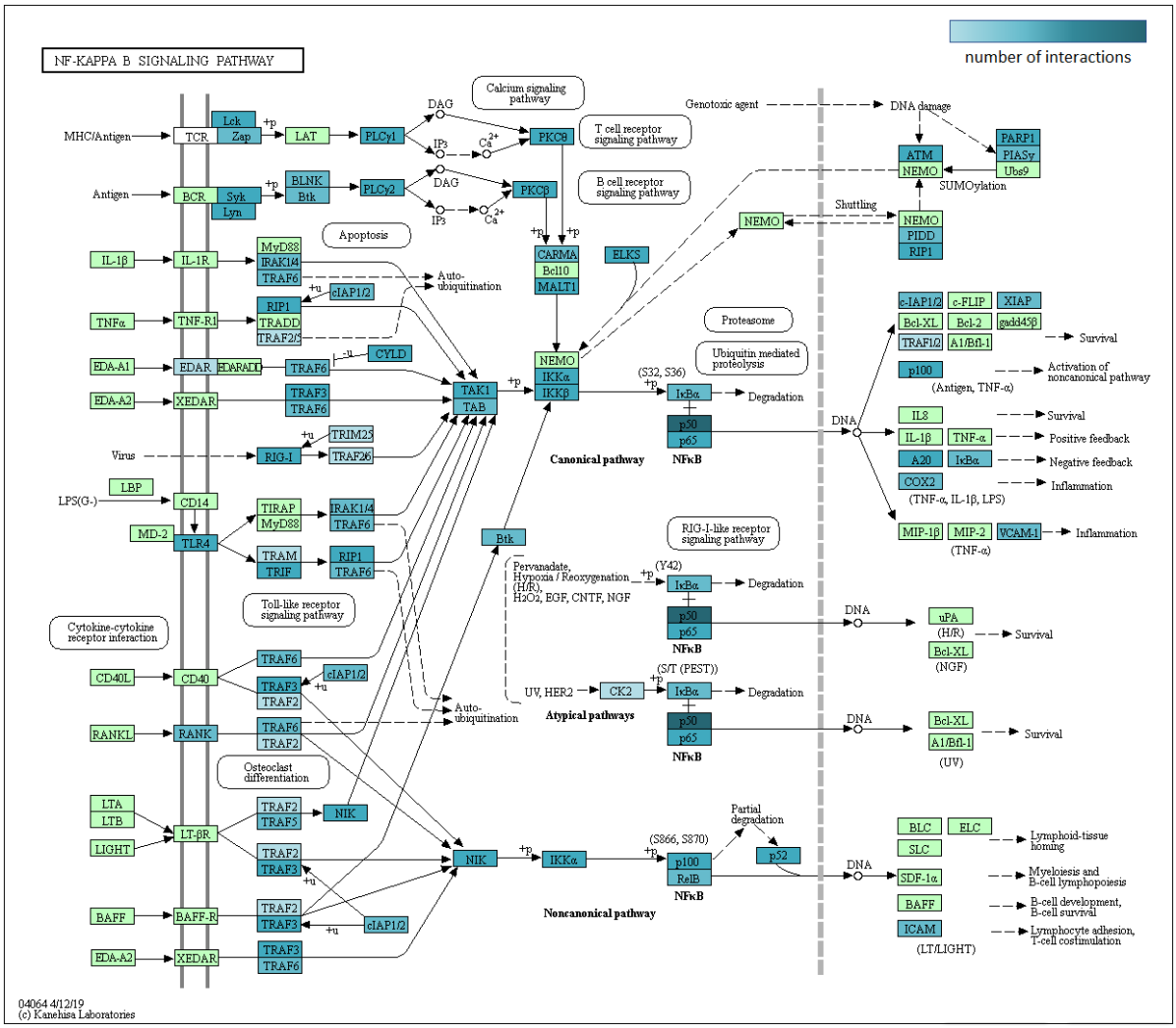
651	RpsO	659917387	CDP49266.1	PFCIRM129_10985	30S ribosomal protein S15	J	Cytoplasmic	Other	0	0	0	1
652	GlgB	659917873	CDP48786.1	PFCIRM129_05075	1,4-alpha-glucan branching enzyme (Glycogen branching enzyme) (BE) (1,4-alpha-D-glucan:1,4-alpha-D-glucan 6-glucosyl-transferase)	G	Cytoplasmic	Other	0	0	0	1

Legend: # - number to indicate the order of presentation. GI: GenInfo Identifier. ACCESSION: accession number. LOCUS TAG: genomic locus tag identification. DESCRIPTION: protein brief description. COG: COG category prediction. LOCALIZATION: subcellular localization prediction. LIPO-PROTEIN: lipoprotein signal prediction, Sec: secretion signal peptide, Lipo: lipoprotein signal peptide, TM: transmembrane, Other: no signals found. Conditions: EV obtention condition, UC_UF: UF-derived EVs purified by UC, UC_YEL: YEL-derived EVs purified by UC, SEC_UF: UF-derived EVs purified by SEC, SEC_YEL: YEL-derived EVs purified by SEC. For each condition: 1 – presence, 0 – absence.

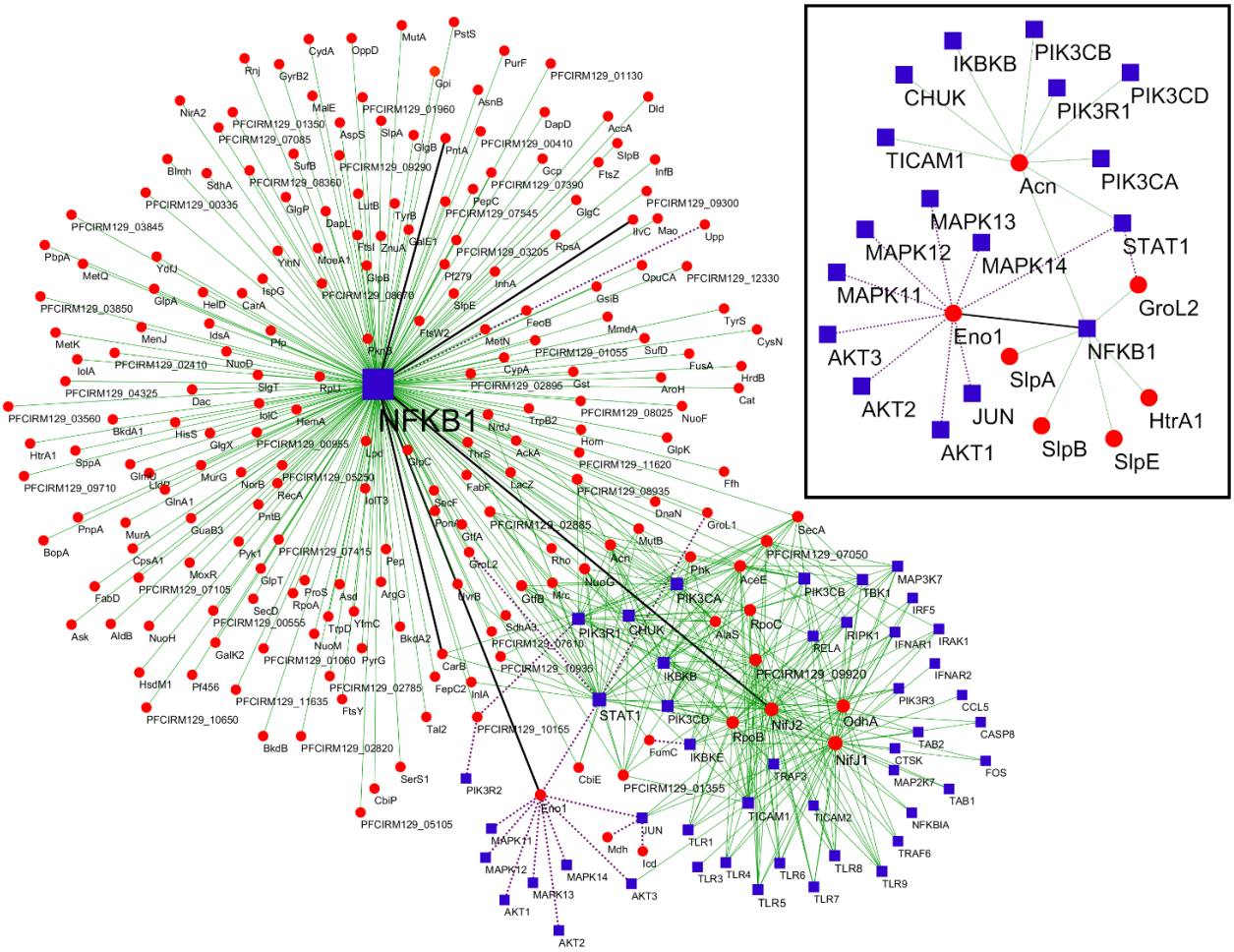
2. Supplementary figures for Research article 1 (Chapter 3)



Supplementary Figure 1. Frequency of predicted interactions mapping to KEGG terms, according to prediction method.

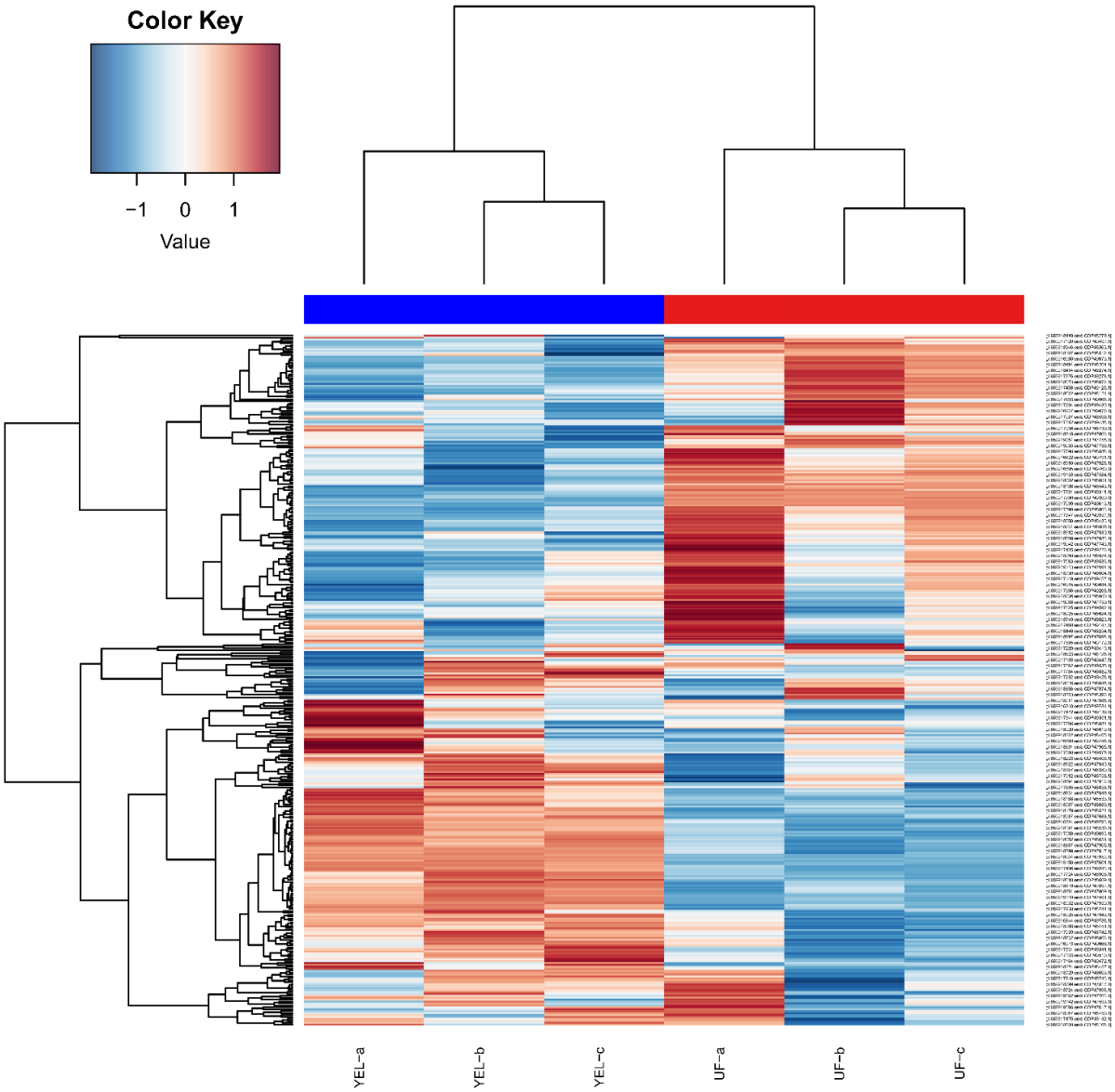


Supplementary Figure 2. Human KEGG NF-κB pathway, highlighting in blue the different human proteins predicted to interact with proteins from the EVs of *P. freudenreichii* CIRM-BIA 129. Non-interacting proteins are in green.



Supplementary Figure 3. Subnetwork of predicted interactions mapping to KEGG toll-like receptors (TLRs) pathway. Blue square nodes represent human proteins, red round nodes represent bacterial proteins, full thin green lines represent intersppi-predicted interactions, dotted purple lines represent interolog-predicted interactions and full thick black lines represent interactions shared by both methods. Inset: selected interactions involving bacterial proteins previously associated to immunomodulatory roles.

3. Supplementary figure for Research article 2 (Chapter 4)



Supplementary Figure 1. Heatmap representing variations in the relative abundance of identified proteins.

4. Research articles as co-author

1. TARTAGLIA NR, NICOLAS A, **RODOVALHO VR**, LUZ BSRD, BRIARD-BION V, KRUPOVA Z, THIERRY A, COSTE F, BUREL A, MARTIN P, JARDIN J, AZEVEDO V, LE LOIR Y, GUÉDON E. Extracellular vesicles produced by human and animal Staphylococcus aureus strains share a highly conserved core proteome. Sci Rep. 2020 May 21;10(1):8467. doi: 10.1038/s41598-020-64952-y.

2. LUZ BSR, NICOLAS A, CHABELSKAYA S, **RODOVALHO VR**, LE LOIR Y, AZEVEDO VAC, FELDEN B, GUÉDON E. Environmental plasticity of the RNA content of Staphylococcus aureus extracellular vesicles. Front Microbiol. 2021; 12:482. doi: 10.3389/fmicb.2021.634226.

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Extracellular vesicles produced by human and animal *Staphylococcus aureus* strains share a highly conserved core proteome

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Staphylococcus aureus **is an important opportunistic pathogen of humans and animals. It produces extracellular vesicles (EVs) that are involved in cellular communication and enable inter-kingdom crosstalk, the delivery of virulence factors and modulation of the host immune response. The protein content of EVs determines their biological functions. Clarifying which proteins are selected, and how, is of crucial value to understanding the role of EVs in pathogenesis and the development of molecular delivery systems. Here, we postulated that *S. aureus* EVs share a common proteome containing components involved in cargo sorting. The EV proteomes of five *S. aureus* strains originating from human, bovine, and ovine hosts were characterised. The clustering of EV proteomes reflected the diversity of the producing strains. A total of 253 proteins were identified, 119 of which composed a core EV proteome with functions in bacterial survival, pathogenesis, and putatively in EV biology. We also identified features in the sequences of EV proteins and the corresponding genes that could account for their packaging into EVs. Our findings corroborate the hypothesis of a selective sorting of proteins into EVs and offer new perspectives concerning the roles of EVs in *S. aureus* pathogenesis in specific host niches.**

Staphylococcus aureus is a Gram-positive opportunistic pathogen that causes a broad spectrum of infections in humans and animals. In humans, these diseases range from superficial skin and soft tissue infections to life-threatening conditions that require hospitalisation and extensive medical support[1,2]. This bacterium is also one of the main causative agents of nosocomial infections. In animals, *S. aureus* is notably responsible for ruminant mastitis, an inflammation of the mammary glands that dramatically affects animal health and welfare, milk quality and the economics of milk production[3]. Mastitis is also the principal reason for the use of antibiotics in dairy herds[4]. The wide range of clinical manifestations of *S. aureus* infections is likely associated with its huge arsenal of virulence factors, which include structural components and extracellular factors such as enzymes and toxins[5]. Despite considerable efforts, the precise mechanisms underlying host adaptation, colonisation and interactions are not yet fully understood[6].

Extracellular vesicles (EVs) are used by many pathogenic bacteria as a secretory route to deliver toxic compounds to infected cells[7,8]. EVs are lipid bilayer nanoparticles that range in size from 20 to 300 nm and are released by almost all cells in all domains of life[9]. In Gram-positive bacteria, they are formed by budding and shedding of the cytoplasmic membrane. They play a pivotal role in cell-to-cell communication through their ability to transport bioactive molecules (proteins, nucleic acids, lipids, metabolites) from donor to recipient cells. The EVs produced by *S. aureus* can mediate the pathogenesis of infection in a variety of ways. They may be cytotoxic to host cells[10–12], induce the production of cytokines[13–18], contribute to biofilm formation[19], mediate antibiotic

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resistance[20] or promote an increase in the *in vivo* survival of *S. aureus*[21]. In addition, the immunogenic and adjuvant properties of *S. aureus* EVs can induce a protective response against *S. aureus* infection, thus offering new alternatives for the design of vaccines and the control of infections[15,17,21,22]. The broad spectrum of activities associated with *S. aureus*-derived EVs, as well as their strain-dependency, is mainly related to their protein content[12]. The EV cargo includes a variety of proteins, such as virulence factors and lipoproteins, some of which are known to be potent antigens[12,17,18,21–24]. The EV protein cargo has been shown to vary as a function of environmental conditions[21] and the producer strains[12,17]. The identification of proteins shared between the EVs of several strains has suggested that protein packaging in EVs results more from a conserved selective mechanism than from a random process[12,18]. However, to date, the molecular mechanisms that drive the recruitment into EVs of these proteins remain unclear, thus limiting our understanding of the pathophysiological relevance of EVs *in vivo* and the development of EV-based applications.

