Extracellular vesicles in S. aureus pathogenesis in a mastitis context

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Ph.D Thesis

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Abstract

Mastitis is an inflammation of the mammary gland associated with worldwide economic losses. *Staphylococcus aureus* is one of the main etiologic agents of mastitis and triggers several clinical manifestations in the host. Although *S. aureus* is extensively studied as an agent of nosocomial infections, the mechanisms involved in *S. aureus* pathogenesis in a mastitis context are not completely understood. Extracellular factors are important components of *S. aureus* virulence. The production of extracellular vesicles (EVs) by *S. aureus* has gained attention in the last years. EVs are nano-sized particles formed by the budding of the bacterial membrane. Nowadays, EVs are accepted as a conserved mechanism of secretion that enables inter-kingdom cellular communication. EVs have been purified from different clinical isolates of *S. aureus* and appear to be a key factor in the infectious process when evaluated using several *in vitro* and *in vivo* models. In this thesis, we purified EVs from phylogenetically distant strains of *S. aureus* isolated from bovine, ovine and human hosts. Ruminant strains were responsible for different degrees of severity of mastitis. *S. aureus* EVs were strain-dependent in terms of yield, proteins cargo and size. Overall, they were enriched with cytoplasmic and membrane-associated proteins, lipoproteins and virulence factors, including toxins (*e.g*. PSMs, δ-hemolysin), factors associated to adhesion and colonization (*e.g*. EbpS, FnBPs), and evasion of host immune system (*e.g*. Sbi, leukocidin). 44 proteins were shared between all strains, defining a core proteome for *S. aureus* EVs. We selected the bovine strain *S. aureus* N305 for further *in vitro* and *in vivo* investigations. Although *S. aureus* N305 EVs were not able to induce cytotoxic effects in bovine mammary epithelial cells (bMEC), they stimulated an immunomodulatory response with an increase of some key inflammatory markers, such as the chemokine IL-8. Purified *S. aureus* N305 EVs were then tested in a murine model of mastitis. Histopathological results were consistent with the levels of cytokine after 24 h of mammary infection. *S. aureus* N305 EVs induced a dose-dependent neutrophil recruitment to the alveolar space that can be correlated to the local levels of chemokines. EVs-induced symptoms were although distinct and milder than those induced by the live N305. These results suggest that EVs play a role in *S. aureus* pathogenesis and can induce an immune response to *S. aureus* mastitis. Overall, this work opens new perspectives in the development of diagnostic tools and vaccine formulations using native or engineered EVs against *S. aureus* mastitis.

Résumé

La mammite est une inflammation de la glande mammaire responsable de pertes lourdes économiques dans le monde. *Staphylococcus aureus* est l'un des principaux agents étiologiques de la mammite et déclenche plusieurs manifestations cliniques chez l'hôte. Bien que *S. aureus* soit largement étudié en tant qu'agent d'infections nosocomiales, les mécanismes impliqués dans la pathogenèse de *S. aureus* dans un contexte mammite sont encore inconnus. Les facteurs extracellulaires sont des composants importants de la virulence de *S. aureus*. La production de vésicules extracellulaires (VEs) par *S. aureus* a attiré l'attention au cours de ces dernières années. Les VEs sont des particules de taille nanométrique formées par le bourgeonnement de la membrane bactérienne. De nos jours, les VEs sont considérées comme un mécanisme de sécrétion conservé qui permet la communication cellulaire inter-royaume. Les VEs ont été purifiées à partir de différents isolats cliniques de *S. aureus* et semblent être un facteur clé dans le processus infectieux lorsqu'ils sont testés en modèles *in vitro* et *in vivo*. Dans cette thèse, nous avons purifié des VEs de souches phylogénétiquement distantes de *S. aureus*, isolées d'hôtes bovins, ovins et humains. Les souches de ruminants étaient responsables de différents degrés de sévérité de mammite. Les caractéristiques de VEs de *S. aureus* étaient souches-dépendantes en termes de rendement, de contenu protéique et de taille. Elles sont enrichies en protéines cytoplasmiques et membranaires, en lipoprotéines et en facteurs de virulence, notamment en toxines (ex. PSM, δ-hémolysine), en facteurs d'adhésion et de colonisation (ex. EbpS, FnBPs) et d'évasion du système immunitaire de l'hôte (ex. Sbi, leucocidine). 44 protéines sont communes à toutes les souches et définissent un protéome cœur pour les VEs de *S. aureus*. Nous avons sélectionné la souche bovine *S. aureus* N305 pour des études fonctionnelles *in vitro* et *in vivo*. Bien que les VEs de *S. aureus* N305 ne soient pas capables d'induire des effets cytotoxiques sur cellules épithéliales mammaires bovines (CEMb), elles induisent une réponse immunomodulatrice avec une augmentation de certains marqueurs inflammatoires clés, tels que la chimiokine IL-8. Des VEs de N305 purifiées ont ensuite été testées dans un modèle murin de mammite. Les résultats histopathologiques étaient en accord avec les taux de cytokines mesurés après 24 h d'infection mammaire. Les VEs de *S. aureus* N305 ont induit un recrutement de neutrophiles dose-dépendant dans l'espace alvéolaire qui peut être corrélé aux taux locaux de chimiokines. Les symptômes induits par les VEs étaient bien distincts et plus légers que ceux induits par la souches N305 vivante. Ces résultats suggèrent que les VEs jouent un rôle dans la pathogenèse de *S. aureus* et peuvent induire une réponse immunitaire

lors de mammite à *S. aureus*. Dans l'ensemble, ces travaux ouvrent de nouvelles perspectives dans le développement d'outils diagnostiques et de formulations de vaccins utilisant des VEs naturels ou artificiels contre la mammite à *S. aureus*.

Resumo

Mastite é uma inflamação da glândula mamária associada a relevantes perdas econômicas mundiais, tendo o *Staphylococcus aureus* como um dos principais agentes etiológicos. Embora esse micro-organismo seja amplamente estudado devido sua importância nosocomial, os mecanismos patogênicos associados à mastite ainda não foram completamente elucidados. Um dos importantes aspectos associados à virulência em microorganismos patogênicos envolve a secreção de proteínas, e devido a isso, a produção de vesículas extracelulares (VEs) tem recebido destaque nos últimos anos. VEs são nanopartículas formadas a partir do brotamento da membrana bacteriana. Atualmente, essas vesículas são aceitas como um conservado sistema de secreção que possibilita uma comunicação celular entre reinos. Assim, este projeto de tese teve como objetivo avaliar VEs em um contexto de mastite. Para tal, vesículas foram purificadas de linhagens de *S. aureus* filogeneticamente distintas e isoladas de diferentes hospedeiros (bovina, ovina e humana). As linhagens isoladas de ruminantes desencadeiam diferentes manifestações clínicas da doença. O rendimento, o conteúdo protéico e o tamanho das VEs foram dependentes da linhagem. De modo geral, as vesículas purificadas foram enriquecidas com proteínas citoplasmáticas, proteínas associadas à membrana, lipoproteínas e fatores de virulência. Dentre esse grupo encontram-se toxinas (PSMs e δ-hemolysin), fatores associados à adesão e colonização (EbpS e FnBPs) e proteínas envolvidas na evasão da resposta imune do hospedeiro (Sbi e leucocidinas). Além dos fatores de virulência, 44 proteínas foram conservadamente idenficadas no conteúdo intravesicular das seis linhagens estudadas. Em seguida, a linhagem bovina *S. aureus* Newbould N305 foi selecionada para os ensaios *in vitro* e *in vivo*. VEs purificadas da linhagem N305 não foram capazes de induzir um efeito citotóxico em células epiteliais mamárias bovinas (CEMb), embora induziram a expressão de alguns marcadores de inflamação *in vitro*, includindo a quimiocina IL-8. Em um modelo murino de mastite, os resultados histopatológicos foram consistentes com os níveis de citocinas dosados após 24h de infecção. VEs purificadas da linhagem N305 induziram um recrutamento de neutrófilos de maneira dose-dependente e correspondente aos níveis locais de quimiocinas. VEs induziram uma resposta distinta e moderada em relação as glândulas mamarias infectadas com a bactéria viva. Esses resultados sugerem a participação das vesículas no processo patogênico desencadeado pelo micro-organismo *S. aureus* em um contexto de mastite e abrem novas perspectivas para o desenvolvimento de ferramentas que visam o diagnóstico e tratamento dessa doença.

List of abbreviations

16S rRNA - 16S ribosomal RNA gene

Acn - Aconitate hydratase A

AD - Atopic dermatitis

AFM - Atomic force microscopy

Ag85 - Antigen 85 complex

agr - Accessory gene regulator

AIP - Autoinducing peptide

ALO - Anthrolysin

AMP - Antimicrobial peptides

AMPs - Antimicrobial peptides

APC - Antigen-presenting cells

asp23 - alkaline shock protein 23

Atl – Bifunctional autolysin

Aur – Aureolysin

BAFF - B-cell activating factor

bap - Biofilm-associated protein gene

Bbp - Bone sialo-binding protein

BCG – Bacille de Calmette et Guérin

BHI - Brain heart infusion

BlaZ - β-lactamase

bMEC - Bovine mammary epithelial cells

C12 - Lauric acid

C14 - Myristic acid

C16 - Palmitic acid

Ca^{2+} - Calcium ion

CA-MRSA – Community-acquired methicillin-resistant *Staphylococcus aureus*

CAPES - Brazilian Federal Agency for Support and Evaluation of Graduate Education

CC - Clonal complexes

cfb - CAMP factor gene

CFP29 - 29-kDa antigen

CFU - Colony forming units

Clfs - Clumping factors (ClfA and ClfB)

CM – Clinical mastitis

CMT - California Mastitis Test

Cn - Collagen

CNIEL - *Centre national interprofessionnel de l'économie laitière*

CNPq - National Council for Scientific and Technological Development

CNS - Coagulase Negative Staphylococci

Coa – Coagulase

COGs - Clusters of Orthologous Groups

covRS - Virulence regulator-sensor operon

CPC70 - Carboxylated polystyrene particles

CPS - Coagulase-Positive Staphylococci

Cryo-ET - Cryo-electron tomography

CWA - Cell wall anchored

CXC chemokines - C-X-C motif

KC (CXCL1) - Keratinocyte chemoattractant

MIP-2 (CXCL2) - Macrophage inflammatory protein

CYT: cytoplasmic proteins

DCs – Dentritic cells

DCT - Dry cow therapy

DEFβ1 - Antimicrobial peptides β defensing-1

DLS - Dynamic light-scattering

DltD - Poly (glycerophosphate chain) D-alanine transfer protein

DMEM - Dulbecco's modified eagle medium

DNA – Deoxyribonucleic acid

DnaK - Chaperone protein

DSCC - Differential somatic cell count

DTT – Dithiothreitol

Eap - Extracellular adhesive protein

EbpS - Elastin binding protein

ECM -Extracellular matrix

eDNA - Extracellular DNA

EDTA - Ethylenediamine tetraacetic acid

EF - Edema factor

Efb - Extracellular fibrinogen binding protein

EF-Tu - Elongation factor Tu

El – Elastin

Emp - Extracellular matrix binding protein

EPS - Extracellular polymeric substances

ESAT-6 – Early secreted antigenic target of 6 kDa

ESBP - Intravesicular family 5 extracellular solute-binding protein

ESI - Electrospray ionization

EspA - ESX-1 secretion-associated protein EspA

ETs - Exfoliative toxins

EU – European Union

EVs - Extracellular vesicles

Fbps (A, B, and C) - Antigen 85 proteins

FDR - False discovery rate

Fg - Fibrinogen

FhuD2 - Ferric-hydroxamate uptake

Fn – Fibronectin

FnBPs - Fibronectin binding proteins (FnBPA and FnBPB)