In eukaryotic cells, the components associated with protein packaging into exosomes, a class of eukaryotic EVs, are found in the exosomes produced by most cell types[25,26]. Likewise, the proteins involved in EV formation in *S. aureus* are also found in the EVs they produce[22,23]. We therefore postulated that *S. aureus* strains might share an EV proteome containing essential components for the cargo selection of proteins into EVs. Considering that secreted proteins are key elements in the virulence and host adaptation of *S. aureus*[27], we suggested that strains isolated from different hosts (human, bovine, ovine) and involved in different types of infection (from mild to severe) offer the best source of diversity to explore cargo selectivity and determine a robust core EV proteome. During this work, we purified EVs released into the culture supernatant of five *S. aureus* strains and identified the whole set of proteins packaged into these EVs. This enabled identification of a core EV proteome. The distribution, functions, physicochemical properties and amino acid composition of the proteins packaged into EVs as a function of different strains were analysed and compared with regard to the host origin of strains, the severity of infections the strains thus produced could cause and the selectivity of protein cargo into EVs.

Results

EV production in *S. aureus* strains of human, bovine and ovine origins. The ability to produce EVs of five *S. aureus* strains isolated from three types of hosts was investigated. These included two bovine strains, Newbould 305 (hereinafter referred to as N305) and RF122, two ovine strains, *S. aureus* O11 and O46, and the highly virulent human methicillin-resistant *S. aureus* (MRSA) strain MW2. *S. aureus* N305, in which EV production had already been demonstrated, was used as a positive control for the EV preparations[18]. All the *S. aureus* strains used in this study produced EVs with classical features, including a nanoscale size, spherical structure and cup-shaped morphology when visualised using negative staining electron microscopy (Fig. 1A)[28,29]. In addition to EVs, cylindrical (nanotube-like) structures were observed in the *S. aureus* N305, RF122, O11 and O46 samples (Supplementary Fig. S1). The size of the EVs secreted was homogeneous for each strain and similar between strains (120–130 nm), with the exception of strain O46 which secreted significantly larger EVs (170 nm, $P < 0.0001$) (Fig. 1B,C). Although the growth conditions and EV preparation methods were the same, the number of EVs harvested from the MW2 and O11 culture supernatants was five to ten times lower than that of the other strains, suggesting that the yield of EV production is a strain-specific feature (Fig. 1D).

Protein composition of *S. aureus* EV proteomes. The proteome of the EVs produced by *S. aureus* O11, O46, RF122, and MW2 was determined by Nano LC-ESI-MS/MS analysis on three biological replicates. The dataset was completed by the vesicular proteome of strain N305, which had been obtained during a previous study using the same experimental procedures[18]. A total of 253 proteins associated with *S. aureus* EVs, and corresponding to the EV pan-proteome of these strains, were identified (Supplementary Table S2). 160, 164, 168, 171 and 218 proteins were associated with EVs produced by *S. aureus* strains RF122, O11, MW2, O46 and N305, respectively. The majority of these 253 proteins were predicted as cytoplasmic (PRED-LIPO, n = 126; CELLO, n = 118; PSORTb, n = 92) or membrane-associated (PSORTb, n = 80; CELLO, n = 65; PRED-LIPO, n = 67) (Fig. 2A–C). About 10% and 20% of EV proteins are predicted to contain a signal peptide I (SignalP, n = 27; PRED-LIPO, n = 20) and a signal peptide II (SignalP, n = 51; PRED-LIPO, n = 50), respectively (Fig. 2B,D). When considered strain-by-strain, the protein localisation was similar to these overall results. Interestingly, although the predicted signal peptide II-containing proteins (i.e. lipoproteins) represented about 2.5% of the whole proteome of the strains studied here, they accounted for 20% of EV proteomes, which was indicative of their relative enrichment in EVs. In agreement, EV lipoproteins represented about 50% of whole cell lipoproteins. Taken together, these results showed that the predicted subcellular localisation and richness of proteins associated with EVs were markedly similar in the five strains analysed.

Functional classification of *S. aureus* EV proteomes. The functional characteristics of the 253 *S. aureus* EV proteins were determined using a Clusters of Orthologous Groups (COG) analysis. Overall, *S. aureus* EV-associated proteins were spread over 18 COGs (Supplementary Table S2 and Fig. 2E). Of note, 30% of EV proteins were assigned to the "function unknown" category (n = 49) or not assigned to any COG categories (n = 27). The majority of the remaining proteins (42%; 74/177) could be assigned to COGs related to the general "metabolism" category; *i.e.* energy production and conversion (n = 24, COG C), inorganic ion transport and metabolism (n = 19, COG P), carbohydrate transport and metabolism (n = 9, COG G) and amino acid transport and metabolism (n = 10, COG E). COG J (translation/ribosomal structure and biogenesis) was the most common category, with 38 occurrences. Finally, cell wall/membrane/envelope biogenesis (n = 28, COG M), defence mechanisms (n = 7, COG V) and intracellular trafficking/secretion/vesicular transport (n = 6, COG U) were the most frequent COGs in the general "cellular processes and signalling" category.

Interestingly, 36% (92/253) of the proteins found in EVs could be regarded as virulence-associated proteins (Table 1 and Supplementary Table S3). In particular, these included toxins (e.g. LukMF' and LukGH leukocidins,

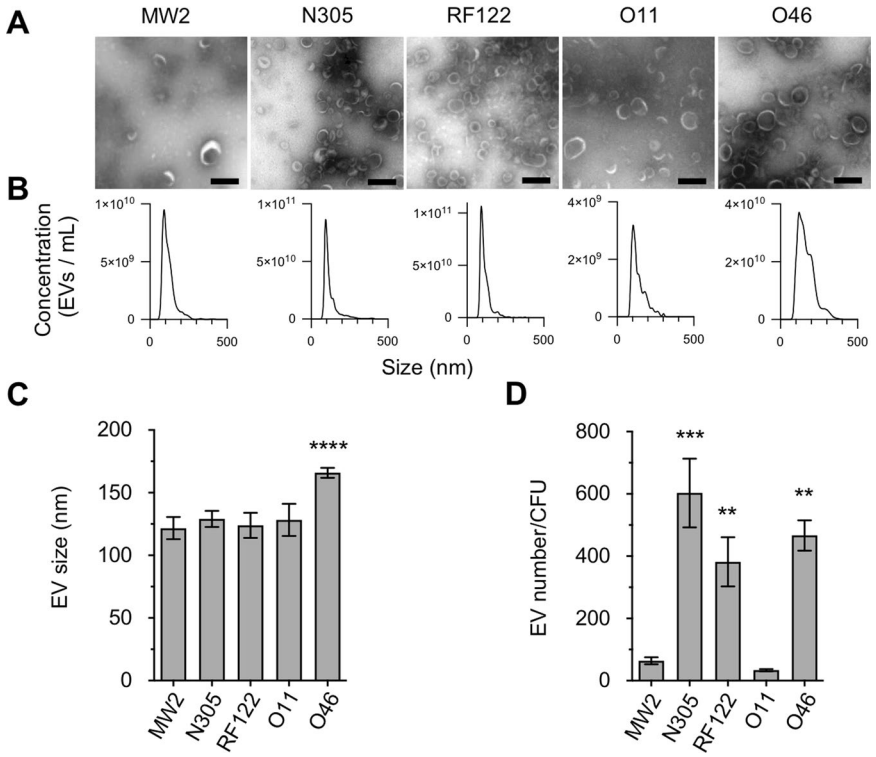

Figure 1. Shape, size and yield of EVs produced by *S. aureus* strains MW2, N305, RF122, O11, and O46. (**A**) Representative electron microscopy images of negatively stained EVs purified from the culture supernatant of *S. aureus* strains MW2, N305, RF122, O11 and O46. Scale bar = 200 nm. (**B**) Representative graphs of the size distribution of EVs purified from culture supernatants of *S. aureus* strains MW2, N305, RF122, O11 and O46. (**C**) Mean size of EVs produced by *S. aureus* strains MW2, N305, RF122, O11 and O46. (**D**) Numbers of EVs produced by *S. aureus* strains MW2, N305, RF122, O11 and O46. Numbers of EVs per milliliter were normalized to numbers of CFU per milliliter calculated from cultures of each strain grown in BHI medium at a time when EVs were recovered. Data are presented as mean ± SD of values obtained from three independent EV replicates. Asterisks indicate statistical significance (one-way ANOVA followed by Dunnett's multiple comparisons test: $^{**}P < 0.01$; $^{***}P < 0.001$) of the mean value for each strain with that of the strain with the lowest mean value (O11).

α- and β-class phenol soluble modulins, δ-haemolysin), adherence proteins (e.g. elastin binding protein) and host immune evasion factors (e.g. Sbi immunoglobulin-binding protein) as well as proteins involved in antibiotic resistance, survival during pathogenesis and lipoproteins. It should be noted that these factors were among the most abundant proteins in the EV proteome (Supplementary Table S2 and Supplementary Table S3). The distribution of EV proteins according to their functional characteristics was similar between strains, although some subtle differences were observed (Fig. 2E). In summary, the *S. aureus* EVs were rich in virulence-associated factors and contained proteins related to metabolic and translation functions.

***S. aureus* EV proteomes reflect strain properties.** To pinpoint potential correlations between strain properties (*i.e.* host of origin, severity of infection) and the protein cargo found in their EVs, heatmap and clustering analyses were performed on EV proteins from the five *S. aureus* strains (Fig. 3). First at all, whatever the dataset and clustering method, biological triplicates clustered together, suggesting that protein loading into an EV is a reproducible and selective process in each *S. aureus* strain. Based on a matrix for the presence/absence of proteins in vesicles (*i.e.* qualitative analysis; n = 253 proteins), heatmap clustering revealed a partial organisation of EVs according to the host origin of the strains (Fig. 3A). EVs from the two ovine strains formed a group, and MW2 and bovine EVs were the nearest and most distant from this group, respectively.

Based on the relative abundance of proteins in EVs (*i.e.* quantitative analysis; n = 70 proteins), their organisation was different and appeared to reflect the severity of the infections that producing strains could cause (Fig. 3B). Indeed, the first cluster contained EV proteins from strains involved in the development of mild mastitis (N305 and O46), while the second cluster was composed of EV proteins from strains involved in gangrenous and severe mastitis (O11 and RF122, respectively). This cluster also included MW2, a human isolate that is responsible for severe soft tissue and bloodstream infections. Several groups of proteins separated strains as a function of the severity of the infections they cause. Two of these contained proteins with an overall lower (group 1) and higher (group 2) relative abundance of the mild strains N305 and O46 (Fig. 3B,C). Group 1 aggregated virulence-associated factors (δ-haemolysin Hld, CamS, MW1483, FtnA, SitA) whereas group 2 mainly included

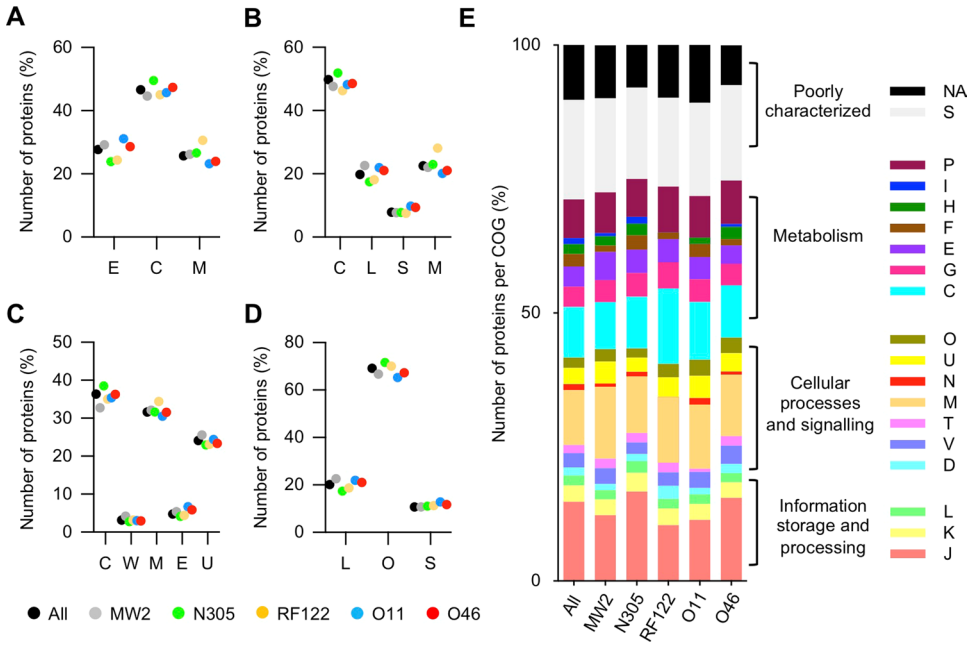

Figure 2. Subcellular localisation and function of *S. aureus* EV proteins. (**A**) CELLO, (**B**) PredLIPO, (**C**) PSORTb, and (**D**) SignalP pipelines were used to predict the subcellular localisation of EV proteins in all the strains tested (pooled dataset; black) and individual strains N305 (green), RF122 (yellow), O11 (blue), O46 (red) and MW2 (grey). C, cytoplasm; E, extracellular; L, lipoprotein; M, membrane; O, other; S, signal peptide I; U, unknown; W, cell-wall. (**E**) Distribution of EV proteins according to their COG classification and strains. C, energy production and conversion; D, cell cycle control/cell division/chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation/ribosomal structure and biogenesis; K, transcription; L, replication/recombination/repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; NA, not available; O, posttranslational modification/protein turnover/chaperones; P, inorganic ion transport and metabolism; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking/secretion/vesicular transport; V, defence mechanisms.

proteins with housekeeping functions. Taken together, these results showed that the EV proteome reflects strain-specific features.

Characterisation of a core EV proteome in *S. aureus*. 119 of the 253 EV proteins identified (47%) were common to all strains, while 20% were strain-specific (Fig. 4 and Supplementary Table S2). The core EV proteome, *i.e.* proteins shared by EVs secreted by all the tested strains, was mostly composed of cytoplasmic proteins (ranging from 36% to 60%, depending on the predictor used) and membrane proteins (ranging from 47 to 51%, depending on the predictor used). 54% of EV proteins with a lipoprotein signal peptide present in the dataset belonged to the core EV proteome (n = 27), which also contained more than 80% of proteins belonging to COG F (nucleotide transport and metabolism, 2/2), G (carbohydrate transport and metabolism, 7/7) and O (post-translational modification/protein turnover/chaperones, 4/5) categories. Other major categories concerned COG C (energy production and conversion, 14/24), J (translation/ribosomal structure and biogenesis, 14/38), M (cell wall/membrane/envelope biogenesis, 16/28) and P (inorganic ion transport and metabolism, 12/19). The core EV proteome notably gathered 59% (54/92) of the previously defined virulence-associated proteins (Table 1 and Supplementary Table S3).

To challenge the core EV proteome established for the MW2, N305, RF122, O11 and O46 strains, its overlap with the EV proteomes of Gram-positive bacteria available in the EVpedia database[30] was analysed (Supplementary Table S4). A large fraction (80%, n = 97) of core EV proteins had homologues present in the EVs of *S. aureus* RN4220. When compared to phylogenetically distant species, proteins belonging to the core EV proteome were also identified, notably in the EVs of *Bacillus subtilis* 168 (n = 43), *Streptococcus pneumoniae* R6 (n = 27), *Streptococcus pyogenes* M1 (n = 38), *Listeria monocytogenes* 10403 S (n = 33), *Mycobacterium tuberculosis* ATCC 25618 (n = 20) and *Clostridium difficile* 630 (n = 25). Nine proteins (the DnaK chaperone protein, GAPDH, ATP synthase subunit beta, Enolase, the Tu elongation factor and four ribosomal proteins) were shared by all of the Gram-positive species analysed.

Patterns, amino acid composition and physicochemical properties of the *S. aureus* EV proteome. The reproducible presence of proteins associated with EVs in biological replicates, and the characterisation of a core EV proteome shared by strains of different host origins, supported the hypothesis regarding the existence of selective processes for cargo sorting. To address these issues, the presence of protein patterns in core

Function[a]	Label	Gene name	MW2	N305	RF122	O11	O46
Host immune evasion							
Immunoglobulin-binding protein	MW2341	sbi	+	+	+	+	+
Bi-component leukocidin LukMF' subunit M	SAB0782	hlyII	−	−	+	+	−
Bi-component leukocidin LukMF' subunit F'	SAB0783	lukF	−	−	+	−	−
Delta-hemolysin	MW1959	hld	+	+	+	+	+
Bi-component leukocidin LukGH subunit G	MW1941	lukG	+	+	+	+	+
Bi-component leukocidin LukGH subunit H	MW1942	lukH	+	+	+	+	+
Phenol-soluble modulin alpha 4 peptide	MW0406.1	psmA4	+	+	+	+	+
Phenol-soluble modulin alpha 3 peptide	MW0406.2	psmA3	+	+	+	+	+
Phenol-soluble modulin alpha 2 peptide	MW0406.3	psmA2	+	+	+	+	+
Phenol-soluble modulin alpha 1 peptide	MW0406.4	psmA1	+	+	+	+	+
Beta-class phenol-soluble modulin	MW1056	psmB1	+	+	+	+	+
Beta-class phenol-soluble modulin	MW1057	psmB2	+	+	+	+	+
Exoenzymes							
Glyceraldehyde-3-phosphate dehydrogenase	MW0734	gapA1	+	+	+	+	+
Sortase	MW2448	srtA	+	+	−	+	+
Adhesion and cell wall anchored surface proteins							
Elastin binding protein	MW1369	ebpS	+	+	+	+	+
Enolase	MW0738	eno	+	+	+	+	+
Extracellular adherence protein Eap/Map	SAO46_01921	map_2	−	−	−	−	+
Proteins related to biofilm production							
Thermonuclease	MW1211	nuc	+	+	+	+	+
Regulatory proteins							
Accessory gene regulator protein D	SAB1921	agrD	−	−	+	−	−
Miscellaneous proteins							
Zinc metalloprotease	MW0466	ftsH	+	+	+	+	+
Serine protease	MW1670	htrA	+	+	+	+	+
Zn-dependent membrane protease	MW1350	yugP	+	+	+	+	+
Bifunctional autolysin	MW0936	atl	+	+	+	+	+
D-alanyl-lipoteichoic acid biosynthesis protein DltD	MW0817	dltD	+	+	+	+	+
Lipoteichoic acid synthase	MW0681	ltaS	−	+	+	−	−
Preprotein translocase subunit SecA	MW0715	secA1	+	+	+	+	+
Preprotein translocase subunit SecDF	MW1587	secDF	+	+	+	+	+

Table 1. Selection of virulence-associated factors found in EVs from *S. aureus* strains MW2, N305, RF122, O11 and 046. [a]adapted from[84].

EV proteins, and the physicochemical properties and amino acid and nucleotide composition of their encoding sequences were assessed. The search for local sequence conservation within at least 3 core EV proteins, done by partial local multiple alignment of sequences with Protomata-Learner, enabled the detection of conservated motifs in only 44 out of the 119 non-redundant (<70% identity) core EV proteins. The most prevalent motif was present in 22 protein sequences, while the others were seen in between three and eight sequences (Supplementary Table S5). Among the 9 most significant motifs, five overlapped with four matches of known motifs or domains from PROSITE database: Lipoprotein signal peptide (PS51257), ATP/GTP-binding site motif A (PS00017), the iron siderophore/cobalamin periplasmic-binding domain (PS50983), and the ATP-binding cassette of ABC transporter (PS50893) (Supplementary Fig. S2 and Supplementary Table S5). The four other motifs had no counterparts in the PROSITE and pfam databases. Interestingly, all the motifs detected were composed of charged or aliphatic amino acid residues.

The overall physicochemical properties of EV proteins were compared to the whole proteomes of the MW2, N305, RF122, O11 and O46 strains (Supplementary Table S6). These analyses showed that the average isoelectric point, the GRAVY (grand average of hydropathy) value and the net charge at pH 7 of proteins packaged into EVs differed significantly ($P < 0.001$) from those of the whole cell proteomes (Fig. 5A and Supplementary Fig. S3). Notably, EV proteins were more globular and more positively charged at a physiological pH. In addition, proteins packaged into EVs significantly ($P < 0.001$) contained more charged, polar and tiny residues and fewer aromatic, aliphatic, hydrophobic and large residues than the whole cell proteome. By contrast, no differences were observed regarding the distribution of proteins according to their number of residues and composition in neutral and small residues, although differences were found at the level of individual amino acids: Ala, Gly and Lys were significantly overrepresented ($P < 0.001$) in EV proteins, whereas Cys, His, Ile, Leu, Phe and Tyr were underrepresented when compared to whole cell proteomes (Fig. 5A and Supplementary Fig. S4). Finally, a comparison carried out on the Codon Adaptation Index (CAI) of each gene revealed that the CAI values of genes coding for EV proteins

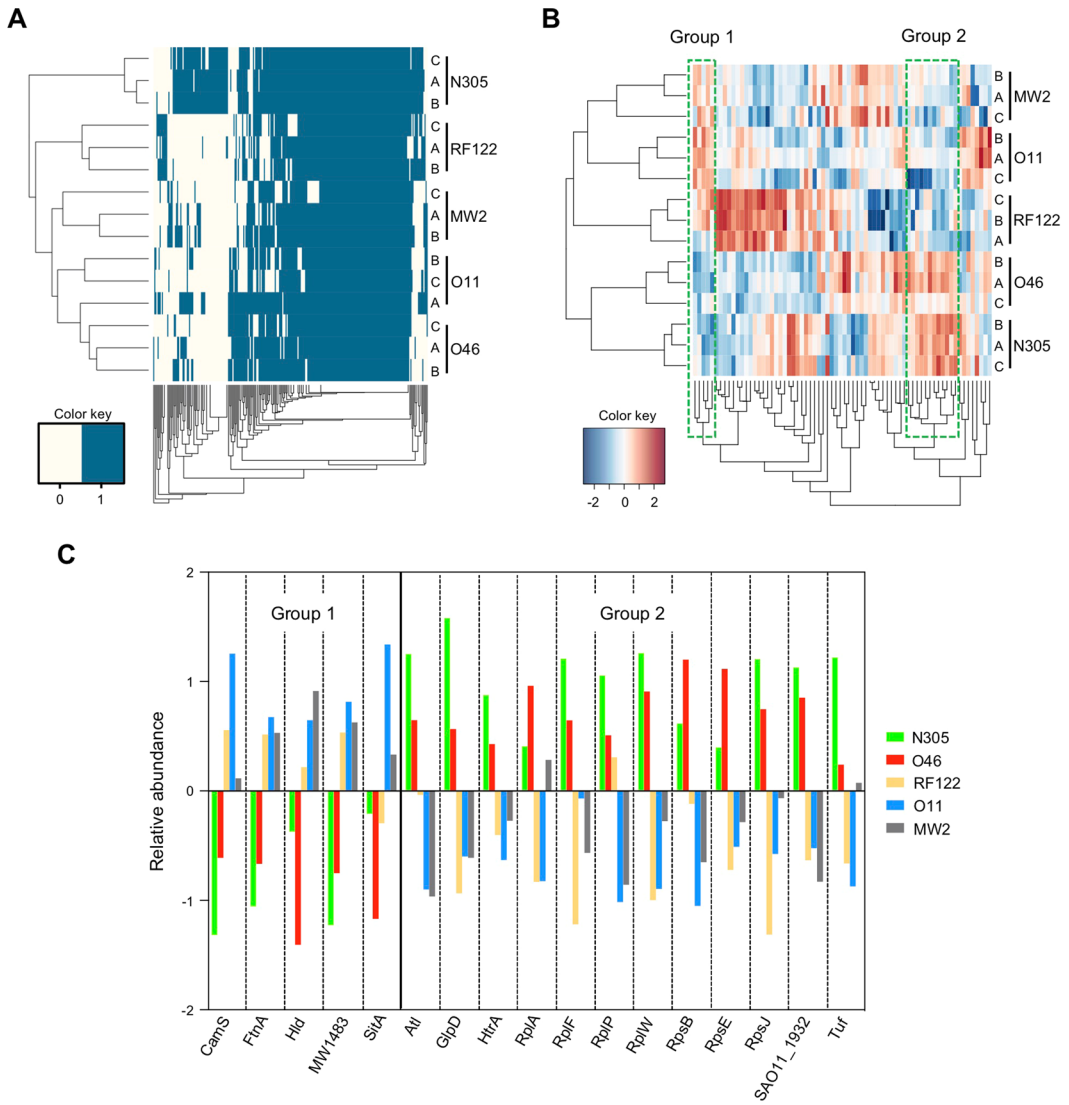

Figure 3. Hierarchical clustering of EV samples. (**A**) Heatmap representation of clustering according to presence/absence of proteins in EV samples. (**B**) Heatmap representation of clustering according to the relative abundance of proteins in EV samples. The group 1 and group 2 proteins with contrasting relative abundance between mild (N305 and O46) and severe (RF122, O11 and MW2) strains are boxed. (**C**) Proteins from group 1 and group 2 and their relative abundance in EVs according to producing strains.

were significantly higher ($P < 0.0001$) compared to those of the whole genome (Fig. 5A). For example, EV proteins in strain MW2 contained 36% more Lys compared to the other cell proteins (10.4% versus 7.6%), and the CAI value of the associated genes was 16% higher (0.68 versus 0.59), although they contained 20% fewer Phe, Ile, and Leu residues.

A discriminant analysis was used to identify the physicochemical properties that best discriminated the proteins according to their detection in EVs or not (Fig. 5B). EV proteins were positively associated with the first axis, which was characterised by charged and polar proteins rich in Glu, Asp and Lys and with high CAI values of the corresponding genes, but were negatively associated with proteins rich in hydrophobic, aliphatic and aromatic amino acids with high GRAVY values.

Overall, these results indicated an absence of conserved motifs within all EV protein sequences, but the existence of specific shared features such as positively charged proteins and high CAI values.

Discussion

The study of EVs produced by *S. aureus* is an emerging field of research, with pioneering work published in the late 2000s[24]. EVs from this bacterium are certainly some of the best documented among Gram-positive bacteria. They might indeed play a role in *S. aureus* pathogenesis and, as such, they offer interesting perspectives in medical applications. The characterisation of the EV protein cargo has been the subject of particular study[12,17,18,21–24]. However, most of this work focused on clinical isolates with emphasis on the contribution of EVs to the pathogenetic process. Moreover, variations to both the growth conditions used to produce EVs and the procedures for

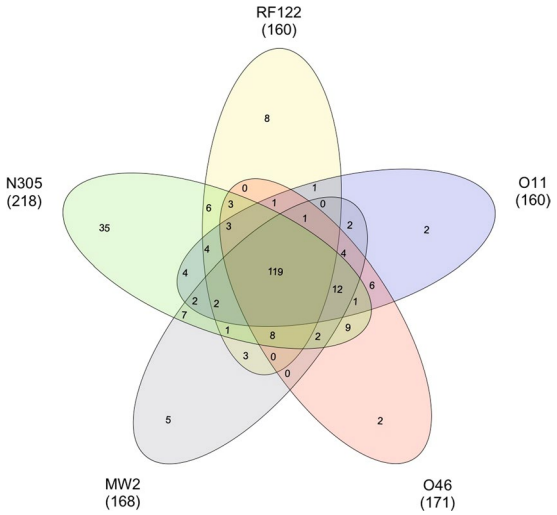

Figure 4. Venn diagram of proteins identified in EVs from *S. aureus* strains MW2, N305, RF122, O11 and O46. The numbers of unique and shared proteins between EVs from several strains are presented.

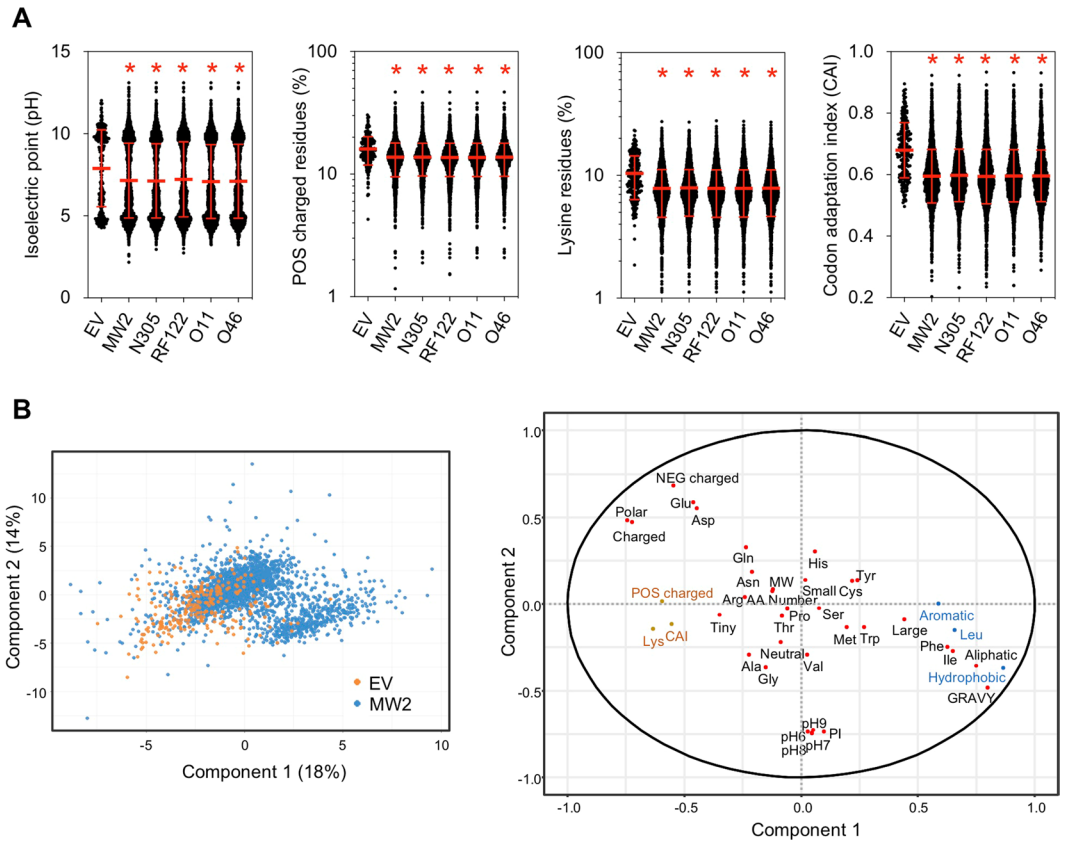

Figure 5. Mean features of EV proteins. (A) Distribution of EV proteins and whole cell proteins of *S. aureus* strains MW2, N305, RF122, O11 and O46 according to their isoelectric point, composition in positively (POS) charged residues, composition in lysine (Lys) residues and the codon adaptation index (CAI) of their corresponding genes. Asterisks indicate statistical significance when compared to the EV group (one-way ANOVA followed by Dunnett's multiple comparisons test: *$P < 0.0001$). (B) Score plot and loading plot of the partial least squares-discriminant analysis (PLS–DA) performed on proteins from *Staphylococcus aureus* MW2 in order to differentiate EV proteins (in orange) from proteins not identified in EV proteins (in blue). Variables are the physicochemical properties of proteins, their amino acid composition and the CAI of each of the corresponding genes. In the loading plot, the most significant features positively and negatively associated with EV proteins are indicated in orange and blue, respectively.

EV preparation and protein identification rarely enabled a reliable comparison of their protein content. Here, we used identical procedures to compare the proteins in EVs. We chose five *S. aureus* strains of diverse host origins (ovine, bovine, human) and causing different types of infection (from mild to severe) to reveal a core EV proteome and gain insight into the mechanisms that drive the selection of proteins to be packaged into EVs. Indeed, understanding how these proteins are selectively packaged offers a possible source of innovation regarding both intervention against pathogenic bacteria and the development of drug delivery systems.

Under our experimental conditions, all these *S. aureus* strains produced and secreted EVs into their surrounding environment. However, some differences between the strains were observed, notably regarding EV production yields and EV size. One remarkable feature was the co-purification of cylindrical (nanotube-like) structures together with EVs in the four animal strain samples. Such nanotubes had previously been observed in Gram-negative and a few Gram-positive bacteria, but with some differences regarding the size of the objects[31]. To the best of our knowledge, ours is the first report to have shown a co-purification of EVs with nanotube-like structures in *S. aureus*. MRSA strains such as MW2 are known to develop a thicker cell wall than methicillin-susceptible *S. aureus* strains[32]. A major barrier to the release of EVs by Gram-positive bacteria is the need to cross a thick cell wall. Whether this accounted for the absence of nanotube-like structures in the MW2 MRSA samples deserves further investigations, as does any biological role they may play.