Foxp3 - Forkhead Box protein 3

FPR2 - Formyl peptide receptor 2

FtsH - ATP-dependent metallopeptidase

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

G-CSF - Granulocyte colony-stimulating factor

GM - Growth medium

H&E - Hematoxylin and eosin

HA-MRSA - Hospital-acquired methicillin-resistant *Staphylococcus aureus*

HbhA - Heparin-binding hemagglutinin

HBSS - Hank's Balanced Salt Solution

Hdl - δ-toxin/Delta-Hemolysin

hFC -High-resolution flow cytometry

Hla - α-toxin/Alpha-hemolysin

Hup - DNA-binding protein HU

HysA - Hyaluronidase protein

Ica - Intercellular adhesion gene

IDF - International dairy federation

Ig - Immunoglobulins

IgBDs - Ig-binding domains

ILs - Interleukins

IMG – Integrated microbial genomes & microbiomes

IMI - Intramammary infection

InlB - Internalin B

INRA - National Institute for Agricultural Research

Institut National de la Recherche Agronomique

Isds - Iron-regulated surface proteins

ISEV - International society for extracellular vesicles

LA-MRSA - Livestock-associated methicillin-resistant *Staphylococcus aureus*

LC-MS/MS – Liquid chromatography-tandem mass spectrometry

LDH - Lactate dehydrogenase

LF - Lethal factor

Lgt - Prolipoprotein diacylglyceryl transferase

LLO - Listeriolysin O

LP - Lipoproteins

LPS - Lipopolysaccharides

LPXTG - Leu-Pro-X-Thr-Gly motif

Lsp - Lipoprotein signal peptidase

LTA - Lipoteichoic acid

LTA - Lipoteichoic acids

MAMPs - Microbe-associated molecular patterns

MAPK - Mitogen-activated protein kinase

MCP-1 - Monocyte chemoattractant protein 1

MEC - Mammary epithelial cells

MG - Mammary gland

Mg^{2+} - Magnesium ion

MGEs - Mobile genetic elements

MHC - Major histocompatibility complex

MLST – Multilocus sequence typing

MMC - Mitomycin C

MntC - Manganese transport protein C

MOI – Multiplicity of infection

mRNA - Messenger RNA

MRSA - Methicillin-resistant *Staphylococcus aureus*

MSCRAMMs - Microbial surface components recognizing adhesive matrix molecules

MSSA - Methicillin-susceptible *Staphylococcus aureus*

MTT - Thiazolyl Blue Tetrazolium Bromide

MVBs - Multivesicular bodies

MVE - Multivesicular endosome

MVs - Membrane vesicles

NaCl - Sodium chloride

NAGase - N-acetyl-β-D-glucosaminidase

NCBI – National Center for Biotechnology Information

ncRNAs - non-coding RNAs

NF-κB - Factor nuclear kappaB

NIPH - Institute of public health

NK - Natural killers

NLRs - Nod-like receptors

NTA - Nanoparticle tracking analysis

OatA - Peptidoglycan-N-acetylmuramate O-acetyltransferase

OD - optical density

OM - Outer membrane

OMPs - Outer membrane proteins

OMVs - Outer membrane vesicles

PA - Protective antigen

PAMPs -Pathogen-associated molecular patterns

PBPs - Penicillin-binding proteins

PBS - Phosphate-buffered saline

pfoA - Perfringolysin O gene

PG - Peptidoglycan

PG - Phosphatidylglycerol

plc - Alpha-toxin gene

Ply - Toxin pneumolysin

PMN - Polymorphonuclear cells

PPIA (peptidyl-prolyl *cis-trans* isomerase A

PQS - Pseudomonas quinoline signal

PRR - Patterns recognition receptors

PrsA - Peptidyl-prolyl cis/trans isomerase

PSA - Polysaccharide acapsular antigen

PSE – Polyethersulfone

PSMs - Phenol-soluble modulins

PTSAg – Pyrogenic toxins superantigens

PVL - Panton-valentine leukocidin

qPCR - Quantitative polymerase chain reaction

RANTES - Regulated on activation, normal T cell expressed and secreted

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RPL19 (ribosomal protein 19)

rRNA - Ribosomal ribonucleic acid

RT-qPCR - Quantitative reverse transcription PCR

Sags - Superantigens

sarR - Staphylococcal accessory regulator protein

Sbi - Immunoglobulin-binding protein

SC - Somatic cells

SCC - Somatic cell count

SCC - Staphylococcal chromosomal cassette

SCC*mec* - Staphylococcal chromosomal cassette mec

ScpA – StaphopainA/Cysteine proteases A

SCVs - Small-colony variants

SdrC - Serine-aspartate repeat protein C

SdrD - Serine-aspartate repeat protein D

SdrE - Serine-aspartate repeat protein E

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Se - Selenium

SEls - SEs-like toxins

SEM - Scanning electron microscopy

SERAM - Secreted expanded repertoire adhesive molecules

SEs - Staphylococcal enterotoxins

sfp – Surfactin production gene

sle1- N-acetylmuramoyl-l-alanine amidase gene

SM - Stimulation medium

Sod - Superoxide dismutase

SP – Supernatant

SpA - Protein A

SPI - signal peptidase I

SPII - signal peptidase II

SrtA - Sortase A

SspA - V8 protease/Serine protease

SspB – StaphopainB/Cysteine proteases B

SSSS - Staphylococcal scalded skin syndrome

SSTI - Skin and soft tissue infections

ST – Sequence types

STLO - Science and Technology of Milk and Eggs

Science & technologie du lait & de l'oeuf

TatA - Twin-arginine translocation TatA

TBS - Tris-Buffered Saline

TCA – Trichloroacetic acid

TEM – Transmission electron microscopy

TFA – Trifluoroacetic acid

TLRs - Toll-like receptors

TMH - N-terminal transmembrane helices

TNF-α - Tumor necrosis factor alpha

Treg - Regulatory T cells

tRNA - Transfer ribonucleic acid

TRPS - Tunable resistive pulse sensing

TSS - Toxic shock syndrome

TSST-1 - Toxic shock syndrome toxin-1

UFMG - Federal University of Minas Gerais

Universidade Federal de Minas Gerais

UniProt - Universal Protein

VISA - Vancomycin-intermediate *Staphylococcus aureus*

Vra E/D - Two-component system involved in nisin resistance

VRSA - Vancomycin-resistant *Staphylococcus aureus*

vWbp - von Willebrand factor binding protein

WHO - World health organization

YWHA - 14-3-3 phospho-serine/phospho-threonine binding protein

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1. Presentation

Collaborations

This thesis was conducted in the frame of an international collaborative program between the Federal University of Minas Gerais (UFMG) (Belo Horizonte, Brazil) and the Food and Agricultural Science University (Agrocampus Ouest) (Rennes, France). It has benefited a multidisciplinary knowledge under the shared direction between France (Microbiology) and Brazil (Genetics of microorganism and biotechnology). In France, the Ph.D. Project was supervised by Dr. Yves Le Loir and Dr. Eric Guédon in the UMR1253 STLO (Science and Technology of Milk and Eggs), a joint research unit of the National Institute for Agricultural Research (INRA) and Agrocampus Ouest. In Brazil, it was supervised by Dr. Vasco Ariston de Carvalho Azevedo, member of the Postgraduate Program of Genetics in the Institute of Biological Sciences.

The laboratories that contributed to this work are listed below:

- **Federal University of Minas gerais (UFMG) – Belo Horizonte, Brazil**
  - Cellular and Molecular Genetics Laboratory, Department of Biological Sciences
- **National Institute for Agricultural Research (INRA), Agrocampus Ouest - Rennes, France**
  - UMR1253 STLO Science and Technology of Milk and Eggs
- **National Institute for Agricultural Research (INRA) – Jouy en Josas, France**
  - UMR1313 Animal Genetics and Integrative Biology (GABI)
- **Excilone, France**
- **Ghent University - Merelbeke, Belgium**
  - Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine.
- **Tokyo University of Agriculture and Technology**
  - Laboratory of Veterinary Internal Medicine, Division of Animal Life Science, Tokyo, Japan.

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2. General introduction

Staphylococcus aureus is a Gram-positive microorganism described as an opportunistic pathogen that mediates human and veterinary infections on a global scale (Schmidt et al., 2017). This pathogen is responsible for both community-acquired and nosocomial infections, which make it a public health menace worldwide (Tong et al., 2015; Wertheim et al., 2005). Hospital-associated infections caused by *S. aureus* strains include respiratory tract infections, bacteremia, and infective endocarditis (Tong et al., 2015; Wertheim et al., 2005). On the other hand, skin and soft tissue infections (SSTI) dominate among community-associated infections (DeLeo et al., 2010; Lowy, 1998). In animal health, this bacterium is one of the most important etiological agents of mastitis, an inflammation of the mammary gland (MG) mostly caused by an intramammary infection (IMI) (Johnzon et al., 2016).

2.1. The dairy industry and the economic context

Since milk is an important nutritional source, its production is of primary importance in world food economy. According to the World Dairy Situation Report (2017), cow's milk production is led by Europe with 33%, Asia (30%) and North and Central America occupy the third position with 18% of the output (Figure 1).

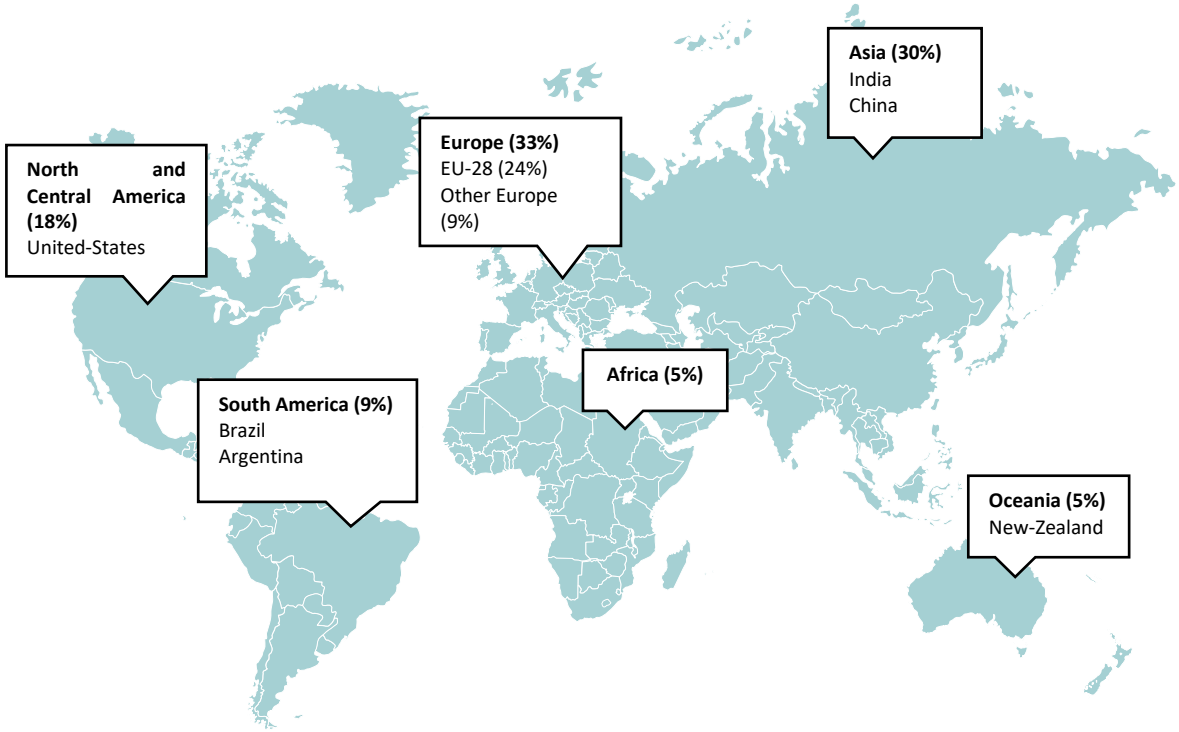

Figure 1. Cow's milk production (Source: The World Dairy Situation 2017).

In the world market, the production and consumption of cow's milk are much more important than those of other animals. In 2016, EU-28 produced approximately 168.3 million tons of milk. Production of cow's milk was 163.0 million tons (96.9 % of all milk produced) while milk from ewes, goats, and buffalos represented 5.4 million tons (3.1 %). The main part of milk produced was delivered to the dairy transformation plants (157.1 million tons), of which 153.2 million tons from cow's milk (Figure 2) (Eurostat, 2017).