The EVs of the five *S. aureus* strains studied here displayed similarities in terms of their cargo proteins, and a core EV proteome containing 119 proteins was identified. As previously shown for clinical isolates[12,17,21–24], *S. aureus* EVs secreted by animal isolates are characterised by the presence of numerous proteins with roles in bacterial survival and pathogenesis. These include toxins (phenol soluble modulins, haemolysin, leukocidin), invasion factors (elastin binding protein, autolysin, extracellular adherence protein), enzymes (nuclease, proteases), surface antigens and immunoglobulin-binding protein. The EV cargo also includes proteins that are involved in metal ion acquisition and allow bacterial cells to circumvent metal ion starvation within the host during infection[33]. No nutritional requirements have yet been described for EVs. Presence of such proteins in the EV proteome thus suggests that EVs act as a source of additional metal ions made available at distance for other invading bacterial cells[34]. Alternatively, they could disturb metal ion acquisition in competing bacterial cells or host cells. The EV cargo also comprises numerous lipoproteins, some of which contribute to the nutrient transport, Toll-like receptor 2 activation and pathogenicity of *S. aureus*[35,36]. Most of these proteins belong to the core EV proteome, indicating that the secretion through EVs of proteins involved in many facets of *S. aureus* pathogenesis represents a common strategy for both animal and human *S. aureus* strains. Moreover, the EVs analysed here also contained antibiotic resistance-associated proteins such as penicillin-binding proteins (in four of the five strains studied) or β-lactamase (in MW2). It was shown that EVs carrying biologically active antibiotic degrading enzymes such as β-lactamases were able to confer transient resistance on susceptible surrounding bacteria[20,37,38]. Indeed, penicillin-binding proteins may act as lures or catching agents and titrate antibiotics in the surrounding medium. Whether these antibiotic resistance-associated proteins do confer transient resistance on distant bacterial cells still needs to be explored and could have interesting implications for the treatment of staphylococcal infections.

A remarkable overlap (>80%) was noted between the core EV proteins that we identified here and the EV proteins previously described in the human *S. aureus* strain RN4220, although we could have expected a fall in the number of shared proteins in the core EV proteome as EVs from more *S. aureus* strains were included. This overlap was even more consistent because different growth conditions and methods for EV preparation and protein identification were used[39]. When compared to EV proteomes from phylogenetically distant Gram-positive bacteria, some proteins also appeared to be conserved. This notably included several moonlighting proteins (e.g. enolase, GAPDH, EF-Tu) with functions in adhesion, membrane fusion, and internalisation[40–43]. Because they are considered as a protein secretion pathway, EVs may thus form part of the mechanism for the sorting of moonlighting proteins[44]. Moreover, conservation of these proteins within the EVs of phylogenetically distant species, including Firmicutes and Actinobacteria, might also account for a generic process involved in intercellular interactions; i.e. the attachment and fusion of EVs to the membrane of targeted cells.

EVs mirror the properties of producing strains despite their compositional and functional similarities so could be considered as potential markers for *S. aureus* infections in both human and veterinary medicine. Indeed, based on their protein content, they can be stratified with respect to the host origin (bovine, ovine, or human) of the strains and the severity of infections they cause. Notably, the relative abundance of proteins in EVs is a criterion that can discriminate them according to the severity of infections. Two groups of proteins with contrasted relative abundances of mild and severe strains were identified during the present study, one of them aggregating several virulence-associated factors (e.g. δ-haemolysin Hld) that was less abundant in mild strains than severe strains. *S. aureus* strains of bovine and ovine origins have evolved to adapt to their animal hosts and differ from human strains at the genotypic and genomic levels[45,46]. In some cases, species-specific traits are also observed at the molecular level, as exemplified for example by the bovine variant of von Willebrand factor-binding protein[47], or ovine-specific exfoliative toxin type E[48]. Here, none of the EV proteins specifically associated with ovine (n = 6), bovine (n = 6) or human (n = 5) strains presented any functions that could be related specifically to their host origin, with the exception of beta-lactamase for strain MW2. Moreover, among the 52 strain-specific EV proteins identified here (i.e. proteins found in the EVs of one strain) only nine were strictly strain-specific, because the others were present in the predicted whole proteome of the other strains. This demonstrates that drivers other than the presence of a protein in the whole proteome can account for the strain-specific cargo of EV proteins.

The hypothesis that specific and conserved rules for protein loading into EVs exist was further supported by the identification of a core EV proteome for a variety of strains and the partial conservation of protein cargo in phylogenetically distant species. The mechanisms that control protein packaging into EVs still remain obscure. Our analysis has provided some information on drivers that might influence this process. We found compositional biases in the nucleotide sequences of genes encoding EV proteins and in the amino acid sequences of EV proteins themselves. When compared to the whole genome, the CAI value of genes coding for EV proteins was significantly

higher. Likewise, EVs contained proteins with more positively charged residues (notably Lys) than in the whole predicted proteome. It is often thought that CAI reflects the level of protein expression, with high CAI values being associated with highly expressed proteins[49–51]. Such a feature might reflect the possible enrichment of highly expressed proteins in EVs. In line with this, almost half of the 50 most abundant whole cytoplasmic proteins of *S. aureus* COL were found in EVs during our study[52]. It seems consistent that the relative abundance of a protein in the whole cell could affect its availability to be packed into EVs: the more abundant a protein, the greater the probability of it being loaded into EVs in the absence of, or in addition to, a selective process. The biases regarding the amino acid composition of EV proteins also suggested the selective packaging of positively charged proteins. In line with this, the theoretical PI value of about half of EV proteins is higher than 8, indicating that they are probably positively charged at a physiological pH. Electrostatic interactions are known to contribute to the subcellular targeting of proteins[53,54]. In particular, the negative charges associated with the inner surface of the membrane can direct cationic and basic proteins to a peripheral membrane localisation[55]. On the other hand, lipid homeostasis of the membrane is a crucial key for EV biogenesis[22,23,56–58]. Notably, an accumulation of negative charges in lipid microdomains contributes to membrane curvature and EV biogenesis. The presence of more negatively charged microdomains at the cytoplasmic surface of the membrane may favour the recruitment of positively charged proteins through electrostatic interactions at the site of EV formation, and hence their selective capture.

Our findings show that EVs act as a "proxy" for producing strains in *S. aureus*, and that host-specific profiles can be seen in the protein composition of EVs as well as in the producing strains. Our analysis of EV protein features suggested that abundance, charge and subcellular localisation could influence the protein availability of the vesicle cargo in *S. aureus*. However, not all proteins with high CAI values, highly expressed or with basic domains were packed within EVs, indicating the existence of a selective process following additional rules. The identification of a core EV proteome offers an opportunity to reveal specific signature(s) (e.g. signals, motifs, domains) in protein sequences that might be characteristic of the molecular machinery (or machineries) dedicated to the selection of proteins into EV cargoes. Despite our efforts, no specific signature could be evidenced in EV protein sequences, except for signal peptide and lipoprotein signal peptide. It is interesting to note that proteins known to be associated with the biogenesis of *S. aureus*-secreted EVs (PSM, autolysin) belong to the core EV proteome. One can suppose that the EV cargo also contains components for the selection of proteins into EVs. Chaperones and protein secretion systems, among others, are good candidates, but they still need to be assessed.

Methods

Bacterial strains and growth conditions. *Staphylococcus aureus* strains used in this study were chosen, based on their representativeness with regard to the clonal complex (CC) associated to a given host (Supplementary Table S1). The CC of the strains used in this study are reportedly dominant (or at least frequent) in *S. aureus* strains associated to human (CC1), ovine (CC130), and bovine (CC97, CC151). Furthermore, the studied strains are well documented with genome sequence and phenotype information publicly available (Supplementary Table S1). *S. aureus* ovine strains O11 and O46 were isolated from gangrenous and subclinical mastitis, respectively, and were shown experimentally to induce severe or mild mastitis in ewes[59,60]. Both were isolated in 2003 in Southeast France and kindly provided by Dr Eric Vautor (ANSES, Sophia Antipolis, France). Bovine strain RF122 was isolated from a case of clinical mastitis[61,62]. Bovine strain Newbould 305 (N305) was shown to reproducibly induce mild and chronic mastitis in bovines[63,64]. RF122 and N305 were kindly provided by Pr Ross J. Fitgerald (Edinburgh Infectious Diseases, Roslin Institute, University of Edinburgh, UK) and Dr Pascal Rainard (ISP, INRAE, Nouzilly France), respectively. The human strain MW2 (ATCC BAA-1707) was isolated from a severe case of hospital acquired infection[65] and is reportedly highly virulent[66]. The human strain MW2 was obtained from the Laboratory of Human Bacterial Pathogenesis, National Institutes of Health (Bethesda, MD, USA). The *S. aureus* strains were grown in Brain Heart Infusion (BHI) medium (Difco, pH 7.4) at 37 °C under agitation (150 rpm). The concentrations and growth phases of the bacteria were estimated from spectrophotometric measurements of optical density at 600 nm (VWR V-1200 spectrophotometer). They were further confirmed routinely by counting the colony forming units (CFU) on BHI agar using the micromethod[67].

Purification of *S. aureus*-secreted EVs from culture supernatants. EVs were purified from *S. aureus* culture supernatants as described previously[18]. Briefly, sub-cultured cells at the end of the exponential phase were diluted in 1 L of fresh BHI medium. The cultures were grown until the early stationary phase to optimise the number of EVs that could be recovered. The cells were then pelleted at 6,000 *g* for 15 min and the supernatant fraction was filtered through a 0.22 µm vacuum filter (PES). The filtrate was concentrated using the Amicon ultrafiltration system (Millipore) with a 100 kDa filter and subjected to ultracentrifugation at 150,000 *g* for 120 min at 4 °C. The pellet was then re-suspended in 8% sucrose in tris-buffered saline (TBS) (150 mM NaCl, 50 mM Tris-Cl, pH 7.5), overlaid with sucrose dilutions ranging from 8% to 68% in TBS and centrifuged at 100,000 *g* for 150 min at 4 °C in a SW 55 Ti rotor (Beckman Coulter). The different density fractions were collected, and those containing a similar number of EVs, with a similar particle-size distribution and protein pattern were pooled, centrifuged at 150,000 *g* for 120 min at 4 °C and re-suspended in TBS. EV fractions and isolated EV samples were routinely verified by electron microscopy and Bradford assay (Bio-Rad) and quantified using Nanoparticle Tracking Analysis before being stored at −20 °C until use.

Electron microscopy. The purity and quality of EV samples were confirmed by negative staining electron microscopy and cryo-electron tomography, as previously described[18]. Images were acquired and analysed at the Rennes Microscopy Imaging Centre platform (MRic MET) (University of Rennes 1, Rennes, France).

Nanoparticle tracking analysis (NTA). NanoSight NS300 (Malvern Instruments, United Kingdom) with a 488 nm laser module and sCMOS camera type were used for all measurements of *S. aureus* EVs. All counts were

performed in replicates of five videos of 60 s for each sample (n = 3) and measured in flow mode using a syringe pump. The EVs were thawed and diluted in TBS until optimum visualisation of a maximum number of vesicles was achieved. All quantifications were performed at a controlled temperature of 25 °C, and the measurement data captured were analysed using NTA 3.3 software (Malvern Instruments). We confirmed that the TBS was free of contamination with any other nanoparticles prior to all measurements.

Identification of proteins in *S. aureus* EVs. Three independent biological replicates of each *S. aureus* purified EV were digested for NanoLC-ESI-MS/MS analysis. Approximately 50 µg of EVs were pelleted at 150,000 *g* for 2 h at 4 °C and suspended in a solution of 6 M Guanidine-HCl (Sigma-Aldrich), 50 mM Tris-HCl (pH 8.0) (VWR) and 2 mM DTT (Sigma-Aldrich). The EVs were heated at 95 °C for 20 min and cooled in 50 mM NH_4HCO_3 (pH 7.8) (Sigma-Aldrich). The samples were then digested in solution using sequencing grade-modified trypsin (Promega) at an enzyme:protein ratio of 1:50 for 15 h at 37 °C, as previously reported[24]. After digestion, the peptides were stored at −20 °C until further analysis. NanoLC-MS/MS experiments were performed as previously described[18]. The peptides were identified from the MS/MS spectra using X!TandemPipeline software[68] and searches performed against the genome sequence of *S. aureus* MW2, N305, RF122, O11 and O46 strains. The database search parameters were specified as follows: trypsin cleavage was used and the peptide mass tolerance was set at 10 ppm for MS and 0.05 Da for MS/MS. Methionine oxidation was selected as a variable modification. For each peptide identified, a minimum e-value lower than 0.05 was considered to be a prerequisite for validation. A minimum of two peptides per protein was imposed, resulting in a false discovery rate (FDR) < 01% for peptide and protein identifications.

For protein quantification, each peptide identified by tandem mass spectrometry was quantified using the free MassChroQ software[69,70] before data treatment and statistical analysis with R software (R 3.2.2, Project for statistical computing). A specific R package called 'MassChroqR' (v0.4.3) was used to automatically filter dubious peptides and group the peptide quantification data into proteins. Two different and complementary analytical methods were used, based on peak counting or XIC (eXtracted Ion Current). For peak counting, variance analysis was performed on proteins with a minimum peak ratio of 1.5 between both culture conditions. Proteins with an adjusted P-value < 0.05 were considered to be significantly different. For XIC based quantifications, normalisation was performed to take account of possible global quantitative variations between LC-MS runs. Peptides shared between different proteins were automatically excluded from the dataset, as were peptides present in fewer than two of the three biological replicates. Missing data were then imputed from a linear regression based on other peptide intensities for the same protein. Analysis of variance was used to determine proteins whose abundance differed significantly between strains.

Bioinformatics analysis. All proteins were searched in the NCBI (https://www.ncbi.nlm.nih.gov/) and UniProt (http://www.uniprot.org/) databases. In-house bash script embedding NCBI's Entrez Direct tools was used to retrieve locus tags from Uniprot id, and PATRIC[71] was used to find locus tags of orthologous genes between the five strains. Several pipelines based on different methods were used to predict EV protein localisations: CELLO[72], PredLipo[73], PsortB version 3.0.2[74] and SignalP 5.0[75]. Clusters of Orthologous Groups of proteins (COGs) were used to categorise *S. aureus* EV proteins[76]. Motifs in protein sequences were discovered using Protomata learner 2.0 (http://tools.genouest.org/tools/protomata/learn/)[77], while matches of known PROSITE motifs and profiles were identified with ScanProsite[78]. Amino acid composition, physicochemical properties, the GRAVY (grand average of hydropathy) value and the theoretical isolectric point of proteins from EVs and whole cells in the N305, RF122, O11, O46 and MW2 strains were computed with COPid[79] and the Sequence Manipulation Suite[80] from genomic data present in the MicroScope platform[81]. The Codon Adaptation Index (CAI) values of each gene were obtained from the MicroScope platform. Moonlighting proteins were identified using the MoonProt database[82]. Comparisons of proteins from the EVs of *S. aureus* strains was performed using InteractiVenn[83].

Statistical analyses. The data were presented as mean ± standard error. The differences between groups were verified using one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test with GraphPad Prism version 8.3.1 for macOS (GraphPad Software, San Diego, California USA). A P-value lower than 0.01 was considered to be significant. A partial least squares discriminant analysis (PLS–DA) was performed using the mixOmics R package to identify the variables that best discriminated the MW2 cell proteins identified in EVs from the other MW2 proteins among the 40 variables that described the amino acid composition and physicochemical properties of the proteins, and the CAI of the corresponding genes. PLS–DA was performed on a dataset made up of 40 variables for the 2759 proteins in *S. aureus* MW2, including 253 EV proteins and 2506 proteins not identified in EVs.

Data availability

Raw data are available at this adress: https://doi.org/10.15454/SMFFWK.

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Author contributions

E.G., Y.L.L. and V.A. conceived and designed the study. N.R.T., V.R.R., B.S.R.L., A.B., V.B.B. and J.J. performed the experiments. N.R.T., A.N., Z.K., F.C., P.M., J.J., A.T., V.R.R., B.S.R.L. and E.G. analysed the data. E.G., A.N., V.R.R., and Y.L.L. interpreted the results. E.G. and Y.L.L. wrote the original draft. E.G., Y.L.L., and V.A. contributed to funding acquisition. All authors contributed to preparation of the manuscript and read and approved the final version.

Competing interests

The authors declare no competing interests.

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Environmental Plasticity of the RNA Content of *Staphylococcus aureus* Extracellular Vesicles

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The roles of bacterial extracellular vesicles (EVs) in cell-to-cell signaling are progressively being unraveled. These membranous spheres released by many living cells carry various macromolecules, some of which influence host-pathogen interactions. Bacterial EVs contain RNA, which may serve in communicating with their infected hosts. *Staphylococcus aureus*, an opportunistic human and animal pathogen, produces EVs whose RNA content is still poorly characterized. Here, we investigated in depth the RNA content of *S. aureus* EVs. A high-throughput RNA sequencing approach identified RNAs in EVs produced by the clinical *S. aureus* strain HG003 under different environmental conditions: early- and late-stationary growth phases, and presence or absence of a sublethal vancomycin concentration. On average, sequences corresponding to 78.0% of the annotated transcripts in HG003 genome were identified in HG003 EVs. However, only ~5% of them were highly covered by reads (≥90% coverage) indicating that a large fraction of EV RNAs, notably mRNAs and sRNAs, were fragmented in EVs. According to growth conditions, from 86 to 273 highly covered RNAs were identified into the EVs. They corresponded to 286 unique RNAs, including 220 mRNAs. They coded for numerous virulence-associated factors (*hld* encoded by the multifunctional sRNA RNAIII, *agrBCD*, *psmβ1*, *sbi*, *spa*, and *isaB*), ribosomal proteins, transcriptional regulators, and metabolic enzymes. Twenty-eight sRNAs were also detected, including *bona fide* RsaC. The presence of 22 RNAs within HG003 EVs was confirmed by reverse transcription quantitative PCR (RT-qPCR) experiments. Several of these 286 RNAs were shown to belong to the same transcriptional units in *S. aureus*. Both nature and abundance of the EV RNAs were dramatically affected depending on the growth phase and the presence of vancomycin, whereas much less variations were found in the pool of cellular RNAs of the parent cells. Moreover, the RNA abundance pattern differed between EVs and EV-producing cells according to the growth conditions. Altogether, our findings show that the environment shapes the RNA cargo of the *S. aureus* EVs. Although the composition of EVs is impacted by the physiological state of the producing cells, our findings suggest a selective packaging of RNAs into EVs, as proposed for EV protein cargo. Our study shedds light to the possible roles of potentially functional RNAs in *S. aureus* EVs, notably in host-pathogen interactions.

Keywords: membrane vesicle, small regulatory RNA, virulence factors, vancomycin, RNA-Seq, extracellular vesicle, RsaC, RNAIII

INTRODUCTION

The release of extracellular vesicles (EVs) by living cells is a well-established phenomenon required for intercellular communications and *trans*-kingdom interactions (Brown et al., 2015; Toyofuku, 2019). These spherical membranous particles vary from 20 to 300 nm in diameter and contain macromolecules such as nucleic acids, proteins, lipids, and small metabolites. Initially considered to be trash bags to eliminate unwanted material outside of the cells, they are now widely recognized as protective delivery shuttles of bioactive molecules from donor to recipient cells (Brown et al., 2015; Kim et al., 2015; Gill et al., 2019). The functional characterization of bacterial EVs is of interest due to their capacities to affect bacteria-host cell interactions and bacterial pathogenesis (Kaparakis-Liaskos and Ferrero, 2015; Tsatsaronis et al., 2018). Although the formation of outer membrane vesicles (OMVs) in Gram-negative bacteria was early documented in 1966 (Work et al., 1966), the formation of such structures was disregarded in Gram-positive bacteria until recently. The production of EVs by a Gram-positive bacterium, *Staphylococcus aureus*, was demonstrated in 2009 and, ever since, numereous studies confirmed EV release by other Gram-positive bacteria (Lee et al., 2009, 2013a; Rivera et al., 2010; Prados-Rosales et al., 2011; Brown et al., 2014; Olaya-Abril et al., 2014; Kim et al., 2016a; Liu et al., 2018b).

Staphylococcus aureus commonly colonizes the skin or nasal tract of vertebrates, without causing disease (Wertheim et al., 2005). However, it is also one of the main opportunistic pathogen in humans, and a frequent cause of multi-drug resistant nosocomial infections (Ziebuhr, 2001). *S. aureus* is responsible for a wide array of diseases, ranging from minor infections in soft tissues to life-threatening diseases, such as sepsis, meningitis, and pneumonia (Salgado-Pabón and Schlievert, 2014; Tong et al., 2015). The type and severity of infections depend on strain-specific virulence factors, mostly expressed from accessory genetic elements (Gill et al., 2011). Secreted and surface-exposed *S. aureus* virulence factors weaken the host immune response, leading to bacterial immune evasion and pathogenesis (Foster, 2005). EVs could be a vehicle for secretion and surface-display of these molecules and, accordingly, recent studies indicate that *S. aureus* EVs carry important bacterial survival and virulence factors, such as β-lactamases, toxins, and proteins involved in adhesion to host cells (Lee et al., 2009; Gurung et al., 2011; Jeon et al., 2016; Askarian et al., 2018; Tartaglia et al., 2018, 2020; Wang et al., 2018).

Biologically active β-lactamase in *S. aureus* EVs can confer a transient resistance against ampicillin to surrounding sensible bacteria (Lee et al., 2013b). Furthermore, the presence of α-hemolysin inside EVs accelerates host cell death (Thay et al., 2013; Hong et al., 2014), and EV-associated exfoliative toxin A (ETA) induces a characteristic toxicity onto human epithelial cells (Jeon et al., 2016). Moreover, *S. aureus*-derived EVs facilitate the induction and exacerbation of skin and pulmonary inflammations (Hong et al., 2011, 2014; Kim et al., 2012; Jun et al., 2017). EVs-associated molecules can be more efficient than cytoplasmic proteins to elicit an immune response and host-cell toxicity (Hong et al., 2014). In response to antibiotics exposures, EVs increase *S. aureus* adhesion and cell aggregation, and contribute to biofilm formation (He et al., 2017). Recent data highlight the importance of EVs in staphylococcal pathogenesis since EVs derived from various human and animal strains of *S. aureus* share a conserved EV proteome (Tartaglia et al., 2020).

The vast majority of functional studies on bacterial EVs, however, challenged their proteome. Regarding the presence of DNAs and RNAs in EVs, most studies have been conducted on Gram-negative bacteria (Perez Vidakovics et al., 2010; Blenkiron et al., 2016; Koeppen et al., 2016; Bitto et al., 2017; Choi et al., 2017; Malabirade et al., 2018; Yu et al., 2018; Han et al., 2019). OMV-associated RNAs can include messenger RNAs (mRNA), transfer RNAs (tRNA), ribosomal RNAs (rRNA), or small regulatory RNAs (sRNA; Biller et al., 2014; Ghosal et al., 2015; Ho et al., 2015; Sjöström et al., 2015; Blenkiron et al., 2016; Koeppen et al., 2016; Choi et al., 2017; Dauros-Singorenko et al., 2018; Liu et al., 2018a; Malabirade et al., 2018; Tsatsaronis et al., 2018; Frantz et al., 2019). EV-associated RNA cargo, notably sRNAs, can influence host-pathogen interactions, cell-to-cell communications, and bacterial pathogenesis (Dauros-Singorenko et al., 2018; Tsatsaronis et al., 2018; Lee, 2019; Ahmadi Badi et al., 2020; Lécrivain and Beckmann, 2020). For instance, OMVs from *Pseudomonas aeruginosa* can transfer an sRNA into the human airway cells, resulting in IL-8 decrease (Koeppen et al., 2016). Likewise, transfection of OMV-associated sRNAs from the periodontal pathogens *Aggregatibacter actinomycetemcomitans*, *Phorphyromonas gingivalis*, and *Trepanema denticola* into human cells reduced host interleukine release (Choi et al., 2017). The presence of RNAs within Gram-positive EVs has been reported for fewer species (Resch et al., 2016; Dauros Singorenko et al., 2017; Frantz et al., 2019; Rodriguez and Kuehn, 2020). Interestingly, Frantz et al. (2019) recently reported that the EV-associated *rli*32 sRNA of *Listeria monocytogenes* can trigger the induction of a type I IFN response in host cells. This finding supports that Gram-positive EVs can also participate to host-pathogen interactions by dedicated vesicular RNAs. Data about RNA cargo in EVs released by *S. aureus* are scarce, with only two recent reports. While the first provided a partial RNA profile of *S. aureus* MSSA476 EVs without functional analyses (Joshi et al., 2021), the second showed that the uncharacterized RNA content of *S. aureus* Newman EVs likely stimulate the potent IFN-β response observed in cultured macrophage cells (Rodriguez and Kuehn, 2020).

As far as we know, our work is the first example that provides a detailed RNA profile associated to EVs from a reference clinical *S. aureus* strain, HG003. The staphylococcal EV RNA cargo was unveiled by high-throughput RNA sequencing from purified EVs after release by cells grown under various environmental conditions. They include early- and late-stationary growth phases, with or without a sublethal concentration of vancomycin, an antibiotic used to treat multidrug-resistant infections and that influences *S. aureus* EV biogenesis and functions (Hsu et al., 2011; He et al., 2017). The RNA cargo from the EVs was analyzed and compared to the RNA content of the HG003 parental cells.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The *S. aureus* strain used in this work was the model strain HG003 (Herbert et al., 2010), a NCTC8325 derivative, isolated in 1960 from a sepsis patient. HG003 contains functional *rsbU* and *tcaR* genes, two global regulators that are missing in the NCTC8325 parent strain. The HG003 genome is well documented (Sassi et al., 2014), and this strain is widely used as a reference to investigate staphylococcal regulation and virulence (Liu et al., 2018a). HG003 strain was pre-inoculated in BHI broth and grown overnight at 37°C under 150 rpm/min agitation, and then inoculated 0.1% in 500 ml of fresh BHI (125 rpm/min, at 37°C) on a 1 L Scott flask. Bacterial cultures were retrieved after 6 h and 12 h for early- and late-stationary phases, respectively, in the presence or absence of a sub-inhibitory concentration (0.5 μg/ml) of vancomycin (**Supplementary Figure S1**).

S. aureus EVs Isolation and Purification

Cultures were submitted to EVs isolation and purification, as previously described (Tartaglia et al., 2018, 2020). In brief, for each condition 1 L of bacterial cell culture was centrifuged at 6,000 × *g* for 15 min and filtered through 0.22 μm Nalgene top filters (Thermo Scientific). Then, the culture supernatant fraction was concentrated around 100-fold using the Amicon ultrafiltration systems (Millipore) with a 100kDa filter, and ultra-centrifuged for 120 min at 150,000 × *g* to eliminate the soluble proteins. Next, the suspended pellet was applied to a discontinuous sucrose gradient (8–68%) and ultra-centrifuged at 100,000 × *g* for 150 min. Fractions containing EVs were recovered and washed in TBS (150 mM NaCl; 50 mM Tris-Cl, pH 7.5) for final ultra-centrifugation at 150,000 × *g* (120 min). At last, EVs were suspended in cold TBS and kept at −80°C until use.

EVs Visualization by Electron Microscopy

Negative staining electron microscopy was performed as previously described (Rodovalho et al., 2020) to investigate the shape and integrity of purified EVs. EVs samples were diluted, and solutions containing between 10^{10} and 10^{11} particles per ml were analyzed. For this, samples were applied to glow-discharged copper EM grids (FF200-Cu) for 30 s, followed by excess solution removal with filter paper. The same process was repeated with 2% uranyl acetate, and samples were observed with a Jeol 1400 transmission electron microscope (JEOL Ltd.), operating at 120 kV.

Determination of EVs Sizes and Concentrations

Nanoparticle Tracking Analysis (NTA) using an sCMOS camera and a Blue488 laser (Nano Sight NS300) was performed to assess EVs size and concentration. For that, samples were diluted into TBS to achieve optimal concentration and submitted to a constant flux generated by a syringe pump (speed 50), at 25°C. Results were retrieved from 5 × 60 s videos recorded with camera level at 15 and threshold at 5, while other parameters were adjusted as necessary.

RNA Extraction From *S. aureus* HG003 Whole Cells and Its Derived EVs

RNA extraction was carried out as similar as possible for both cell and EV samples. Bacterial RNA extraction was performed from 10 ml culture pellet. The samples were mixed with glass beads in 300 μl lysis buffer (0.5% SDS w/v, 30 mM sodium acetate; 1 mM EDTA) and 400 μl phenol (acid buffered at pH 5.0) at 65°C. Mechanical lysis was accomplished with 2 cycles of 30 s in Precellys at 6,500 rpm. For EV sample RNA extraction, particles isolated from the equivalent of 800 ml bacterial culture were mixed with 300 μl of lysis buffer and 400 μl phenol at 65°C, the same volumes used for cell RNA extraction. Since EVs lack the thick layer of peptidoglycan (PGN) found in the bacterial cell wall, mechanic lysis was not necessary and was achieved with lysis buffer. EV and EV-producing cell samples were incubated for 10 min at 65°C, being homogenized by vortex every minute. Next, samples were centrifuged during 10 min 13,000 rpm, 4°C, and the upper phase was recovered to a new tube. All samples were mixed with additional 400 μl of phenol at 65°C, and the previous steps were repeated. Then, 400 μl of phenol:chloroform 1:1 was added, followed by two times addition of 400 μl pure chloroform, repeating the step of upper phase recovery, mixture and centrifugation (5 min at 13,000 rpm, 4°C). Subsequently, 1.5 volumes of ice-cold 100% ethanol and 10% volume of NaAc were added and the mix was stored at −20°C overnight. Samples were centrifuged at 13,000 rpm for 30 min at 4°C, and the pellets were washed twice with 1 ml of cold 70% ethanol. Finally, the pellets were dried with a SpeedVac concentrator for 2 min and dissolved in RNase-free water. The quality and quantity of the RNAs were verified by Nano Drop, agarose gel, and Bioanalyzer (Agilent). Samples were kept at −80°C until use. No RNase treatment was applied to bacterial cell or to EV samples before RNA extraction.

RNA Sequencing

The RNA samples were sent to ViroScan3D® (Lyon, France) for DNA removal, ribosomal RNA depletion and RNA sequencing. Total RNA samples were submitted to a DNase treatment with RNase-Free DNase Set (Qiagen) according to manufacturer's instructions. Then, the samples quantified using the Quantifluor RNA system (Promega), and qualified using RNA Nano Chip on Bioanalyzer 2100 (Agilent) for the EV-producing cell samples, and on the SS RNA system on Fragment Analyzer (AATI) for the EVs samples. RNA samples were then submitted to the standard protocol Ovation Universal Prokaryotic RNA-Seq, Nugen, Anydeplete rRNA, library preparation. RNA quantity used for library preparation are displayed in **Supplementary Table S1**. The quality of libraries was assessed with the Quantifluor DNA system (Promega) and qualified with the HS-NGS system on Fragment Analyzer (Aati). The insert mean size of the libraries was 0.34 kp for the EV-producing cell samples, and 0.45 kp for

the EV samples (**Supplementary Table S1**). Sequencing was performed with Illumina, NextSeq500, 75 cycles, single-read, High Output. For each experimental condition, three biological replicates were sequenced. EV-producing cell samples ranged from 9 to 27 million pair-end reads per sample, and EV samples ranged from 30 to 67 million pair-end reads per sample. Reads mapping to the reference genome ranged from 8 to 26 million, and from 0.38 to 27 million reads for EV-producing cells and EV samples, respectively. Basic statistics of the RNA-Seq data are displayed in **Supplementary Table S1**.

Transcriptome Analysis

The reads were cleaned and trimmed with Trim-Galore (Martin, 2011) using the default parameters. Reads were mapped with Bowtie2 (Langmead and Salzberg, 2013) in local mode against two staphylococcal genomes used as references: the NCTC 8325 (NC_007795.1) reference genome with sRNA annotation from SRD (Sassi et al., 2015) and the HG003 genome (GCA_000736455.1) for the non-annotated genes in NCTC8325 genome. Genes were counted with FeatureCounts (Liao et al., 2014) with the strand, the multi mapping, and the overlapping options.

A list of differentially expressed RNAs was obtained by EdgeR (Robinson et al., 2009) embedded in SARTools (Varet et al., 2016). The threshold of statistical significance was set to 0.05, with the adjustment method of Benjamini-Hochberg. RNA coverage was calculated with Bedtools coverage (Quinlan and Hall, 2010). RNAs with ≥ 90% coverage in at least one EV condition were kept for further indepth analysis. RNA coverage visualization was performed with the Integrative Viewer Software (IGV; Thorvaldsdóttir et al., 2013) on a log scale.

Subcellular location prediction was performed with SurfG+ (Barinov et al., 2009). Clusters of Orthologous Groups (COGs) and KEGG categories were obtained using the eggNOG-mapper v2 web tool (Huerta-cepas et al., 2017, 2019). Functional enrichment analysis was performed with g:Profiler web-server (Raudvere et al., 2019; Reimand et al., 2019). A maximum value of p 0.05 was set as a threshold for significative categories.

A timepoint clustering study was conducted with the R package maSigPro (Conesa et al., 2006; Nueda et al., 2014) on highly covered EV RNAs with normalized counts by EdgeR. In this analysis vancomycin treatment is not taken into consideration. The threshold of statistical significance was set to 0.05, with the adjustment method of Benjamini-Hochberg.

RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) was used to validate RNA-seq results. EVs were isolated from the cell-free supernatants of three new independent *S. aureus* cultures at late-stationary growth phases (12 h) in the absence of vancomycin. EV RNAs were purified as mentioned above. Around 1.5 µg of RNAs was treated with DNAse I (Amplification Grade, Invitrogen) according to manufacturer's instructions. cDNA synthesis was performed with the high capacity cDNA Reverse Transcription kit (Applied Biosystems). The primers used for quantitative PCR (qPCR) are listed in **Supplementary Table S2** and were designed using eprimer3 software (EMBOSS). qPCR was carried out in a 16 µl volume containing 15 ng cDNA, specific primers (300 nM), and 8 µl IQ™ SYBR Green Supermix (Bio-Rad). Reactions were run on a CFX96 real-time system (Bio-Rad, France) using the following cycling parameters: DNA polymerase activation and DNA denaturation 95°C for 5 min, 40 cycles of denaturation at 94°C for 15 s, and extension at 60°C for 30 s. Melting curve analysis was included to check the amplification of single PCR products. Samples setups included biological triplicates and technical duplicates as well as negative controls corresponding to qPCR reactions performed without cDNA (cDNA negative control) and from RT reactions obtained from EV RNAs without reverse transcriptase (RT negative control). Results were analyzed with the GFX Manager software and Ct values were determined. Results with Ct equal or above 40 were considered negative and only experiments with $\Delta Ct \geq 4$ between negative controls and RT samples were considered.