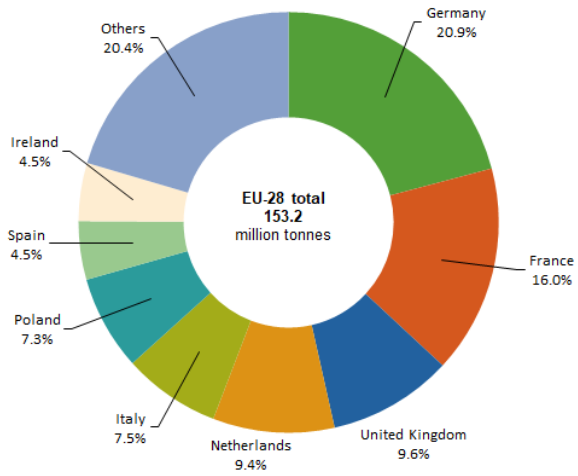

Figure 2. Collection of cows' milk by dairy transformation plants, 2016 (% share of EU-28 total, based on tons) (Source: Eurostat - data extracted in October 2017).

The dairy industry is an important economic sector in France, the second European producer behind Germany and the eighth producer in the world (CNIEL/Eurostat). Cow's milk is consumed mainly in the form of cheeses (34.3%), milk fat (19.8%), milk powder (15.8%), packaged milk (9.8%), yogurt and milk desserts (6.4%), packaged creams (7.9%), whey powder (3.1%) and casein and caseinates (2.2%) (CNIEL, 2018). Brazil is the biggest milk producer in South America mainly in the Southeast and South regions (CNIEL, 2018; USDA, 2017). The Annual Dairy Report 2017 forecasts an increase of 1.8 % in milk production in 2018 when compared to 23.5 million tons produced in 2017 (USDA, 2017).

Nowadays, milk production, like other agricultural sectors, faces many challenges, in terms of social demand on the reduction of inputs (*e.g.* antibiotics), better animal welfare, and sustainability.

2.2. New challenges of the milk sector

Clinical mastitis (CM) is one of the most frequent and costly diseases affecting dairy cows (Halasa et al., 2007; Jamali et al., 2018). Mastitis constitutes a worldwide economic problem concerning milk production. This infectious process can affect quality, quantity, and properties of the milk produced by the affected mammary quarters (Middleton et al., 2014). The economic consequences of mastitis also involve veterinary healthcare, animals treatments, diagnosis and replacement or culling of the infected animals (Middleton et al., 2014). Mastitis is also the first cause of antibiotic use in dairy cattle herds (Jamali et al., 2018).

Economic estimates of the impact of mastitis are closely associated with the country, type of mastitis, treatment, and preventive measures (Halasa et al., 2007; Middleton et al., 2014). According to Jamali et al. (2018), the incidence rate of clinical mastitis ranges from 13 to 40 cases/100 cow years in different countries and housing types (Jamali et al., 2018). After Halasa et al. (2007), clinical mastitis costs $367 per cow per year on average, whereas the average cost of subclinical mastitis is $130 per cow per year (Halasa et al., 2007). Regarding prophylaxis, the average cost of mastitis prevention was $33 per cow per year (Halasa et al., 2007). These data highlight the advantages of prevention over treatment for both economic aspects and animal welfare.

Beside the above-mentioned economic aspects, infections associated with antibiotic-resistant strains of *S. aureus* have reached epidemic proportions globally (Levy and Marshall, 2004). Recently, World Health Organization (WHO) published a list of priority pathogens in terms of urgency in the development of new antibiotics, which classified three categories (critical, high and medium) of threat to human health. *S. aureus* (methicillin-resistant, vancomycin-intermediate, and resistant) was classified as a high priority, together with vancomycin-resistant *Enterococcus faecium* and clarithromycin-resistant *Helicobacter pylori.*

Furthermore, mastitis in cattle can have severe consequences on human health, mainly associated with the production of staphylococcal enterotoxins (SEs) that are secreted and remain stable in the milk (Martins et al., 2015). More than half of the *S. aureus* strains isolated from infected mammary gland were described as positive for enterotoxin genes (Le Loir et al., 2003; Merz et al., 2016). The consumption of milk products contaminated by enterotoxigenic *S. aureus* has been associated with many staphylococcal food poisoning outbreaks around the world (Hennekinne et al., 2012).

Altogether, the economic problems involving herd infections and the need to improve animal welfare and agricultural production without antimicrobial selection justify the efforts to better understand and prevent mastitis.

3. Chapter 1. Literature review

This chapter aims at providing a literature review of the microorganism *Staphylococcus aureus* and the mechanisms of pathogenesis associated with intramammary infections (IMI) in bovines. This section is subdivided into three parts to better explain the background of the Ph.D. project: the mastitis context, *S. aureus,* and the mechanisms of pathogenicity, including the secretion by extracellular vesicles.

3.1. Mastitis

3.1.1. General considerations

Mastitis is an inflammation of the MG that affects humans and other animals, including bovines and small ruminants, and can be described as a multifactorial disease in relation to the etiological agents involved. Apart from its infectious nature, mechanical, physical and chemical traumas may also induce an inflammation of the MG (Le Maréchal et al., 2011a; Oviedo-Boyso et al., 2007). In most cases, mastitis is due to a bacterial infection. The microorganisms associated with this infection can be classified as environmental and contagious and they are characterized by triggering different clinical manifestations of the disease (Bradley, 2002). Contagious microorganisms are adapted to survive and multiply within the MG of the host and can spread in the herd (Bradley, 2002; Dego et al., 2002). This group includes *Staphylococcus aureus*, *Streptococcus agalactiae*, several *Mycoplama* and *Arcanobacterium spp*. (Bradley, 2002; Oviedo-Boyso et al., 2007). In contrast, environmental microorganisms are normally present in the animal environment, which facilitates the contamination of the teat and udder (Bradley, 2002; Oviedo-Boyso et al., 2007). This group comprises *Escherichia coli, Streptococcus uberis, Streptococcus dysgalactiae, Klebsiella pneumoniae* and *Bacillus spp*. (Oviedo-Boyso et al., 2007). In general, the main mastitis-causing pathogens are *S. aureus*, *E. coli* and *S. uberis* (Gomes et al., 2016; Keane et al., 2013). *S. aureus*, as a mastitis pathogen, can develop several clinical manifestations, which range from asymptomatic subclinical mastitis to severe clinical mastitis, with gangrene, severe tissue damage, and local and systemic symptoms (Margarita and Elena, 2012).

S. aureus and *E. coli* are the most prevalent bacteria that induce CM in cattle and elicit different immune responses in the host (Bannerman et al., 2004). *E. coli* mastitis normally results in an acute and severe infection that can result in loss of the infected animal (Hagiwara et al., 2014). In contrast, *S. aureus* disease starts with an acute phase and is often associated with chronic and subclinical infections (Sutra and Poutrel, 1994). The terminology for

classifying these different clinical manifestations of mastitis according to the International Dairy Federation (IDF) is summarized in table 1. These clinical differences correlate with different levels of cytokines dosed in milk after bacterial challenge. By comparison with *E. coli*, *S. aureus* induces a limited cytokine response in vitro (Deplanche et al., 2016) and in vivo in cow intramammary infections (Bannerman et al., 2004).

Table 1. Definition of the types of mastitis concerning the severity of symptoms.

Mastitis type	Definition[1]
Subclinical mastitis (SM); Pre-clinical mastitis	✓ Inflammation of the mammary gland that is not visible and requires a diagnostic test for detection. The diagnostic test most used is measurement of milk somatic cell count. ✓ Subclinical mastitis is the most prevalent form of the disease.
Clinical mastitis (CM)	✓ Udder inflammation characterized by visible abnormalities in the milk and or udder. Severity of clinical cases should be described as mild, moderate, or severe.
Mild clinical mastitis	✓ Observable abnormalities in milk, generally clots or flakes with little or no signs of swelling of the mammary gland or systemic illness. ✓ Preferred terminology when describing severity of clinical cases.
Moderate clinical mastitis	✓ Visible abnormal milk accompanied by swelling in the affected mammary quarter with an absence of systemic signs of illness. ✓ The terminology is preferred when describing the severity of clinical symptoms.
Severe clinical mastitis	✓ Udder inflammation characterized by sudden onset with grave systemic and local symptoms. ✓ This terminology is preferred to peracute clinical mastitis.

[1]: International Dairy Federation: Suggested Interpretation of Mastitis Terminology. Bull Int Dairy Fed 2011, 448 (revision of Bull Int. Dairy Fed 1999, 338)

As exposed in table 1, subclinical mastitis is characterized by an elevation in somatic cell count (SCC) in the milk obtained from an infected quarter, with a predominance of neutrophils and epithelial cells. The increase of SCC in milk is thus the main marker for the detection and diagnosis of mastitis (Viguier et al., 2009). In contrast, clinical mastitis is

characterized by visible abnormalities in milk with an aqueous appearance, flocculation, and formation of clots. In the udder, presence of the cardinal signs of inflammation, as pain, warm, swelling, redness and sensitivity to touch characterize the infectious process. Severe clinical mastitis can become a gangrenous infection with consequent destruction of tissues and loss of the infected udder (Bradley, 2002; Le Maréchal et al., 2011b; Oviedo-Boyso et al., 2007). Beside the quantitative aspect (decrease in milk yield), the milk composition and quality is different from those of healthy milk. On one hand, mastitis milk is characterized by an increase in free fatty acids, whey proteins, serum albumin, transferrin, lactoferrin, immunoglobulin G or noncasein nitrogen. On the other hand, it is characterized by a decrease in lactose, and total caseins (Le Maréchal et al., 2011c). Altogether, these characteristics determine a production with less favorable organoleptic properties and with a lower "cheeseability" (Le Maréchal et al., 2011c; Viguier et al., 2009).

3.1.2. *S. aureus* mastitis

S. aureus is one of the main etiological agents of bovine mastitis in different countries, associated with subclinical and clinical forms. It was reported with prevalence from 5% up to 70% of cows and 90% of herds affected worldwide (Zecconi and Scali, 2013). Subclinical mastitis is described as 15 to 40 times more prevalent than clinical mastitis, and is characterized by a long duration and usually precedes the clinical form (Hoque et al., 2018). In small ruminants, *S. aureus* is mainly related to clinical mastitis, but is also able to induce cases of subclinical mastitis, while Coagulase Negative Staphylococci (CNS; e.g., *Staphylococcus hiycus*, *S. epidermidis*, *S. equorum*, *S. saprophyticius*, *S. simulans*, *S. chromogenes*) are more frequently isolated from subclinical cases (Bergonier et al., 2003; Martins et al., 2015; Merz et al., 2016; Vautor et al., 2009).

The association between host factors and strain features mainly determines the severity of the infection and the subsequent inflammatory response (Le Maréchal et al., 2011a). Briefly, the variability of clinical manifestations in mastitis may be associated with genetically intrinsic characteristics of the bacteria (repertoire of virulence factors), as well as with the host immune factors that make it more or less predisposed to infections (Vautor et al., 2009). Indeed, this variability can be observed in the same dairy herd, which suggests the holistic relation in this type of infection. Furthermore, a frustrating aspect of *S. aureus* mastitis is the recurrence. Cows that already experienced clinical mastitis are more prone to develop new infection (Jamali et al., 2018). Besides clinical mastitis, *S. aureus* subclinical

mastitis still tends to become chronic and persist for long periods (Wallemacq et al., 2010; Abebe et al., 2016).

3.1.3. Mammary gland defenses

In this part, we will address various aspects of the MG defenses against infections. Each paragraph will start with generalities about MG defenses and whenever relevant, MG defenses against *S. aureus* mastitis will be presented in more details.