RESULTS

S. aureus HG003 Produces EVs in Different Growth Conditions

Extracellular vesicles secreted by HG003 were isolated from the cell-free supernatants of bacterial cultures at early- and late-stationary growth phases (6 and 12 h, respectively), as well as in the absence (V-) and presence (V+) of a sublethal concentration of vancomycin (0.5 µg/ml). For that purpose, we used centrifugation, filtration, and density gradient ultracentrifugation, the standard method for EV isolation and purification at high purity (Yamada et al., 2012; Dauros Singorenko et al., 2017). EV homogeneity and integrity were evaluated by both negative staining electron microscopy and by NTA. Electron micrographs of purified EVs revealed typical nano-sized vesicular structures, with cup-shaped forms in all tested conditions (Raposo and Stoorvogel, 2013; **Figure 1A** and **Supplementary Figure S2**). NTA analyses showed a typical profile of particles for all EV samples (**Figure 1B** and **Supplementary Figure S2**). A significant increase of approximately 55% in EV diameter was observed in those purified from late-stationary phase cultures, compared to early-stationary phase cultures (for both 6V− vs. 12V− and 6V+ vs. 12V+), whereas no significant difference was observed in the absence or presence of vancomycin (6V− vs. 6V+ and 12V− vs. 12V+, **Figure 1C**). EV yield is essentially similar at the two growth phases, irrespective to the presence/absence of vancomycin (**Figure 1D**). In summary, *S. aureus* HG003 releases EVs with variable diameters depending on the growth phase. A sublethal concentration of vancomycin, however, does not impact the EV morphology, concentration, or diameter.

The *S. aureus* EVs Harbor all RNA Functional Classes

Total RNA was extracted from HG003 EVs to investigate their compositions. The quality of the RNA preparations was checked and validated, and the samples sequenced. RNA-seq data were

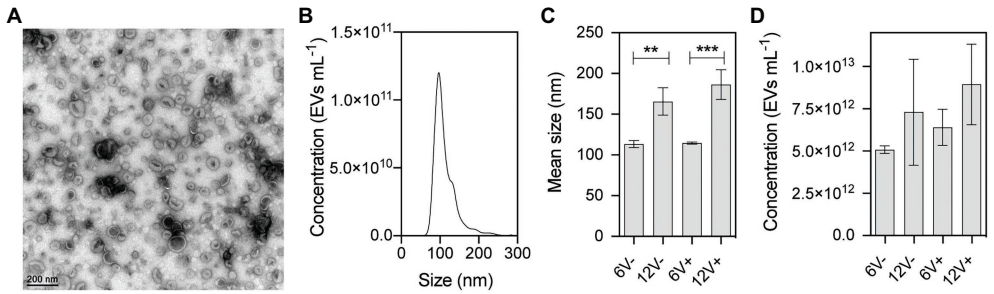

FIGURE 1 | Physical characterization of purified *Staphylococcus aureus* HG003-secreted extracellular vesicles (EVs). **(A)** Representative electron microscopy image of negatively stained HG003 EVs. **(B)** Representative graph of the EV size distribution. **(C)** Mean EV sizes. **(D)** EV yields. Data were obtained from three independent EV replicates. Asterisks indicate statistical significance (one-way ANOVA followed by Tukey's multiple comparisons test: $^{**}p < 0.01$; $^{***}p < 0.001$). Early- and late-stationary growth phases (6 and 12, respectively) in the absence (V–) or presence (V+) of vancomycin.

compared between the different growth conditions, and with those obtained from parental HG003 cells that produced the EVs in each condition (i.e., the EV-producing cells). Around 2649 ± 238 RNAs annotated in the HG003 genome were identified in the EVs according to growth conditions, with an average count of over five reads per RNA in each condition, whereas 3120 ± 35 annotated RNAs were identified within the EV-producing cells (**Supplementary Tables S3** and **S4**). All the four main RNA functional classes (tRNAs, rRNAs, mRNAs, and sRNAs) were identified in both the purified EVs and the EV-producing cells (**Figure 2A**). In both the EVs and the EV-producing cells, ~84% of the mapped RNAs corresponded to protein-coding genes (mRNAs). The sRNAs were the second most abundant mapped RNA class (~12% of the reads). The remaining 4% of the mapped RNAs are tRNAs and residual rRNAs. Note that most of the rRNAs were voluntarily removed during the RNA purification. Most of the annotated mRNAs, tRNAs, and residual rRNAs were identified in the EV-producing parental cells (from 86 to 96%), while this value dropped to 79.8 ± 2.7% for sRNAs (**Figure 2B**). These percentages were slightly lower for EV samples (from 72 to 89%), although they remained high for the sRNAs (59.1 ± 10.2%). All these RNAs detected in the purified EVs prompted us to check their coverages, to evaluate their integrity.

The *S. aureus* Purified EVs Contain Both Fragmented and Intact RNAs From Various Functional Classes

For the EV-producing cells, the median values of mRNAs, tRNAs, and residual rRNAs coverages were between 95 and 100%, and were 74 and 92% for the sRNAs (**Figure 3A** and **Supplementary Table S4**), implying that those RNAs were mainly intact, and not degraded. The coverage profile was drastically different for the RNAs recovered from the purified EVs. While the coverage of the residual rRNAs varied from 83 to 92%, the median coverage values for the other RNA functional classes ranged from 61 to 92% for the tRNAs, 15 to 47% for the mRNAs, and 4 to 20% for the sRNAs (**Figure 3A**). These lower coverages suggested that a substantial fraction of mRNAs and sRNAs were fragmented in the EVs compared to the EV-producing cells. To analyze potentially functional RNAs in EVs, only RNAs with a coverage ≥90% were considered for further analysis (**Supplementary Table S5**). The distribution of the newly filtered RNAs was depicted in **Figures 3B**,**C**. Such a harsh quality criterion impacted mainly the RNAs from the EVs, and particularly mRNAs and sRNAs. Only 3.5 ± 2.7 and 3.4 ± 1.2% of annotated mRNAs and sRNAs, respectively, were identified within EVs with such a threshold, while 67.9 ± 7.2% and 34.9 ± 5.0% of annotated mRNAs and sRNA were identified, respectively, for EV-producing cells (**Figure 3C**). Compared to the parental cells, the EVs were slightly depleted into mRNAs (68.0 ± 7.0% for the EVs vs. 88.2 ± 0.3% in the EV-producing cells) but, interestingly, were enriched for the other RNA functional classes including the sRNAs (14.3 ± 3.2% for the EVs vs. 8.6 ± 0.4% in the EV-producing cells, **Figure 3B**).

Functional Characterization of the RNAs From the EVs

According to experimental conditions, from 86 to 273 RNAs with a ≥90% coverage and an average count of over 5 reads per RNA were identified within EVs from *S. aureus* HG003 (**Figure 4A** and **Supplementary Table S5**). They corresponded to 286 unique RNAs and were either mRNAs (220), tRNAs (28), residual rRNAs (10), and sRNAs (28). The presence of some of these transcrits associated with HG003 EVs and corresponding either to mRNAs or sRNAs was confirmed by RT-qPCR on RNAs extracted from three independent biological replicates (**Figure 5**). Among the mapped mRNAs, most were implicated in translation, ribosomal structure and biogenesis (17.5%, COG J), energy production and conversion (13.6%, COG C), carbohydrate transport and metabolism (COG G, 7.9%), transcription (5.2%, COG K), and cell wall/membrane/envelope biogenesis (5.2%, COG M; **Figure 4B**). Several COG and KEGG categories were notably enriched ($p < 0.05$) in the EVs compared to the EV-producing bacteria (**Figure 4C**). mRNAs expressing proteins with a cytoplasmic location prediction were more represented in the EVs (79.5%) than into the producing cells (72.9%; **Figure 4D**). Interestingly, EVs contained several mRNAs coding for virulence-associated proteins such as the immune evasion protein A and Sbi, the Atl autolysin,

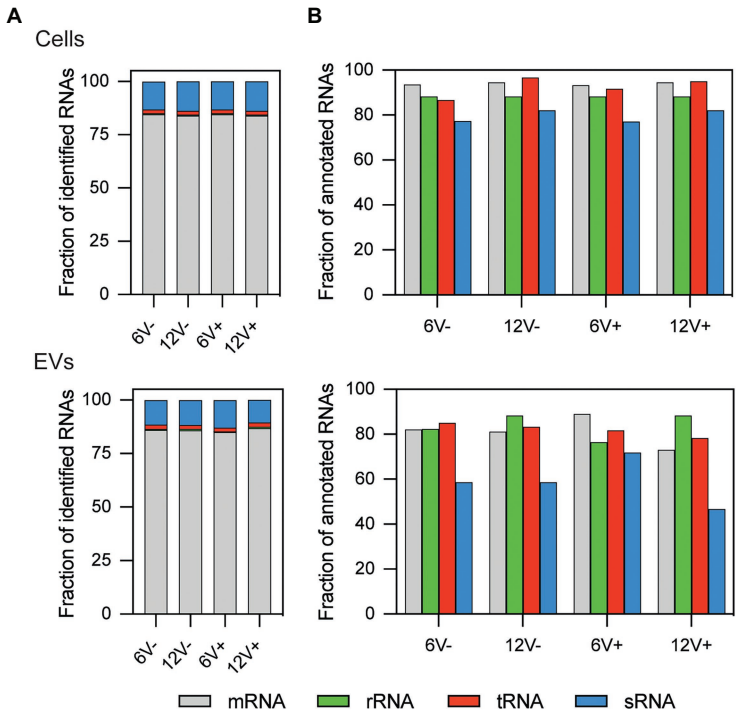

FIGURE 2 | Relative RNA composition of *S. aureus* HG003 and its secreted EVs. Individual colored bars represent the relative amount of each RNA class for mapped reads **(A)** and annotated RNAs **(B)**. RNA-Seq data is the average of three independent replicates. Number of reads have been normalized with EdgeR. RNA classes are defined from the *S. aureus* genome annotation NCTC8325/HG003. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V–) or presence (V+) of vancomycin.

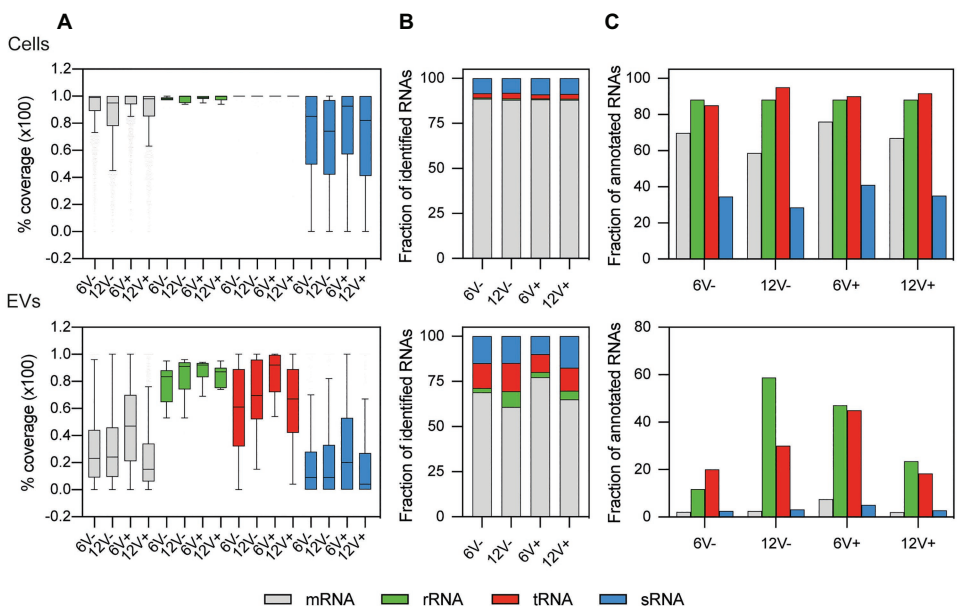

FIGURE 3 | Relative composition of highly covered RNAs from *S. aureus* HG003 and its secreted EVs. Colors represent the relative amount of each RNA class. **(A)** Percentage of RNA median coverage. Distribution of newly filtered RNAs with ≥90% coverage were plotted for **(B)** mapped reads **(C)** and annotated RNA. Number of reads have been normalized with EdgeR. RNA classes are defined from the *S. aureus* genome annotation NCTC 8325/HG003. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V–) or presence (V+) of vancomycin.

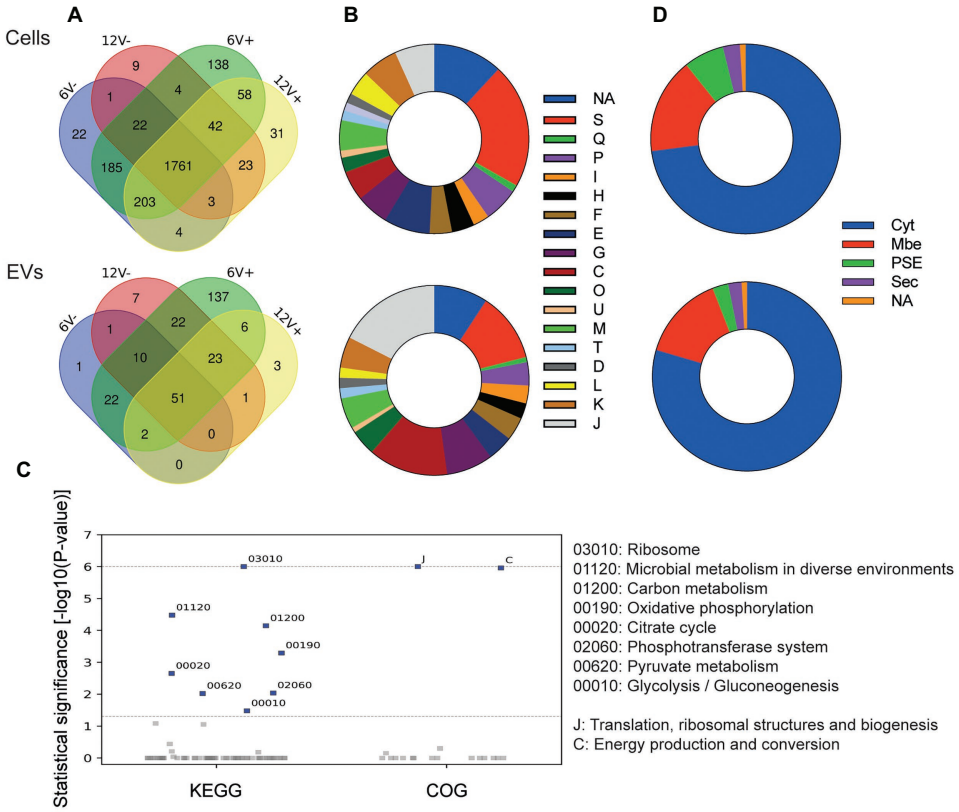

FIGURE 4 | *Staphylococcus aureus* HG003 EV RNA cargo and its modulation by different growth conditions. **(A)** Venn diagrams of RNA composition in EV-producing cells (upper panel) and EVs (lower panel) from different growth conditions. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V-) or presence (V+) of vancomycin. **(B)** Prediction of Clusters of Orthologous Groups (COG) categories for mRNAs: NA, not predicted; S, function unknown; Q, secondary metabolites biosynthesis, transport, and catabolism; P, inorganic ion transport and metabolism; I, lipid transport and metabolism; H, coenzyme transport and metabolism; F, nucleotide transport and metabolism; E, amino acid transport and metabolism; G, carbohydrate transport and metabolism; C, energy production and conversion; O, post-translational modification, protein turnover, and chaperones; U, intracellular trafficking, secretion, and vesicular transport; M, cell wall/membrane/envelope biogenesis; T, signal transduction mechanisms; V, defense mechanisms; D, cell cycle control, cell division, chromosome partitioning; L, replication, recombination and repair; K, transcription; J, translation, ribosomal structure and biogenesis. **(C)** COG and KEGG categories enriched ($p < 0.05$) in EVs compared to EV-producing cells. **(D)** Subcellular localization of proteins encoded by mRNAs as predicted by SurfG+: Cyt, cytoplasmatic; Mbe, membrane; PSE, surface-exposed; Sec, secreted; NA, Not predicted.

the Hld δ-hemolysin encoded by the multifunctional sRNA RNAIII, the PSMβ1 Phenol Soluble Modulin, the FntA iron-storage ferritin, and the MntABC iron ABC transporter. Among the 20 tRNAs annotated in the genome, 15 were identified into the EVs ($tRNA^{His}$, $tRNA^{Asn}$, $tRNA^{Glu}$, $tRNA^{Arg}$, and $tRNA^{Asp}$ were absent). Five copies of the 16S and 23S rRNAs were also detected, implying that our rRNA depletion procedure was incomplete. Finally, 28 annotated potential sRNAs were detected within EVs, and among the 50 or so *bona fide* sRNAs defined for the HG003 strain (Liu et al., 2018a), only RsaC was identified with a ≥90% coverage in this study. Note that despite encoding the highly covered Hld transcript, the sRNA RNAIII presented only 71% gene coverage and therefore was excluded from analysis. Around 196 out of the 286 EV-associated RNAs colocalized at the same loci onto the HG003 chromosome, to form 42 clusters of 2 to 29 contiguous genes that were experimentally shown to belong to the same transcriptional units (Mäder et al., 2016; **Supplementary Table S6**). Among these transcriptional units, 17 displayed a RNA-Seq coverage ≥90% across the entire operon in both the EVs and the EV-producing bacteria. **Figure 6** illustrates the sequencing coverage of various contiguous genes within the EVs and the EV-producing cells. Long mRNA operons, up to ~14,000 nucleotides, were detected as fully covered by reads into the purified EVs, supporting the presence of highly covered RNAs and operons as full-length transcripts.