3.1.3.1. Anatomic and microbial barrier

The udder is the organ responsible for milk production and a healthy animal is necessary for a safe production and good yields (Ezzat Alnakip et al., 2014). Milk is produced by the cells lining the mammary alveoli deep within the MG (Blowey and Edmondson, 2010). After birth, milk production varies according to the animal species and is characterized by being more intense in the first weeks post-partum. In between two consecutive lactations, the cows go through a dry period that includes the time between halting of milk removal (milk stasis) and the subsequent calving (Leelahapongsathon et al., 2016). In dairy cattle herds, the dry period generally lasts 45 to 50 days. If less than 40 days, then milk yield decreases in the next lactation.

The compartmentalization of udders in four quarters (mammary complexes) in cow and two in small ruminants help to limit the spread of infection between compartments. Furthermore, the udder is strongly supported by ligaments to keep teats vertical and to prevent a contamination by contact with surfaces (Figure 3A) (Blowey and Edmondson, 2010). These constitute the first natural defense mechanisms of ruminants against a bacterial infection.

The main entry for pathogens in the udder is the teat canal. Pathogens can ascend through the teat cistern until the lumen of the MG (Figure 3B). However, the teat canal is closed by sphincters and constitutes the first physical barrier against the infection by microorganisms (Ezzat Alnakip et al., 2014). In the internal end of the canal, Furstenberg's Rosette has leukocytes that also can influence the susceptibility of MG to infections. Besides, the bacteria must combat the antimicrobial factors and the keratin layer in the MG environment. This layer originates from the stratified squamous epithelium and it is able to prevent the migration of bacteria in the teat canal, as it contains esterified and non-esterified fatty acids (myristic, palmitoleic and linoleic) that act as bacteriostats and help the MG in fighting infection (Craven and Williams, 1985; Paulrud, 2005; Sordillo and Streicher, 2002).

Furthermore, the microbiota associated with teat canal and cistern may constitute a microbiological barrier against pathogens. Indeed, it was recently shown by our team that healthy quarters exhibited a higher bacteria diversity than quarters that had already underwent mastitis. Its composition in terms of bacterial species was also different with regard to the sensitivity to mastitis (Falentin et al., 2016).

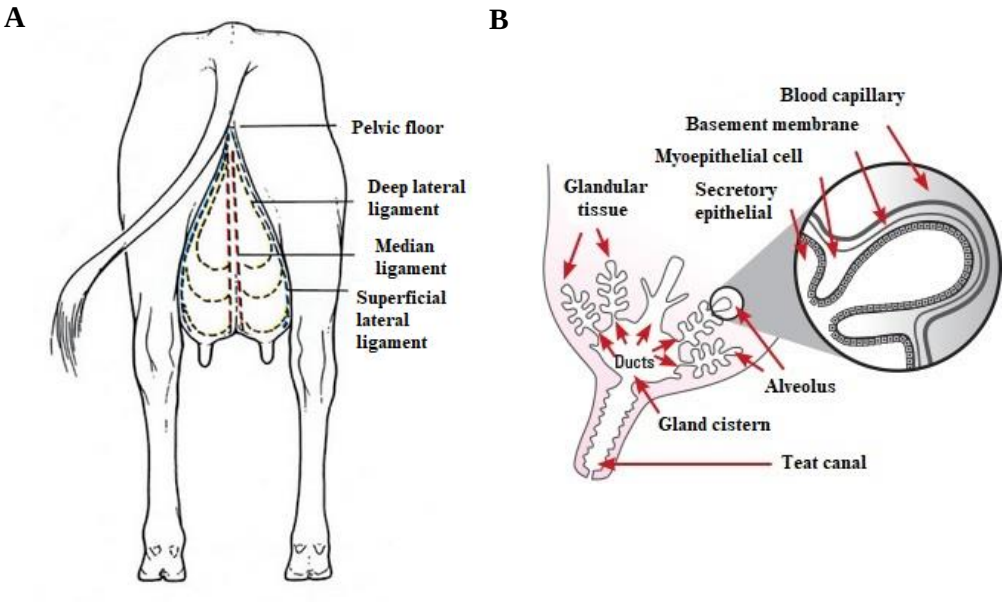

Figure 3. Mammary gland in ruminants. A) The suspension of the udder (Source: adapted from Blowey and Edmondon, 2010); B) Structure of the mammary gland.

Among the factors that influence the susceptibility of the animal, stand out the breed, nutritional status, stage of lactation and parity (older cows) and milk production (Oviedo-Boyso et al., 2007). The gestation, birth, lactation and physical stresses of parturition affects the hormonal and metabolic profiles of the animal, which allow an increase in the prevalence of this disease in the periparturient period (Mallard et al., 1998; Wagter et al., 2000). Furthermore, fluid accumulation inside of the MG results in increase of intramammary pressure and vulnerability (Viguier et al., 2009). In addition, milking increases this vulnerability once that keratin layer is flush out and the sphincter requires approximately 2h to reestablish its contracted form giving thus way to pathogen ascendant contamination (Viguier et al., 2009).

3.1.3.2. Innate immune response

In spite of these mechanisms, if the pathogen ascends the gland lumen, it can colonize, multiply and establish an infectious process (Figure 4). However, the bacteria must also elude cellular and humoral host immune defense in the udder (Viguier et al., 2009).

Somatic cells (SC) consist of different types of cells, including neutrophils, macrophages, lymphocytes, eosinophils and mammary epithelial cells (MEC) (Kehrli and Shuster, 1994). They are described as key cellular effectors in the mammary innate immune system that are recruited from the bloodstream after stimulus and provide rapid defense against invading microorganisms (Rainard and Riollet, 2006; Wellnitz et al., 2010). In the healthy mammary gland, the predominant cells are macrophages (Lee et al., 1980), which following an infection can recognize the microorganism and stimulate the migration and bactericidal activity of neutrophils through the production of inflammatory mediators, such as cytokines (TNF-α and IL-1β), prostaglandins, and leukotrienes (Oviedo-Boyso et al., 2007; Sordillo, 2005). In this initial phase, a fast induction of the innate immune response occurs, mainly mediated by neutrophils, macrophages, natural killers (NK) and cytokines (Brenaut et al., 2014; Oviedo-Boyso et al., 2007).

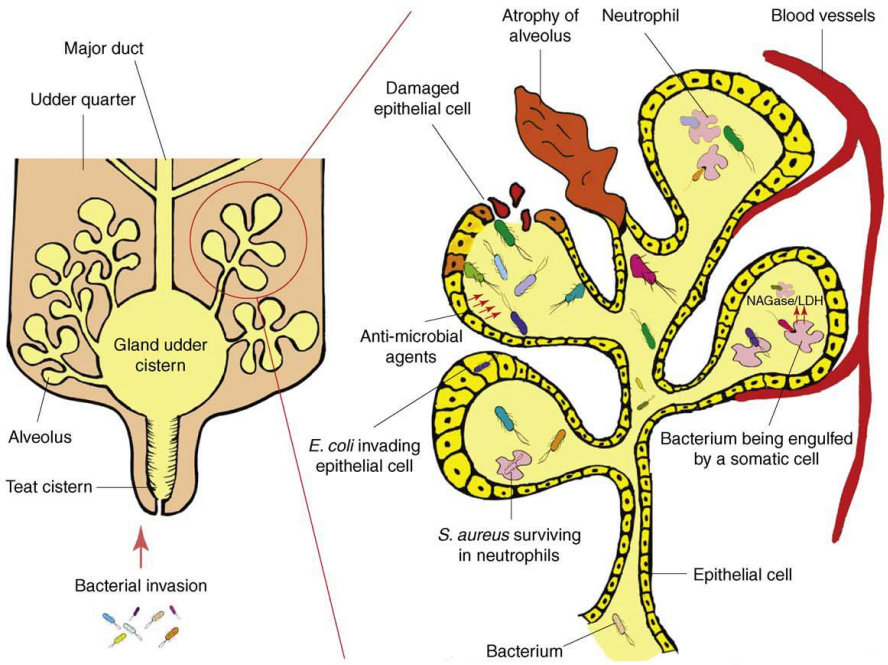

Figure 4. Mastitis development in an infected udder. Bacteria invade the udder and ascend through the canal of the teat until the cistern of the gland. In the mammary alveoli, the recognition of the bacteria leads to a stimulation of the immune system with neutrophilic recruitment. The pathogens adhere and internalize in

mammary epithelial cells, which leads to progressive tissue damage. This progression leads to milk contamination and may result in loss of anatomical integrity of the mammary gland (Viguier et al., 2009).

Bacterial mastitis can be influenced by specific factors produced by the infecting bacteria (Zecconi et al., 2006) and the constituents of cells wall, including lipoteichoic acids (LTA), peptidoglycan (PG) and lipoproteins (LP) are classical examples of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Sukhithasri et al., 2013). These MAMPs are recognized by patterns recognition receptors (PRR), such as Toll-like receptors (TLRs), which induce the production of cytokines and endogenous mediators to combat the pathogenic microorganism (Oviedo-Boyso et al., 2007; Sordillo and Streicher, 2002). Activated TLRs stimulate the signaling pathway that leads to NF-κB activation and subsequently production of cytokines (Takeda and Akira, 2001). Briefly, the pro-inflammatory cytokines produced induce the expression of adhesion molecules in epithelial cells and consequently promote chemotaxis of neutrophils. Hence, neutrophils are the first immune cells to arrive on the focus of inflammation after margination of cells circulating followed by interaction and movement through endothelial cells in the blood vessels (Lahouassa et al., 2007). Once infected, the rise of SCC exceeds 200,000 cells per mL and has its origin in transendothelial migration (Oviedo-Boyso et al., 2007). Polymorphonuclear cells (PMN) can reach up to 95% in cow milk from infected quarters (Damm et al., 2017; Paape et al., 1979). Phagocytosis of *S. aureus* triggers production of reactive oxygen species (ROS), antimicrobial peptides (AMPs) and defensins and release of bactericidal granule components which leads to a neutrophil programmed self-death (Guerra et al., 2017; Oviedo-Boyso et al., 2007; Sordillo and Streicher, 2002).

Progressively, the MG tissue becomes infected (Figure 4). The progression of the infection leads to tissue damage with a loss of the anatomical integrity of the alveolus with breaches of the blood-milk barrier. This consequentially results in contamination of the milk with extracellular fluids. In this way, milk exhibits visible characteristics as clots and flakes accompanied by the swelling and reddening of the udder (Viguier et al., 2009; Zhao and Lacasse, 2008).

3.1.3.3. Adaptive immune response

The second line of the defense against the infection consists in the stimulation of the humoral and cellular responses in the host, with a consequent symptomatology in the infected MG (Oviedo-Boyso et al., 2007; Viguier et al., 2009). Lymphocytes play an important role as

mediators of cellular immune response and can directly or indirectly be associated to the fluctuations in the overall production of antibodies, cytokines, and hormones in the MG (Van Kampen and Mallard, 1997).

Lymphocytes B secrete immunoglobulins (Ig) or antibodies, such as IgG, IgM, and IgA involved in the MG immune response (Korhonen et al., 2000). IgG1 is predominant in the milk of a healthy mammary gland while an increase in IgG2 is observed during intramammary infections (Sordillo, 2005). IgGs are mainly involved in the activation of complement-mediated bacteriolytic reactions and bacteria opsonization. Further, IgA is mainly involved in the agglutination and neutralization of the invading bacteria, preventing the spread of the pathogenic agent in the MG (Korhonen et al., 2000).

Bovine milk T lymphocyte cells consist of CD4+ T-helper and CD8+ T cells, which include both T-cytotoxic and suppressor T cells. The latter group is predominant in the healthy bovine mammary gland (Mehrzad et al., 2008). During bovine *S. aureus* mastitis, the increase in T cells in milk is due to an increase in activated CD8+ (Park et al., 1993). During the inflammatory response in MG, CD4+ cell become the predominant phenotype (Riollet et al., 2000). The presentation of antigens from *S. aureus* to CD4+ T cells and subsequent secretion of certain cytokines play an important role in activating B lymphocytes, T lymphocytes, macrophages and various other cells (Riollet et al., 2000).

3.1.4. Diagnosis

Early detection of mastitis is a prerequisite for an effective treatment and therefore for minimizing the impact on milk production and the use of antibiotics. Currently, detection of mastitis can be performed through somatic cell counts (SCC), enzymatic analysis and California Mastitis Test (CMT). In the main milk producing countries, SCC is the most accepted standard for measuring the quality of milk produced and is a good indicator of mammary gland health. In this context, SCC above 200,000 cells per mL or some positive score in CMT constitute a great indicator of infected quarter (Rysanek et al., 2007; Viguier et al., 2009; Santana et al., 2013). Recently, a new parameter for cell differentiation in individual cow milk samples using flow cytometry called differential somatic cell count **(**DSCC) parameter was described (Damm et al., 2017). This method enables to discriminate the proportion of immune cell population in milk, which confers advantages in determining the udder health status (Damm et al., 2017).

CMT is a test that indirectly evaluates the amount of somatic cells in milk samples. The CMT reagent is a bromocresol-purple-containing detergent that breaks down the cell membrane of somatic cells and reacts with the nucleic acid to form a gel-like matrix, which viscosity corresponds to the leukocytes number present in the milk (Viguier et al., 2009). Colorimetric and fluorimetric assays are also used to detect enzymes, such as N-acetyl-β-D-glucosaminidase (NAGase) and lactate dehydrogenase (LDH), which are in high concentration in milk from the infected udder. These enzymes are described as a good indicator of tissue damage in the MG. Some other tests that can be performed for the detection of MG infection include electrical conductivity and tamis test. The first evaluates an increase in milk conductance due to the elevation in levels of ions, such as sodium, potassium, calcium, magnesium, and chloride during inflammation (Viguier et al., 2009). The second is basically the evaluation of the milk in a mug with a dark background (Tamis) which allows visualizing clots, blood, and aqueous samples (Souto et al., 2010; Viguier et al., 2009)

3.1.5. Control

3.1.5.1. Prevention

Prevention and control are still the most effective strategy against infection and propagation of the pathogens in the herd and, in the long-term, confers benefits on animal production and health. Poor milking hygiene has been associated with increased SCC, which confers a reduced production and quality of the milk. To overcome this issue, the best measure consists of a good pre-milking hygiene routine that can considerably decrease the infection rate in the herd (Gleeson et al., 2009). One of these methods consists in pre-dipping the teat with disinfectant products. This reduces the bacterial population on teat skin prior to milking (Gleeson et al., 2009). Furthermore, herd management practices are also relevant for mastitis control. This comprises, for instance, the reduction of the pathogen transmission by optimization of herd conditions with segregation and culling decisions, wash of the udder, consistent feeding, and ventilation status of the farm (Ali et al., 2014; Down et al., 2016; Krömker and Leimbach, 2017). In addition, nutritional stages, trace minerals, and vitamins can also influence the animal immune responses and udder health. For instance, Vitamin E and selenium (Se) are important components of the antioxidant defenses of cells and a deficiency in cows ration results in impaired PMNs activity (O'Rourke, 2009).

Genetic correlation between the appearance of clinical mastitis and SCC can also be used to a proper selection of dairy cattle with higher resistance to this type of infection

(Jattawa et al., 2012; Shook, 1993). Besides, a strategy used to reduce the incidence of infections, as well as to prevent the emergence of new cases during the first weeks of dry period, consists in the administration of antibiotic following the last milking of the lactation, called dry cow therapy (DCT) (NMC, 2007; Lents et al., 2008). Indeed, the dry period between two lactations is a crucial time for udder health.

3.1.5.2. Treatment

Current treatments involve antimicrobial therapy for the reduction of IMI and although it is useful for infection control, it constitutes a public health concern because of the emergence and the spread of antimicrobial resistance in the livestock and the community (da Silva et al., 2004; Zhao and Lacasse, 2008; Krömker and Leimbach, 2017). According to the World Health Organization (WHO), a better use of antimicrobial agents in food-producing animals is necessary, since the use of antibiotics is associated with the emergence of bacterial resistance and subsequent dissemination through out the human food chain. Another important aspect involved in the use of antimicrobial agents is the gradual reduction in cure rate of infected animals (WHO, 2017; Tenhagen et al., 2006).

The herd periodic monitoring with sensitivity tests *in vitro* is necessary for the selection of an effective antibiotic therapy. Usually, in udder infections caused by *S. aureus*, treatment using β-lactam is widely employed albeit other classes of antibiotics can also be used (Barkema et al., 2006; Haveri et al., 2005; Tenhagen et al., 2006).

Vaccination constitutes an alternative to the indiscriminate use of antibiotics in the herd. However, the formulations currently available still have limited efficacy in the field. The main strategies used in the development of vaccines and some commercial formulations are summarized in table 2.

Table 2. Strategies used to the development of a vaccine against Staphylococcal mastitis and some examples of commercial vaccines.

Vaccines	Composition	Reference
Whole organism		
One or more killed strains	Endemic strains	Lee et al., 2005
Attenuated vaccines	Live-attenuated *S. aureus*	Watson, 1984
		Gómez et al., 1998
Subunit vaccine		
Toxoids	Inactivated toxins	Adhikari et al., 2015;
		Watson et al., 1996

Surface proteins	Variety of purified surface antigenic determinants	Scali et al., 2015 Scarpa et al., 2010
Capsular polysaccharides	Capsular polysaccharide antigen extracted	Yoshida et al., 1984 Prenafeta et al., 2010
Commercial vaccines		
Starvac®	Polyvalent vaccine containing *E. coli* J5 and *S. aureus* strain SP 140	Schukken et al., 2014
BestVac®	Herd-specific vaccine Three Staphylococcus aureus strains obtained from mastitis milk samples of the herd, icaD- and icA-positive polysaccharides	Freick et al., 2016
MastiVac I	Three strains of S. aureus (VLVL8407; ZO3984 and BS449) which were isolated from clinical and sub-clinical cases of bovine mastitis.	Leitner et al., 2003
Lysigin®	Bacterin formed of five *S. aureus* strains	Middleton et al., 2006

Another strategy for preventing or even treating mastitis in dairy cows consists the use of probiotic strains. According to the World Health Organization (WHO), probiotics are “live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host” (Morelli and Capurso, 2012). In the mastitis context, intramammary infusions of live *Lactococcus lactis* DPC3147 showed potential in the treatment of mastitis when compared to antibiotic therapy (Klostermann et al., 2008). This result was involved with the ability of probiotic bacteria to trigger an influx of polymorphonuclear cells (PMN) in the mammary environment (Crispie et al., 2008).

Besides, *Lactococcus lactis* V7 also showed an inhibitory effect against adhesion and internalization of *E. coli* and *S. aureus* in bovine mammary epithelial cells (bMEC) (Assis et al., 2015). Similar antagonism was observed in vitro using the probiotic strain *L. casei* BL23 (Bouchard et al., 2013). This effect was mediated by bacterial cell surfaces once the sortase mutants had reduced rates in the inhibition of *S. aureus* internalization (Souza et al., 2017).

3.2. Staphylococcus aureus

3.2.1. Staphylococcus spp.

Staphylococcus aureus was initially discovered by Alexander Ogston in a microscopic observation of the pus from an infected tissue (Ogston, 1881). However, the genus *Staphylococcus* was officially described in 1884 by Anton Rosenbach (Rosenbach, 1884) and includes Gram-positive cocci that were associated to different infectious process. Nowadays, this genus comprises 52 species and 28 subspecies conforming the list of Prokaryotic names with standing in nomenclature (Parte, 2018; Schleifer and Bell, 2015). *Staphylococcus* generally range in size from 0.5 to 1.5μm; it is non-spore forming, immobile, and facultative anaerobes albeit they grow best in the presence of oxygen (Schleifer and Bell, 2015). The species can be identified in microbiology laboratories based on conventional biochemical and physiological assays (Hennekinne et al., 2012; Ishii, 2006).

Staphylococci are divided into a group that produces coagulase (Coagulase-Positive Staphylococci - CPS), including *S. aureus*, and that do not (Coagulase-Negative Staphylococci - CNS). This latter group comprises *S. epidermidis*, *S. saprophyticus*, *S. schleiferi*, *S. lugdunensis*, *S. haemolyticus*, among others. CNS species colonize the skin and mucous membranes of humans and animals and are less frequently involved in clinically manifested infections (Becker et al., 2014). However, some of them have particular pathogenic potential such as *S. lugdunensis* and *S. saprophyticus* (Peters et al., 2017). In addition, some species (*e.g. S. xylosus* and *S. carnosus*) have technological potential in fermented products, such as cheese and sausage, due to their aromatic, pigmentary, and color abilities (Coton et al., 2010; Irlinger, 2008).

3.2.2. Staphylococcus aureus

S. aureus is by far the member of most medical interest (Ishii, 2006). An overview of some events involving this microorganism is shown in table 3. *S. aureus* is classified as positive-catalase and negative-oxidase and has the capacity to resist high concentrations of sodium chloride (up to 15%). This bacteria can grow in a wide range of temperatures (7°C a 48,5°C) and pH (4.2 a 9.3, with the optimum being 6-7) (Hennekinne et al., 2012; Le Loir et al., 2003).

Table 3. Some events involving *S. aureus* research (adapted from Wertheim et al., 2005).

Year	Event	Reference
1880	Alexander Ogston identifies micrococci in purulent infections	Ogston, 1881
1884	Genus *Staphylococcus* described by Anton Rosenbach	Rosenbach, 1884
1928	Penicillin as antimicrobial agent	Fleming, 1929
1931	Association between nasal colonization and furunculosis discovered	Solberg, 1965
1942	Penicillin-resistant *S. aureus* reported	Rammelkamp and Maxon, 1942
1944	Introduction of phage typing	Fisk and Mordvin, 1944
1952	Association between nasal colonization of *S. aureus* and infection with the same strain determined by phage typing	Valentine and Hall-Smith, 1952 Atkins and Marks, 1952
1961	Meticillin-resistant (MRSA) reported	Jevons, 1961
1991	Pulsed field gel electrophoresis used for genotyping *S. aureus*	Prévost et al., 1991
1994	Identification of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)	Patti et al., 1994
1998	CA-MRSA	Chambers, 2001
1972	MRSA reported in bovine mastitis	Devriese et al., 1972
1997	Vancomycin-intermediate *S. aureus* (VISA) reported	Hiramatsu et al., 1997
2000	Multilocus sequence typing developed for studying clonality of *S. aureus*	Enright et al., 2000
2001	Whole genome of *S. aureus* sequenced (*S. aureus* Mu50 and N315)	Kuroda et al., 2001
2001	80% of bacteraemic *S. aureus* isolates are endogenous	von Eiff et al., 2001
2001	Increase in community-onset MRSA infections	Chambers, 2001

2002	Vancomycin-resistant *S. aureus* (VRSA) reported	Chang et al., 2003
2011	MRSA bearing a mecC gene discovered in a dairy cattle	García-Álvarez et al., 2011

3.2.3. Biology of *S. aureus*

The primary host and ecological niche of *S. aureus* is the human body, where *S. aureus* colonizes regions as nostrils, axillae, perineum, vagina, and rectum, which correspond to its natural reservoir (Sibbald et al., 2006). Approximately 20% (range 12-30%) of the human population are persistent carriers in the moist squamous epithelium of the anterior nasal cavity (Foster et al., 2014; Kluytmans et al., 1997; Wertheim et al., 2005). Besides, approximately 30% (range 16-70%) are intermittent carriers while about 50% (range 16-69%) were described as non-carriers (Wertheim et al., 2005). Once the host defenses are breached (*e.g.* surgery), the colonization state considerably raises the risk of developing an infection (Gordon and Lowy, 2008; Popov et al., 2014). Interestingly, the mortality rate from hospital-acquired *S. aureus* bacteremia is higher in non-carriers compared with carriers (Wertheim et al., 2004). *S. aureus* is also found in non-human hosts. It can indeed colonize skin and mucous membranes of many warm-blooded animals, including chickens, pig, sheep, cows, and heifers, among others (Peton and Le Loir, 2014).