EV RNA Composition Varies With Growth Conditions

The RNA composition of the purified EVs was compared between early- and late-stationary phases, and with or without vancomycin. Eighteen percentage (n = 51) of all detected RNAs with ≥90% coverage were common to all the EV samples (**Figure 4A** and **Supplementary Table S5**), implying that the RNA content of the EVs highly varied according to the growth conditions. The percentage of RNAs shared by all the EV-producing cell samples, however, was much higher (70%, n = 1761). The shared RNAs among the EVs included mRNAs expressing virulence factors

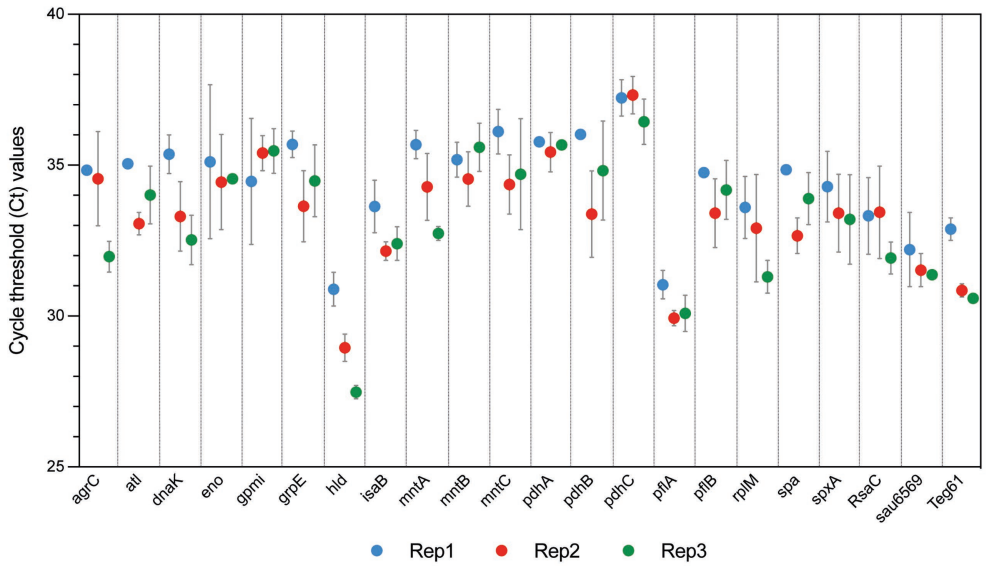

FIGURE 5 | Reverse transcription quantitative PCR (RT-qPCR) validation of *S. aureus* HG003 EV RNAs. RT-qPCR experiments were performed from RNAs extracted from EV samples isolated from the cell-free supernatants of three independent *S. aureus* cultures at late-stationary growth phases (12 h) in the absence of vancomycin (Rep1, Rep2, and Rep3). Quantitative PCR (qPCR) successfully amplified the coding-sequence of 19 mRNAs, and 3 sRNAs. Samples setups included biological triplicates (Rep1, Rep2, and Rep3) and technical duplicates as well as negative controls corresponding to qPCR reactions performed without cDNA, and from RT reactions performed without reverse transcriptase enzyme. Ct values are expressed as mean ± SD from two independent technical replicates performed in triplicates.

(Atl and Spa), metabolic enzymes (pyruvate dehydrogenase and cytochrome c oxidase complexes, glycolytic enzymes) and transcriptional regulators (SpxA, CggR, and GlnR), as well as RNAs involved in translation (ribosomal proteins, rRNAs, and tRNAs; **Supplementary Table S5**). The 51 common RNAs also included 9 potential sRNAs, notably RsaC involved in *S. aureus* oxidative stress adaptation and nutritional immunity (Lalaouna et al., 2019).

For both the EVs and EV-producing cells, more RNAs were detected at 6 h (275 and 2443 for EVs and EV-producing cells, respectively) than at 12 h (126 and 2161 for EVs and EV-producing cells, respectively, **Figure 4A**). Likewise, more RNAs were detected in the EVs in the presence (277 and 2,474 for EVs and EV-producing cells, respectively) than in the absence of vancomycin (140 and 2,279 for EVs and EV-producing cells, respectively; **Figure 4A**), indicating that the antibiotic modifies the RNA cargo of the EVs. These results also highlighted that the growth phase and the antibiotic stress impacted mostly the RNA content of the EVs, but much less that of the parental cells. Indeed, when we considered the RNAs detected in only one condition (i.e., specific RNAs), their fractions were higher in the EVs than in EV-producing cells, and that for all the tested conditions. For example, 58% of RNAs found within EVs at 6 h were specific to this condition, while specific RNAs represented only 14% of all RNAs detected at 6 h in the EV-producing cells.

EV RNA Abundance Varies With Growth Conditions

In addition to the qualitative variations observed, significant differences (Padj < 0.05) in EV RNA abundance between the experimental conditions were also detected (**Supplementary Table S7**). Among the 286 EV-associated RNAs, 110 were differentially abundant between two conditions. Variations were detected at all times and in the absence or presence of vancomycin, although the growth phase appeared to have a greater impact on RNA abundance (75 and 64 differentially abundant RNAs were detected between early- and late-stationary phase with or without vancomycin, respectively), than the antibiotic stress (8 and 9 differentially abundant RNAs were detected between presence and absence of vancomycin in early- and late-stationary phase, respectively; **Supplementary Table S7**). A selection of RNAs with a modulation of their abundance according to the growth conditions is displayed in **Figure 7**. The most modulated RNAs into EVs produced from the two growth conditions were mRNAs coding for virulence-associated factors, such as *agrB*, *agrC*, *agrD*, *psmB1*, and *hld* with a 30- to 1300-fold change, two potential annotated sRNAs, srn_0560, and srn_1000 with a 16- and 190-fold change, respectively, $tRNA^{Gly}$ (SAOUHSC_T00025) and $tRNA^{Thr}$ (SAOUHSC_T00054), with fold changes greater than 16. Among the differentially abundant RNAs according to the growth phase, 32 were detected both in presence and absence of vancomycin, with similar fold changes highlighting their reproducible variations into EVs across different environmental conditions (**Figure 7** and **Supplementary Table S7**).

Differentially expressed RNAs were also detected for the EV-producing cells when their expression was compared between early- and late-stationary phase both in absence (n = 136) and in presence of vancomycin (n = 147), which was expected since bacterial transcription differs qualitatively

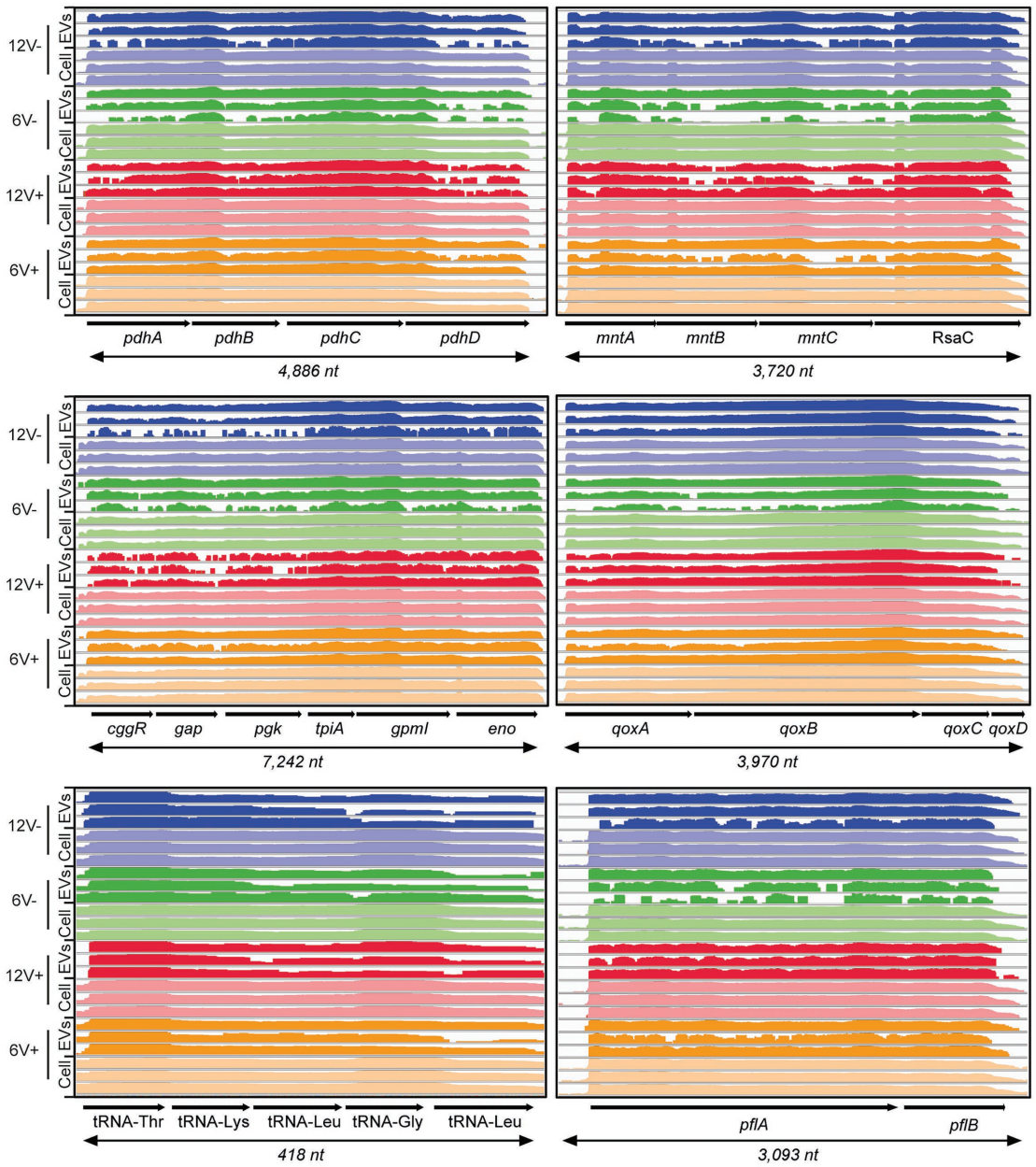

FIGURE 6 | HG003 EVs contain long mRNA transcripts. Comparison between operon coverages between EV-producing cell and EV samples in different conditions. Early- and late-stationary growth phases (6 and 12, respectively) in absence (V-) or presence (V+) of vancomycin. RNA coverage is visualized with Integrative Viewer Software (IGV) in log scale.

and quantitatively when facing different growth conditions (**Supplementary Table S8**). Note that no significantly differentially expressed RNAs were detected according to the presence of vancomycin. As observed previously for the EV RNA content, the growth conditions, particularly the growth phase, impacted mostly the RNA abundance of the EVs, but much less than of the parental cells. Indeed, 38% of RNAs detected within EVs displayed changes in their abundance between conditions, while the fraction of modulated RNAs counted for only 2% of the RNAs in the EV-producing cells. The abundance pattern of several RNAs differed between the EVs and the EV-producing cells according to the growth conditions (**Figure 7**). While some RNAs such as *agrBCD*, *psmβ1*, and *hld* mRNAs displayed the same variations of their abundance pattern in EVs and EV-producing cells regardless the growth conditions. Others, such as *spa* and RsaC, were differentially abundant between the EVs and the EV-producing cells.

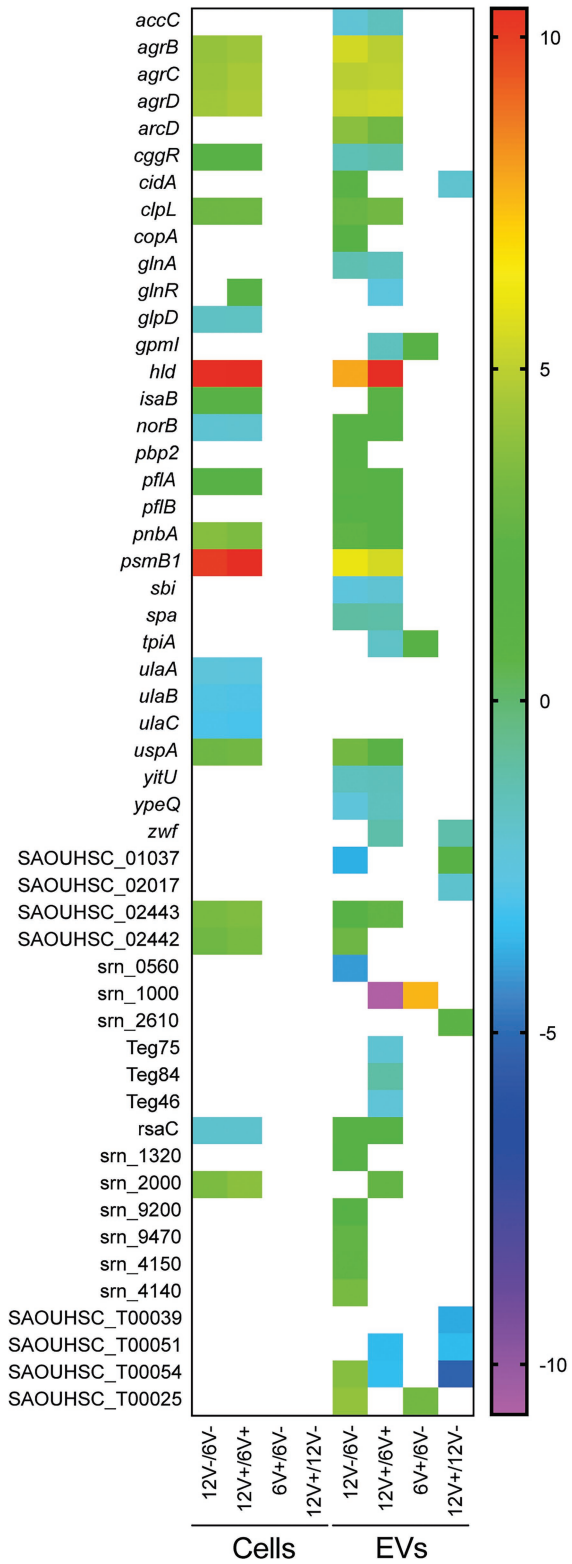

FIGURE 7 | Differential RNA abundance between the EV-producing cells and the EVs. Colored square shows different RNA abundance patterns between

(Continued)

FIGURE 7 | EV-producing cells and EVs with a log2 fold change. Comparisons comprised data with at least one of the two samples containing ≥90% coverage. The log2 fold change is displayed as colored squares from −2 (purple) to 10 (red). Early- and late-stationary growth phases (6 and 12, respectively) in absence (V−) or presence (V+) of vancomycin.

Timepoint Clustering Analysis Reveals Different RNA Abundance Profiles Between the EVs and EV Producing Cells

To evaluate the influence of the growth phase on EV and EV producing cell RNA composition, a negative binomial-based approach, with the R package maSigPro (Conesa et al., 2006) was applied. Briefly, maSigPro provides a differentially expressed transcript analysis of serial data between experimental groups (e.g., EV and EV producing cells). maSigPro was applied to the 286 highly covered EV RNAs and identified 91 RNAs with significant temporal profile changes (Padj < 0.05). RNAs were clustered according to their expression profiles (**Supplementary Table S9**). **Figure 8** shows the six RNA clusters obtained. Three clusters grouped transcripts with a similar expression profile between EV and EV-producing cells: cluster 4 with 8 RNAs (including, e.g., *agrBD* and *arcC2*) and cluster 3 with 18 RNAs (including, e.g., *hld* and *psmβ1*) contained more abundant transcripts over time in both EVs and EV-producing cells, whereas cluster 6 with 14 RNAs (including, e.g., *fusA*, *tuf*, and *secY1*) contained less abundant transcripts at 12 h than at 6 h in both. Interestingly, the three other clusters grouped RNAs that showed opposite expression profiles over time in EVs and EV-producing cells. Cluster 1 with 10 RNAs (including, e.g., RsaC and *pdhA*), and cluster 2 with 24 RNAs (including, e.g., *ldh1*, *qoxABC*, and *rpoBC*) grouped, similarly, more abundant transcripts at 12 h in EVs, and less abundant transcripts at 12 h in EV-producing cells. On the contrary, cluster 5 with 17 RNAs (including, e.g., *ccgR* and *sbi*) grouped less abundant transcripts in EVs and more abundant transcripts in EV-producing cells at 12 h. Altogether, this analysis highlighted that the transcript expression pattern could temporally differ between EVs and EV-producing cells.

DISCUSSION

Extracellular vesicles are universal carriers of macromolecules including extracellular RNAs all along from bacteria, archaea, and fungi to protists. Recent investigations on bacterial EV biogenesis, release, and trafficking showed their functional importance for bacterial communication and survival (Tsatsaronis et al., 2018). Information regarding *S. aureus* EV RNA cargo, however, is lagging behind. Here, we report the first exploratory work on EVs released by *S. aureus* HG003, the characterization of its EV RNA cargo under different conditions, and an indepth transcriptomic comparison between the EVs and the EV-producing cells.

Environmental conditions, such as growth phase and environmental stresses, reportedly influence the production, content and functions of EVs (Tashiro et al., 2010; Kim et al., 2016b; Orench-Rivera and Kuehn, 2016; Askarian et al., 2018;

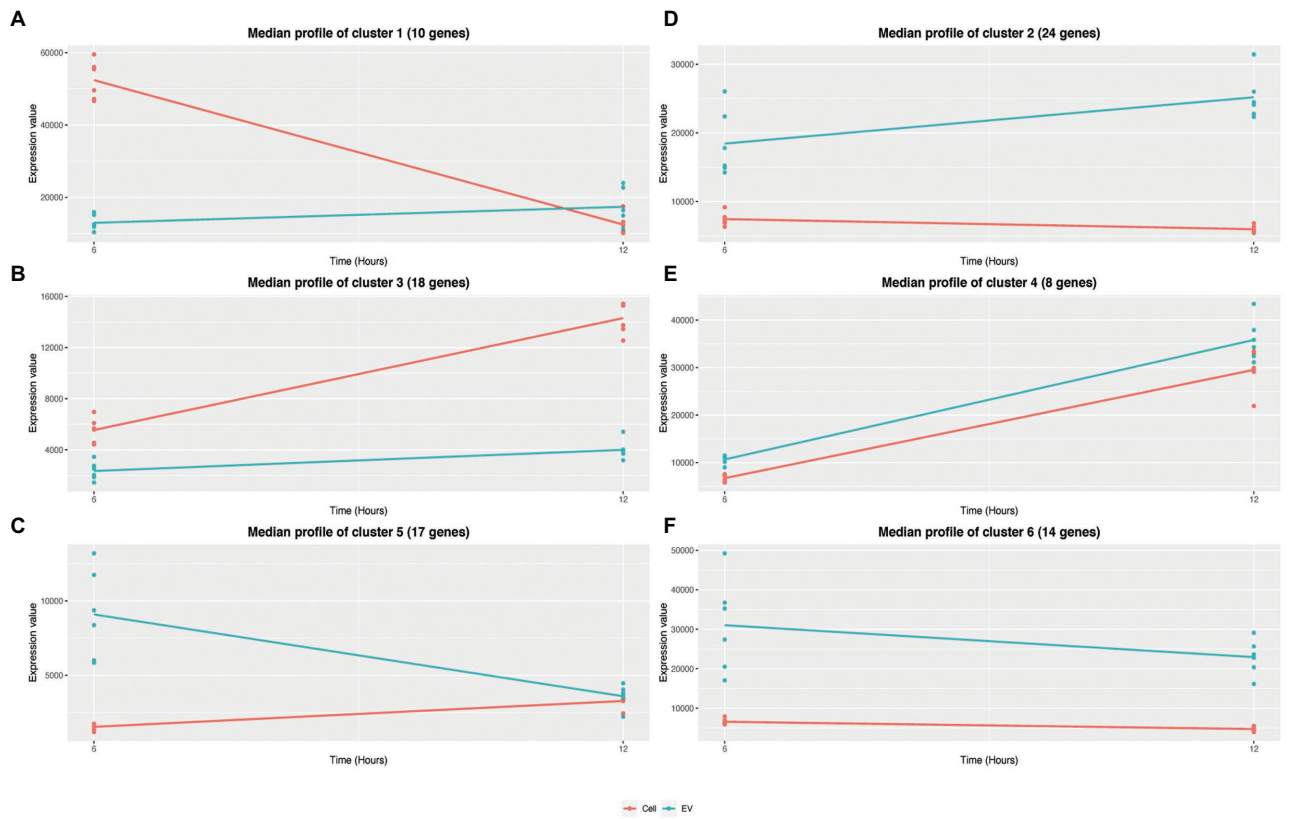

FIGURE 8 | Timepoint clustering of expression profiles between EVs and EV-producing cells. The analyses were performed with the R package maSigPro from normalized counts by EdgeR. Statistical significance ($p < 0.05$) was adjusted with Benjamini-Hochberg method. Median expression values are calculated at 6 and 12 h for EVs (blue) and EV-producing cells (red) for the six clusters (from **A** to **F**).

Yun et al., 2018; Andreoni et al., 2019). Here, to investigate the impact of environmental changes on *S. aureus* EV production, the selected conditions were the early- and late-stationary growth phases, with or without a sublethal concentration of vancomycin that does not impact growth. Differences were observed regarding EV sizes. EVs derived from the late-stationary growth phase were larger than those collected during the early-stationary phase. This can be due to cell-wall morphology and peptidoglycan structure that are characteristics of the growth stage in *S. aureus* (Zhou and Cegelski, 2012). A correlation between the degree of peptidoglycan cross-linking to the cell wall stiffness and EVs release was observed for both Gram-negative and Gram-positive bacteria (Zhou et al., 1998; Deatherage et al., 2009; Schrempf and Merling, 2015; Schwechheimer et al., 2015; Wang et al., 2018). Notably, sub-inhibitory concentrations of penicillin decreases peptidoglycan cross-linking, triggering an increase in *S. aureus* EV yields and sizes (Wang et al., 2018). Here, we show that sublethal concentrations of vancomycin, an antibiotic that also targets peptidoglycan synthesis in *S. aureus*, does not impact either EV morphology or EV production yields, but does change their RNA content in terms of composition and abundance. Vancomycin affects *S. aureus* EV activity starting at 1 μg/ml (He et al., 2017). The sub-inhibitory vancomycin concentration used here (0.5 μg/ml) is probably too low to detect any changes in EV morphology and production.

All RNA classes are detected by RNA-Seq within HG003-derived EVs. These include rRNAs, which were still detected, suggesting that the rRNA depletion carried out here was incomplete. In the absence of filtering by coverage of sequencing data, on average, 78.0 ± 7.0% of the annotated transcripts in HG003 genome were present in the EVs (91.8 ± 1.0% for EV-producing cells). Of these, a large portion of mapped RNAs corresponded to mRNAs. These results are consistent with RNA-Seq data obtained with similar criteria from OMVs in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) that harbor around 73% of the annotated transcripts including up to 86% of mRNAs according to growth conditions (Malabirade et al., 2018). A recent study addressing the sRNA content of EVs also pointed out that mRNAs are the more abundant RNA species in EVs derived from *S. aureus* strain MSSA476 after rRNA depletion (Joshi et al., 2021). On the contrary, studies with *Escherichia coli* revealed that EVs were enriched mainly with short RNAs, such as tRNAs (Ghosal et al., 2015; Blenkiron et al., 2016). Nevertheless, these variations may be a result of different RNA extraction and library preparation protocols, or simply correspond to singular characteristics of EVs derived from different bacterial species. As expected, data

filtering of RNAs with ≥90% coverage decreased the number of detected RNAs. However, the RNA content of the EVs, particularly the mRNAs and sRNAs (only 5.1 ± 2.8% of EV RNAs initially detected are still identified after the filtering), was much more affected than that of the EV-producing cells (69.0 ± 7.90% of EV-producing cell RNAs are still detected after the filtering). The low RNA coverage in the HG003 EVs might perhaps reflect the absence of transcription within EVs and, thus, the progressive degradation of a substantial fraction of the EVs-associated RNAs after their formation and/or during their purification. These findings are consistent with a recent report showing that the predominant RNA type in EVs from *S. aureus* Newman is <300-nucleotide long (Rodriguez and Kuehn, 2020). The presence of numerous processed or degraded RNAs could be a common feature of the bacterial EVs, as Gram-negative *S. Typhimurium* OMV-associated RNAs are also processed or degraded (Malabirade et al., 2018). Since one of the primary physiological functions attributed to EVs is the removal of unwanted materials from cells, such as misfolded or degraded proteins (McBroom and Kuehn, 2007), the *S. aureus* EVs may also help removing the degraded RNAs from the bacteria (Groot and Lee, 2020).

Two hundred and eighty-six highly covered RNAs can be identified within HG003 EVs. That number of highly covered EV RNAs varies according to growth conditions, from 86 in late-stationay growth phase with vancomycin to 273 in early-stationay growth phase also with vancomycin. As expected, all the EV transcrits are also detected in the EV-producing cells. Among them, 51 transcripts are shared by EVs collected in all tested conditions. The 286 highly covered EV RNAs encompass short transcripts, such as tRNAs (~75 nucleotides), mRNAs (*psmβ1*, 135 nucleotides; *agrD*, 141 nucleotides), and some sRNAs (Teg84, 79 nucleotides; Teg46, 124 nucleotides). Yet, these highly covered EV RNAs also comprise long transcripts, including 15 mRNAs with lengths >2,000 nucleotides (e.g., *pbp2*, *copA*, *rpoB*, *rpoC*, and *atl*). 67% of these RNAs are organized into 42 gene clusters that are co-transcribed in *S. aureus* (Mäder et al., 2016). Among them, 17 are full-length transcripts across entire operons in HG003 EVs, with lengths up to ~14,000 nucleotides. As observed for *S. Typhimurium* OMV RNAs (Malabirade et al., 2018), these findings support that highly covered RNAs are present as full-length transcripts. These RNAs belong to all annotated classes of RNAs. The mRNAs from the EVs encode proteins involved in transcription, translation, energy production and conversion, carbohydrate metabolism, and cell wall biogenesis. In addition to these housekeeping functions, EVs also harbors mRNAs encoding virulence-associated proteins, such as the *agr* operon responsible of quorum-sensing, autolysin Atl, protein A (Spa), immunoglobulin-binding protein (Sbi), immunodominant staphylococcal antigen B (IsaB), δ hemolysin (Hld) encoded by the multifunctional sRNA RNAIII, and the PSMβ1 phenol-soluble modulin, as well as several iron acquisition systems. Besides, most tRNA species are detected within the EVs, as well as residual rRNAs and 28 annotated sRNAs, including *bona-fide* RsaC involved in *S. aureus* oxidative stress adaptation and nutritional immunity (Lalaouna et al., 2019). Note that RsaC and RNAIII were also detected within *S. aureus* EVs from strain MSSA476 (Joshi et al., 2021), suggesting their wider occurence in staphylococcal EVs.

The presence of full-length, functional RNAs in EVs raises the question of their biological roles. EVs are produced to transport bioactive molecules to interact and communicate with other cells. So far, most studies on *S. aureus* EVs investigated the protein cargo. Therefore, the broad spectrum of activities associated with *S. aureus*-derived EVs was related to their protein content (Jeon et al., 2016). In some *S. aureus* strains, EVs carry β-lactams that confer transient resistance to ampicillin-susceptible *E. coli* and *S. aureus* (Lee et al., 2013b). Likewise, mycobactin-containing *Mycobacterium tuberculosis* EVs can deliver iron to strains deficient for iron-uptake (Prados-Rosales et al., 2014). The delivery of full-length mRNAs *via* EVs to the surrounding bacterial cells, notably those involved into energetic and metabolic functions, could improve their responses to environmental stimuli to fasten their adaptation. Likewise, rRNAs and tRNAs could boost translation in EV recipient bacterial cells and improve their fitness. In bacteria, sRNAs fine tune target gene expression, usually at the posttranscriptional level in response to changes in the environment, including antibiotic resistance and tolerance (Mediati et al., 2021). Most bacteria encode dozens of sRNAs that are transcribed as independent transcripts or processed from mRNAs. The presence of sRNAs with regulatory roles within *S. aureus* EVs could be a relocation strategy in other surrounding bacteria that need more of these sRNAs for adaptation and infection spreading and/or to coordinate bacterial group adaptation, activities and behaviors. The transfer of RsaC could enhance the concentration of that riboregulator to other *S. aureus* cells when intracellular, especially helping survival within the phagolysosome if the *S. aureus* EVs are internalized by the host cells together with the bacteria. Transfer of functional RNAs to bacterial cells that do not encode the corresponding genes in their genome could also be part of a transient horizontal phenotype acquisition, which could be of use during infection to disseminate specific virulence-associated factors through the bacterial community. Finally, beside interactions between bacterial cells, *S. aureus* EV associated RNAs, notably sRNAs, may be involved in the host-pathogen interactions (Eberle et al., 2009; Li and Chen, 2012; Furuse et al., 2014; Sha et al., 2014; Koeppen et al., 2016; Westermann et al., 2016; Choi et al., 2017; Frantz et al., 2019; Han et al., 2019; Lee, 2019; Rodriguez and Kuehn, 2020). The 28 potential annotated sRNA detected within HG003 EVs are potential candidates for further functional characterization, especially during *S. aureus*-host cell interactions. *S. aureus* secreted EVs elicit immune responses that mimic those of the EV-producing cells (Gurung et al., 2011; Hong et al., 2011, 2014; Kim et al., 2012, 2019; Thay et al., 2013; Choi et al., 2015; Jeon et al., 2016; Jun et al., 2017; Askarian et al., 2018; Tartaglia et al., 2018; Wang et al., 2018, 2020; Rodriguez and Kuehn, 2020). Strikingly, within their RNA cargo, several mRNAs encode immunomodulatory proteins, as Sbi, Spa and PSMβ1, and may participate into the immune response triggered by the protein cargo if they are ultimately translated. Yet, such functions remain to be demonstrated for the RNA cargo of

S. aureus EVs. mRNAs expressing PSMβ and hemolysin δ toxins from the EVs, if translated into recipient bacteria or host cells, could perhaps facilitate staphylococcal intracellular survival, but this hypothesis should be experimentally challenged.

The RNA cargo of HG003 EVs, in both identity and abundance, depends on the growth conditions. Similarly, the EV RNA cargo of *S. Typhimurium* is also sensitive to environmental changes indicating that it reflects the bacterial adaptation to its environment (Malabirade et al., 2018). It could be a faster way to transfer information of changes perceived by one cell to surrounding cells even before they sensed the environmental stimuli in order to quickly promote group adaptation. We found, however, that the vancomycin treatment had less impact on RNA abundance compared to the growth phase. Although the composition of HG003 EVs represented the intracellular state of the bacterial transcriptome through global packaging, two main findings, however, reinforce the concept of a potential selective packaging of RNAs into EVs, as proposed for its protein cargo (Haurat et al., 2011; Cahill et al., 2015; Tartaglia et al., 2020). First, we measured an enrichment for several functional and subcellular localization RNA categories in EVs when compared to EV-producing cells. Second, the relative abundance of several RNAs between two environmental conditions was different in the EVs and the EV-producing cells. Other studies also found that some RNA populations were enriched in EVs from Gram-negative and Gram-positive pathogenic bacteria (Ghosal et al., 2015; Koeppen et al., 2016; Resch et al., 2016; Malabirade et al., 2018; Malge et al., 2018; Han et al., 2019; Langlete et al., 2019; Zhang et al., 2020). This notably includes sRNAs, which can play regulatory activity in the host (Koeppen et al., 2016; Malabirade et al., 2018; Langlete et al., 2019; Zhang et al., 2020). The enrichment of RNAs associated with bacterial diseases in EVs derived from many pathogenic bacteria reinforces the physiopathological role of these structures in host-pathogen interaction and host cell invasion, which could be borne by their RNA cargo as well as by their protein cargo. The selective mechanisms of EV RNA content packaging have not yet been elucidated. It has been proposed that RNA packing into EVs could depend on RNA size and location (eg., nearby EVs formation site), as well as on their affinity for other molecules (eg., membrane proteins; Langlete et al., 2019). Nevertheless, such enrichment results should be interpreted with carefulness. Indeed, they could also reflect a difference in RNA half-lives between EVs and EV-producing cells, as well as a difference in RNase activity (Langlete et al., 2019; Lécrivain and Beckmann, 2020), pointing out that RNAs with longer half-lives could be protected from degradation, leading to an artifactual accumulation in the EVs over time.

In summary, our exploratory work provides novel insights in *S. aureus* EVs by the characterization of its RNA cargo and paves the way for further functional studies. Mainly, it sheds light on the possible roles of EV RNA cargo in intra- and inter-species communication, in the virulence and pathogenesis of *S. aureus*, and as trash bags for degraded RNAs. The study of bacterial EV RNA cargo is an emerging area of research. Evidently, as with all emerging fields, each advance raises further questions: Are the full-length RNAs in HG003 EVs functional, and do they possess similar functions than in the bacterial cytoplasm? What are the rules for RNA sorting into HG003 EVs? What are the roles and functions of the *S. aureus* EV RNA cargo? These exciting questions, among others, should be adressed in further studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, https://www.ebi.ac.uk/ena/browser/view/PRJEB40502.

AUTHOR CONTRIBUTIONS

BSRL, BF, SC, YL, VA, and EG conceived and designed the experiments. VA and EG supervised the study. BSRL and SC performed the experiments. AN performed computational analysis. BSRL, BF, SC, VRR, AN, and EG analyzed the data. VA, YL, and EG contributed to funding acquisition. BSRL and EG wrote the original draft. All authors contributed to data interpretation, drafting the manuscript, critically revising the manuscript, and approving its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.634226/full#supplementary-material

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