3.2.3.1. Molecular determinants of virulence and pathogenicity

S. aureus produces an extensive arsenal of virulence factors, with both structural and secreted products playing a role in the pathogenesis. New insights have been obtained after the release of the first genome sequences of the clinical strains *S. aureus* Mu50 and N315 (Kuroda et al., 2001). The identification of mobile genetic elements (MGEs) and pathogenicity islands highlighted the complexity of this genome and the plasticity of this microorganism (Kuroda et al., 2001). Interestingly, although classified as an opportunistic pathogen, some isolates of *S. aureus* are described as more prone to cause disease due to the repertoire of virulence factors that enable a successful host colonization and spread (Feil et al., 2003). Consequently, the gravity of *S. aureus* infections can be associated with the arsenal of factors, including adhesins, exoenzymes, toxins and immune-modulating proteins that it produces (Gordon and Lowy, 2008). An overview of these determinants of pathogenicity is shown in table 4.

Table 4. Some of the main determinants of *S. aureus* virulence and pathogenicity.

Proteins	Function	Reference
Cell-wall anchored proteins		
MSCRAMMs		
Fibronectin binding proteins (FnBPs)	Adhesion to extracellular matrix (ECM) of the host	Burke et al., 2011; Clarke and Foster, 2006; Geoghegan and Foster, 2017
Clumping factor A (ClfA)	Adhesion to immobilized fribrinogen Immune evasion T cell activator	Ganesh et al., 2008; Geoghegan and Foster, 2017; Lacey et al., 2017
Clumping factor B (ClfB)	Adhesion to squamous cells	Corrigan et al., 2009; Foster et al., 2014
Collagen binding protein	Adhesion to collagen-rich tissue	Clarke and Foster, 2006; Zong et al., 2005
bone sialo-binding protein (Bbp)	Adhesion to ECM (fibrinogen)	Vazquez et al., 2011
Serine-aspartate repeat protein C (SdrC)	Adhesion to squamous cells	Corrigan et al., 2009; Foster et al., 2014
Serine-aspartate repeat protein D (SdrD)	Adhesion to squamous cells	Corrigan et al., 2009; Foster et al., 2014
Serine-aspartate repeat protein E (SdrE)	Adhesin Immune evasion; degradation of C3b	Clarke and Foster, 2006; Foster et al., 2014; Sharp et al., 2012
Protein A (SpA)	Binds Fc domain of immunoglobulin, von Willebrand factor and TNFR-1; Binds complement protein C3 and promotes C3–C3b conversion	Atkins et al., 2008; Clarke and Foster, 2006
Iron-regulated surface proteins (Isds)	Heme uptake and iron acquisition	Clarke and Foster, 2006
SERAM		
Extracellular adhesive protein (Eap)	MHC-II analog protein; adhesion to *S. aureus* cells and host cells; involved in	Chavakis et al., 2002; Johnson et al., 2008

	biofilm formation	
Extracellular fibrinogen binding protein (Efb)	Binds fibrinogen; inhibits C3 and C5 convertases; binds complement C3	Koch et al., 2012; Palma et al., 1996
Coagulase (Coa)	Binds and activates prothrombin; promotes conversion of fibrinogen to fibrin	McAdow et al., 2012
Extracellular matrix binding protein (Emp)	Binds extracellular matrix of host cells (high affinity for vitronectin); involved in biofilm formation	Clarke and Foster, 2006; Johnson et al., 2008
von Willebrand factor binding protein (vWbp)	Binds and activates prothrombin; binds fibrinogen and vW factor	McAdow et al., 2012
Lipoproteins	Recognized by Toll-like receptors (antigenicity) Antibiotic resistance Substrate-binding proteins in transporter systems Adhesion, protein secretion and folding Modulation of inflammatory processes Translocation of virulence factors	Kovacs-Simon et al., 2011
Exoenzymes		
Staphopains (ScpA and SspB)	Papain-like cysteine proteolytic enzyme Cleave proteins including elastin, collagen, fibrinogen, fibronectin and kininogen	Park et al., 2011
Aureolysin (Aur)	Cleaves both complement protein C3 and the antimicrobial peptide LL-37 Participate in the processing of a glutamyl endopeptidase SspA	Laarman et al., 2011; Sieprawska-Lupa et al., 2004; Nickerson et al., 2007
Hyaluronidase protein (HysA)	Enzyme that cleaves hyaluronic acid in the ECM	Makris, 2004
Exotoxins		
Superantigens (PTSAg)		
Staphylococcal enterotoxins (SEs)		

Enterotoxins (SEs)	Gastroenteric toxicity; Immunomodulation via superantigen activity	Le Loir and Hennekinne, 2014; Wu et al., 2016
SEs-like toxins (SEls)	Homology with SE Gastroenteric toxicity not demonstrated Immunomodulation via superantigen activity	Le Loir and Hennekinne, 2014; Ortega et al., 2010
Toxic shock syndrome toxin-1	Superantigens activity with toxic effects on endothelial cells	Dinges et al., 2000
Exfoliative toxins (ETs)	Gluamate-specific serine proteases that digest desmoglein 1	Mariutti et al., 2017; Nishifuji et al., 2008
Hemolysin		
α-hemolysin	Cytolytic pore-forming toxin	Dinges et al., 2000; Otto, 2014
β-hemolysin	Sphingomyelinase with cytolytic activity	Dinges et al., 2000; Otto, 2014
Phenol soluble modulins	Pore-forming toxins or surfactant activity	Cheung et al., 2014
Leucotoxins	Kill leukocytes; bi-component pore-forming leucotoxins	Dumont et al., 2011; Nocadello et al., 2016

3.2.3.2. Virulence regulation systems

Regulation of staphylococcal virulence factors plays a central role in the pathogenesis process and involves a complex network of interacting factors (Bronner et al., 2004). Many of the large repertoire of virulence factors produced by *S. aureus* is under the control of the accessory gene regulator (agr) quorum sensing system. This system corresponds to one of the regulatory mechanism that ensures timely adaptation of staphylococcal physiology to the environment and probably is the most important mechanism involved in the control of *S. aureus* pathogenesis (Bronner et al., 2004; Le and Otto, 2015).

The *agr* operon is an integrated system organized around two adjacent and divergent promoters, P2 and P3, and generates two primary transcripts, RNAII and RNAIII, respectively (Figure 5). RNAII encodes AgrB, AgrD, AgrC and AgrA. *agrD* encodes the precursor of the thiolactone peptides known as autoinducing peptide (AIP) that is processed

by a multifunctional endopeptidase and chaperone AgrB. AgrC and AgrA comprise a two-component signal transduction system, which AgrC is the membrane histidine kinase (sensor) and AgrA is the response regulator. Briefly, the *agr* system is activated when the extracellular AIP concentration reaches a threshold that is directly linked to cell density. Upon binding AIP, it occurs an autophosphorylation of the cytoplasmic histidine kinase domain of AgrC and transphosphorylation of AgrA, which in turn activates the transcription from P2 and P3 promoters in addition to other transcriptional targets. RNAIII is a posttranscriptional regulator of genes involved in virulence and corresponds to the major effector of *agr* system (Bronner et al., 2004; Gordon et al., 2013; Tan et al., 2018).

S. aureus produces cell-wall associated factors in the first stages of infection, allowing the tissue attachment and evasion from the host immune system. Then, secretion of exoproteins is initiated once the population reaches the late exponential phase while the production of cell wall-associated proteins is down-regulated (Wang and Muir, 2016). This mechanism is orchestrated by the *agr* system that up-regulates *S. aureus* toxins and exoenzymes and down-regulates the expression of surface proteins genes (Gordon et al., 2013).

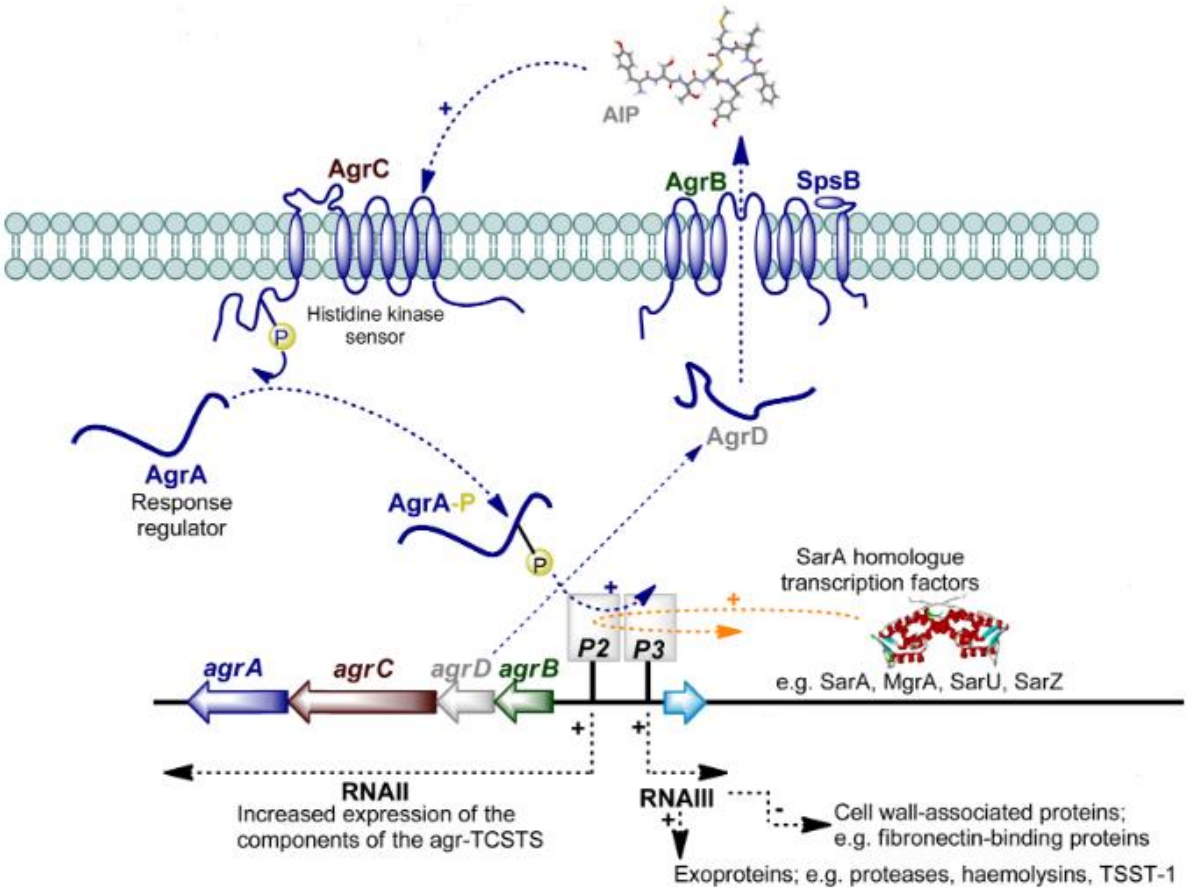

Figure 5. Schematic of the agr system and SarA protein family. Autoinducer peptide **(**AIP) signal is produced from AgrD after processing by AgrB. When reaching up certain threshold, AIP stimulates the AgrA-AgrC two-component signal transduction system that phosphorylates AgrA. Once phosphorylated, AgrA stimulates P2 that results in auto-feedback regulation. AgrA also increases the transcription of RNAIII which acts in most of the Agr targets. The SarA protein family regroups transcriptional regulators that can activate or repress RNAIII **(**TCSTS: two component signal transduction system; TSST-1: *Staphylococcus aureus* toxic shock syndrome toxin-1**)** (Gordon et al., 2013).

The *agr* system corresponds to the most characterized two-component signal transduction system of *S. aureus*. Additionally, *S. aureus* has numerous transcriptional regulator families that have been demonstrated to be involved in the expression of virulence factors, including the most investigated SarA protein family. SarA constitutes the prototype member of this family that also includes SarR, SarS, SarT, SarU, SarV, SarX, SarZ, Rot and MgrA (Cheung et al., 2008; Gordon et al., 2013). These transcriptional regulators can also drive up- or down-regulation the expression of numerous virulence factors via both *agr*-dependent (Figure 5) or *agr*-independent mechanisms (Gordon et al., 2013).

3.2.3.3. Virulence factors

3.2.3.3.1. Surface proteins

S. aureus adheres to artificial surfaces or to host cells via direct interaction or through bridging molecules such as extracellular matrix components (ECM) and plasma proteins. This adhesion step plays a crucial role in the development of the disease (Heilmann, 2011; Heilmann et al., 2005). This occurs due to the *S. aureus* production of a broad range of surface proteins. In most cases, these proteins are cell wall anchored (CWA), i.e. they are covalently linked to the cell wall peptidoglycan. The most prevalent group of *S. aureus* CWA proteins is the Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Foster et al., 2014). MSCRAMMs mediate the bacterial adherence to host cells by binding molecules such as collagen (Cn), fibronectin (Fn), elastin (El) and fibrinogen (Fg) (Gordon and Lowy, 2008; Keane et al., 2007). Further, this adhesion may be followed by proliferation of the bacteria, leading to the formation of complex structures called biofilm (Gordon and Lowy, 2008).

Most MSCRAMMs are covalently connected to cell wall peptidoglycan by the membrane-associated enzyme sortase that recognizes the conserved Leu-Pro-X-Thr-Gly (LPXTG) motif at the C terminus and links the carboxyl-group of threonine and the amino-group of the pentaglycine cell wall cross-bridge (Mazmanian et al., 1999, 2001). MSCRAMMs are expressed during the logarithmic phase of growth, which allows the initial colonization of the host tissue (Gordon and Lowy, 2008). *S. aureus* has two fibronectin binding proteins (FnBPA and FnBPB), two fibrinogen binding proteins called clumping factors (ClfA and ClfB) and the *cna* gene, which encodes the collagen binding protein, described as an important adhesion-associated virulence factor (Que et al., 2001; Zecconi and Scali, 2013). In addition to its adhesion role, ClfA can also capture and activate the complement regulatory protease factor I, which results in enhanced degradation of complement component C3b (Hair et al., 2008). SdrC, SdrD, SdrE, and bone sialo-binding protein (Bbp) are also grouped in the same family of MSCRAMMs, however, some of them have additional functions other than promoting adhesion (Table 4) (Foster et al., 2014).

Another important surface protein associated with pathogenicity is protein A (SpA), encoded by the *spa* gene. SpA is a CWA protein that comprises five Ig-binding domains (IgBDs) in the N-terminal region. This protein allows *S. aureus* to evade the immune response of the host due to its ability to bind to the Fc portion of immunoglobulin G (IgG), preventing phagocytosis of bacterial cells and classical pathway of complement fixation (Atkins et al.,

2008). Sbi is a second immunoglobulin-binding protein from *S. aureus* that comprises two IgG-binding domains similar to those found in SpA; however, Sbi is found both extracellularly and bound to the cell wall. This small protein also helps *S. aureus* to evade the complement system by interaction with the IgG Fc moiety (Smith et al., 2012).

Another group of CWA proteins includes iron-regulated surface (Isd) proteins, involved in heme capture from hemoglobin and bacterial survival under iron restriction (Foster et al., 2014). In the MG, the presence of eukaryotic iron-binding proteins such as lactoferrin and transferrin reduces the free iron available to levels insufficient for the bacterial growth. Basic cellular metabolic activities indeed require this metal. *S. aureus* is capable to sequestrate the iron attached to transferrin through secreted siderophores, which are iron chelating agents that play an important role during the infection (Andrews et al., 2003; Dale et al., 2004; Lacasse et al., 2008).

Besides the surface-associated proteins, *S. aureus* can also produce non-covalently linked adhesins that were described to induce immunomodulatory effects in the host cells (Chavakis et al., 2005; Kohler et al., 2014). This group is called SERAM (Secreted Expanded Repertoire Adhesive Molecules) and is formed by extracellular adhesive protein (Eap), the extracellular fibrinogen binding protein (Efb), coagulase (Coa), and the extracellular matrix binding protein (Emp) (Chavakis et al., 2002, 2005). Coagulase and von Willebrand factor binding protein (vWbp) are the two proteins secreted by *S. aureus* that promote coagulation cascade during host infection (McAdow et al., 2012).

3.2.3.3.1.1. Lipoproteins

Bacterial lipoproteins are membrane proteins that are covalently modified with a lipidic moiety at their N-terminal cysteine residue (Biagini et al., 2015). Gram-positive lipoproteins are processed by the prolipoprotein diacylglyceryl transferase (Lgt) and the lipoprotein signal peptidase (Lsp). Firstly, Lgt recognizes a lipobox motif in the C-terminal region of the signal peptide and transfers a diacylglyceryl moiety to the cysteine residue of the lipobox. Then, the Lsp cleaves the signal peptide and the lipidic moiety remains inserted in the membrane lipid bilayer (Sutcliffe and Harrington, 2002). In Gram-positive bacteria, lipoproteins are associated with antibiotic resistance, such as substrate-binding proteins in transporter systems, adhesion, protein secretion and folding, sporulation and germination and bacterial conjugation, modulation of inflammatory processes and translocation of virulence factors into host cell. Due to these properties, they have been described as virulence factors.

(Kovacs-Simon et al., 2011; Sutcliffe and Russell, 1995). The ability to be sensed by Toll-like receptors classifies them as potential PAMPs and attractive vaccine candidates (Biagini et al., 2015).

3.2.3.3.2. Exoenzymes

During the infection, *S. aureus* is also able to produce numerous enzymes, such as proteases, lipases, and elastases, which allow the invasion of and induce damage to the host tissues. Furthermore, theses exoenzymes can be involved in processes that degrade extracellular matrix proteins and induce vascular permeability (Gordon and Lowy, 2008; Park et al., 2011). Among these exoenzymes are serine proteases, such as V8 protease (SspA), metalloproteinase aureolysin (Aur), staphopain cysteine proteases A (ScpA), B (SspB), as well as hyaluronidase protein (HysA) (Ibberson et al., 2014; Nickerson et al., 2010; Park et al., 2011).

3.2.3.3.3. Exotoxins

Once the colonization is established, *S. aureus* produces a wide variety of exotoxins that contribute to its ability to spread and cause disease in the host.

3.2.3.3.3.1. Enterotoxins (SEs)

These toxins comprise a group of 27 serologically distinct gastrointestinal staphylococcal enterotoxins (SEs), including the most common SEA and SEB, associated with clinical manifestation as emesis with or without diarrhea (Staphylococcal food poisoning) (Dinges et al., 2000; Hennekinne et al., 2012; Ono et al., 2008; Pinchuk et al., 2010; Wu et al., 2016; Zhang et al., 2018). Variants in the emetic reactions in primate models or proteins that have not been tested in this model are appointed as SE-like toxins (SE*l*) (Lina et al., 2004). SEs genes can be carried by plasmids, phages or genomic islands and the expression of most of them are under the control of the *agr* system (Le Loir and Hennekinne, 2014). The enterotoxins belong to the group of pyrogenic toxin superantigens (PTSAg) that also includes the toxic shock syndrome toxin-1 (TSST-1). PTSAg are able to bind major histocompatibility complex (MHC) class II and directly cause a polyclonal proliferation of T cells and massive release of chemokines and pro-inflammatory cytokines (Lowy, 1998; Tinelli et al., 2014). Unlike conventional antigens, superantigens do not need to be processed by antigen-presenting cells (APC) before being presented to T cells (Thomas et al., 2009).

3.2.3.3.3.2. Exfoliative toxins (ETs)

Exfoliative toxins, whose main isoforms are ETA, ETB, and ETD, constitute epidermolytic toxins produced by *Staphylococcus* and associated with bullous impetigo and staphylococcal scalded skin syndrome (SSSS) in the host (Amagai et al., 2002; Nishifuji et al., 2008). The epidermal desquamation results from the cleavage of desmoglein 1, a keratinocyte cell-to-cell adhesion molecule (Mariutti et al., 2017). These toxins are further described in a book chapter (cf. Annexes 4) in this manuscript.

3.2.3.3.3.3. α-hemolysin

S. aureus produces a series of cytolytic proteins that can cause lysis in host red and white blood cells (Otto, 2014). The α-toxin (α-hemolysin) (Hla), the best known of the group of hemolysins, is a pore-forming toxin (attack host cells by permeabilizing their cell membrane) for erythrocytes and other cell types, as epithelial, monocytes, T and B cells (Nygaard et al., 2012). α-hemolysin monomers are secreted by *S. aureus* and integrate into the host cell membrane, where they form cylindrical heptamers which results in the formation of the pore (Vandenesch et al., 2012).

3.2.3.3.3.4. β-hemolysin

Beta-hemolysin is a sphingomyelinase which hydrolysis sphingomyelin in the host plasma membrane. This toxin is highly active against animal erythrocytes, as sheep and bovine, but also has leukotoxic properties (Dinges et al., 2000).

3.2.3.3.3.5. δ-hemolysin and phenol soluble modulins

Delta-Hemolysin (delta-toxin) (Hdl) is a member of the phenol-soluble modulins (PSMs) (Wang et al., 2007), a family of short, amphipathic peptides produced by *S. aureus* and other Staphylococci (Cheung et al., 2014). PSMs can be classified according to their length. The α-type PSM are 20-25 amino acids in length (four PSMα encoded in the *psmα* locus) while β-type have about 43-45 amino acids (two PSMβ encoded in the *psmβ* locus). To date, *S. aureus* produces 7 *S. aureus* PSMs have been described and named PSMα1 - α4 and PSMβ1 - PSMβ2, and the *S. aureus* δ-toxin (Wang et al., 2007). Delta-toxin belongs to the α-type and is encoded within RNAIII, the effector molecule of the *agr* system (Cheung et al.,

2014; Wang et al., 2007). PSMs have cytolytic activity toward many host eukaryotic cell types, including PMNs, stimulate inflammatory responses and they have been associated with biofilm development (Cheung et al., 2014). PSMs interact with the specific formyl peptide receptor 2 (FPR2) to trigger an inflammatory response, however, the cytolytic activity was described as receptor-independent (Kretschmer et al., 2010). PSM β-types were described as less cytolytic than PSM α-types. *S. aureus* PSMα3 and *S. epidermidis* PSMδ are the most potent cytolytic PSMs described to date (Cheung et al., 2014; Wang et al., 2007). Recently, our team showed that PSMα were also able to interact with the host cell cycle by inducing a delay in the G2/M phase transition (Deplanche et al., 2015) and to impair interleukin expression in bovine mammary epithelial cells (Deplanche et al., 2016).

3.2.3.3.3.6. γ-hemolysin and leukocidins

Leucotoxins are a group of other exotoxins produced by *S. aureus* that are structurally similar to alpha-toxin (beta-barrel forming family) and that are associated with host immune system evasion. These proteins are bi-components S and F (for slow- and fast-eluted component) pore-forming toxins that act synergically for pore formation in the membrane of phagocytic cells (Dinges et al., 2000; Nocadello et al., 2016; Rainard and Riollet, 2003). The S component binds to a specific proteinaceous receptor on the cell surface and the subsequent association of F component results in a loss of membrane integrity, pore formation, and lysis (Barrio et al., 2006).

To date, six bi-component leukocidins have been described in *S. aureus*: (1): HlgAB; (2) HlgCB: γ-hemolysin corresponding to two combinations of a S component (HlgA or HlgC) with a F component (HlgB); (3) LukSF-PV; (4) LukAB/HG; (5) LukED and (6) LukMF (Nocadello et al., 2016). Among them, γ-hemolysins (HlgAB and HlgCB) and LukAB (also known as LukGH) are most common, because of their location in the core genome (Vrieling et al., 2016).

3.2.4. *S. aureus* and pathogenesis

S. aureus can cause a wide range of diseases, from superficial skin lesions such as abscesses and impetigo to invasive and more serious infections such as endocarditis, osteomyelitis, septic arthritis, and pneumonia (Tong et al., 2015). Some of them are related to a specific production of a single virulence factor, such as enterotoxins in staphylococcal food poising or the TSST-1 in toxic shock syndrome (TSS) (Dinges et al., 2000). However, some

of these infections result from multifactorial factors, including septicemia and mastitis in humans and animals, respectively (Dego et al., 2002; Girard and Ely, 2007). In general, every strain of *S. aureus* can become a life-threatening pathogen depending on host clinical conditions (Wertheim et al., 2005). An important feature of this bacterium is the encoding of some toxins in the core genome or in highly conserved genomic islands, such as α-toxin and phenol-soluble modulins (PSMs), which suggest they are produced by virtually all *S. aureus* strains (Cheung et al., 2012; Peschel and Otto, 2013).

3.2.4.1. Colonization and tissue invasion

3.2.4.1.1. Adhesion and internalization

The *S. aureus* pathogenicity is closely related to its capacity to bind directly to the extracellular matrix or host cells mainly mediated by microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (Heilmann, 2011). The main MSCRAMMs involved in this host cell adhesion is the fibronectin (Fn) binding proteins (FnBPs) that form a bridge with α5β1 integrin on the cellular side (Josse et al., 2017; Sinha et al., 1999). This triggers an intracellular signaling cascade that leads to remodeling of the actin cytoskeleton and internalization of the bacteria (Ridley et al., 2012). High affinity and specificity of FnBPs for Fn are necessary for adhesion and invasion in endothelial cells, although the efficiency of uptake can differ between cell types (Edwards et al., 2010; Josse et al., 2017; Ridley et al., 2012). *S. aureus* can also adhere and invade non-professional phagocytic cells, such as epithelial, endothelial, fibroblasts, and osteoblasts (Hébert et al., 2000; Josse et al., 2017; Kintarak et al., 2004). In the mastitis context, adhesion and internalization were demonstrated in vitro using bovine mammary epithelial cells (bMEC) (Brouillette et al., 2003; Hébert et al., 2000; Souza et al., 2017). These initial interactions can be affected by the endogenous microbiota, which might express inhibitory properties against pathogens (Woodward et al., 1987). This relation was observed in nasal cavities of healthy hospital staff members in which colonization with corynebacteria was shown to determine low rates of *S. aureus* (Uehara et al., 2000). This competition was also observed in vitro using lactic acid bacteria isolated from bovine mammary microbiota (Bouchard et al., 2015).

3.2.4.1.2. Intracellular survival

To date, the mechanism by which *S. aureus* persists in its host is still not fully understood. However, *S. aureus* has been described as a facultative intracellular pathogen able

to survive and persist intracellularly (Fraunholz and Sinha, 2012). In consequence, an intracellular niche might constitute a reservoir for chronic or relapsing staphylococcal infections (Garzoni and Kelley, 2009). *S. aureus* can interact with integrins and adhere in non-phagocytic cells such as the bovine mammary epithelial cells (bMEC) of the MG with subsequent internalization (Figure 6), which corresponds to an important mechanism of evasion from host immune responses (Bouchard et al., 2013; Sibbald et al., 2006).

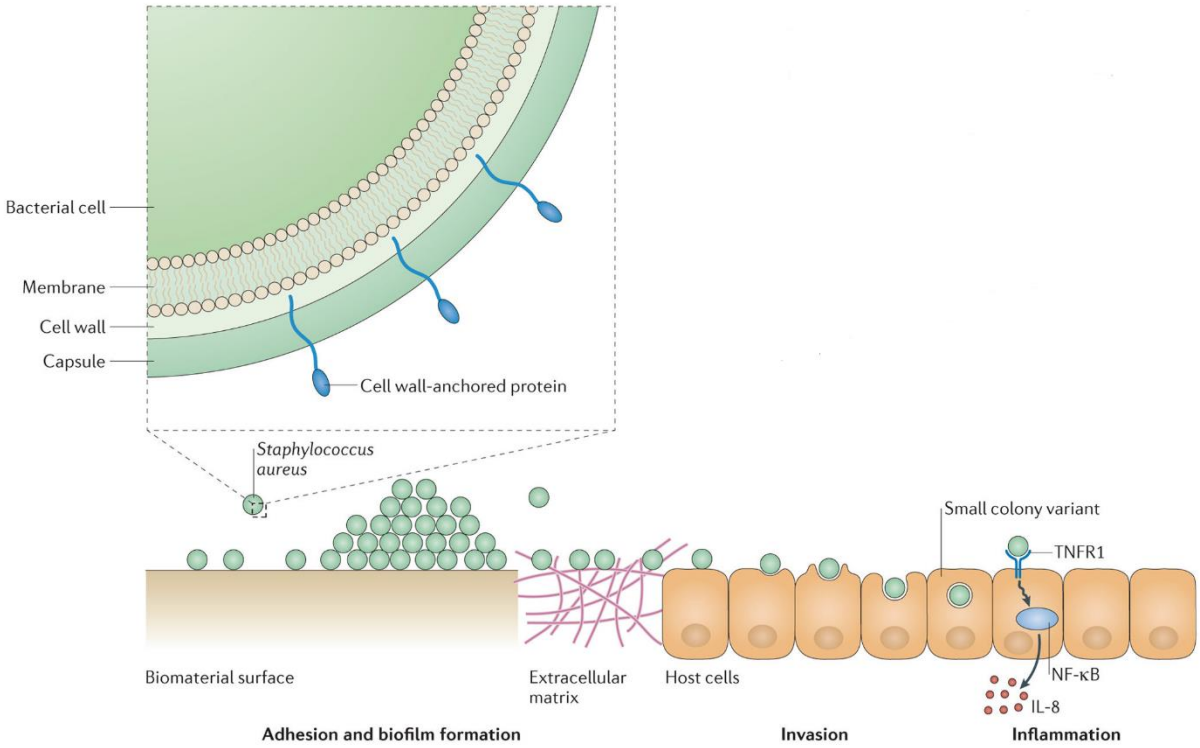

Figure 6. Pathogenic mechanisms that offer some advantages to the spread and survival of *S. aureus*. *S. aureus* cell wall-anchored (CWA) proteins are associated with interaction and recognition by host cells, which enable the adhesion to the extracellular matrix and consequent biofilm formation. When recognized by host receptors, *S. aureus* may stimulate the production of cytokines and pro-inflammatory chemokines. *S. aureus* can be internalized by the host cells, causing cell apoptosis, or can remain dormant in a state known as small colony variants (adapted from Foster et al., 2014).

3.2.4.1.3. Small-colony variants (SCVs)

SCVs are a subpopulation of *S. aureus* with a phenotype characterized by small size colonies, slow growth rate and particular biochemical and morphological properties. *S. aureus* persistent and relapsing infections are mainly associated with this phenotype. It enables to "hide" inside host cells without causing significant damage and confers resistance against host immune responses and antimicrobial therapy (Gordon and Lowy, 2008; Sendi and Proctor,

2009). When back in favorable conditions, *S. aureus* can revert to the virulent wild-type phenotype, which possibly results in recurrent infections (Proctor et al., 2006). Beside chronic mastitis, *S. aureus* SCVs formation was also associated with intracellular persistence in rhinosinusitis and osteomyelitis (von Eiff et al., 1997; Tan et al., 2014).

3.2.4.1.4. Biofilms

When bacteria adhere to a surface, they grow and form complex communities referred to as biofilms (Figure 6). Bacterial biofilms consist of packed bacteria within extracellular polymeric substances (EPS) containing polysaccharides, extracellular DNA, and proteins that are difficult to penetrate and disrupt (Manning and Kuehn, 2013). This organization confers selective advantages for the bacteria under environmental conditions (*e.g.* resistance to antimicrobial agents and to desiccation) (Costerton et al., 1999). Interestingly, most of the mastitis-associated strains have the capacity to organize and form biofilm, which can suggest a correlation with the high recurrence of this infection in the udder (Szweda et al., 2012). In the nosocomial environment, biofilm-associated *S. aureus* induce infections, such as intravascular catheter-related sepsis and infective endocarditis, that lead to high morbidity (Archer et al., 2011; Silva-Santana et al., 2016).

3.2.4.1.5. Antimicrobial resistance

S. aureus is the best opportunistic pathogen to exemplify the adaptive evolution of bacteria in the antibiotic era (Table 2). Its broad resistance to practically all of the early antibiotic classes is mediated almost exclusively by determinants acquired via horizontal DNA transfer. The scarcity of effective treatment against *S. aureus* led to high mortality in hospitals until the introduction of penicillin in the 1940s (Chain et al., 1940). The antimicrobial agent penicillin binds to staphylococcal penicillin-binding proteins (PBP), resulting in the inactivation of an essential transpeptidase which inhibits bacterial cell wall synthesis. *S. aureus* resistance is due to the production of an enzyme called beta-lactamase that hydrolyzes the amide bond of the beta-lactam ring, resulting in functional loss of the antibiotic. The production of this enzyme is encoded by the structural gene blaZ (Pantosti et al., 2007). However, as early as 1942, the first case of penicillin-resistant staphylococci was described (Table 2) (Rammelkamp and Maxon, 1942). Methicillin (semisynthetic β-lactam) was then introduced in 1961 and it was rapidly followed by cases of methicillin-resistant isolates (MRSA) (Jevons, 1961). Methicillin resistance is due to the expression of an

additional penicillin-binding protein (PBP2a), with reduced affinity for beta-lactams. This protein is a product of the *mec*A or *mec*C genes carried in a staphylococcal chromosomal cassette mec (SCC*mec*) element (Kaya et al., 2018; Pantosti et al., 2007). Genetic rearrangements of SCC*mec* element can result in variant elements, indicated by Roman numerals SCC*mec* types (I to XIII) (Baig et al., 2018). Initially described in a British hospital, Hospital-acquired (HA-MRSA) isolates quickly reached a global scale (Lowy, 2003) and subsequently being recognized in the communities (CA-MRSA) (Chambers, 2001). The emergence of CA-MRSA infections in healthy individuals suggested an increase in the *S. aureus* pathogenicity (Liu, 2009). In addition, CA-MRSA carries smaller SCC*mec* types and spread more easily when compared to HA-MRSA (Aires-de-Sousa, 2017).

Vancomycin was then introduced in the treatment of infections caused by methicillin-resistant bacteria (Levine, 2006). In 1997, the first report of vancomycin intermediate-resistant *S. aureus* (VISA) came from Japan (Hiramatsu et al., 1997), with reduced susceptibility as result from changes in bacterial peptidoglycan biosynthesis. However, full resistance to this antibiotic (VRSA) was further reported from strains that acquired by conjugation the *van*A operon from vancomycin-resistant *Enterococcus faecalis* (Ahmad, 2018; Lowy, 2003).

3.2.5. *S. aureus* isolates from mastitis

Comparative analysis between genomes of different *S. aureus* strains allowed to observe host-specific genotypes that emerged after a host jump between human and animal hosts (Guinane et al., 2010). This passage then led to an adaptive evolution with a genomic heterogeneity between strains. However, *S. aureus* populations are clonal and isolates belonging to the same lineage are strongly conserved, even when separated by time and space (Lindsay, 2014).

Typing technique using molecular biology, like Multi Locus Sequence Typing (MLST), enables determining the sequence types (ST) of each strain, that are grouped into clonal complexes by their similarity to a central genotype (Smyth et al., 2009). The majority of ruminants isolates from mastitis are represented by few clonal complexes, including CC97, CC705 (ST151), CC130, and CC126 in cows and CC133 in small ruminants (sheep and goat) (Figure 7) (Fitzgerald, 2012; Guinane et al., 2010). Furthermore, isolates of the CC133 can also be occasionally associated with IMI in cows (Smyth et al., 2009); however, some subtypes, as ST151, were exclusively associated with bovine mastitis and have not been

detected among humans (Fitzgerald, 2012; Guinane et al., 2010; Sakwinska et al., 2011), suggesting some kind of specialization in terms of host. For instance, bovine-adapted genotype belonging to CC8 were reported in Switzerland and was described as the result of a host shift from humans to cows with loss of genes necessary for human carriage (Sakwinska et al., 2011).

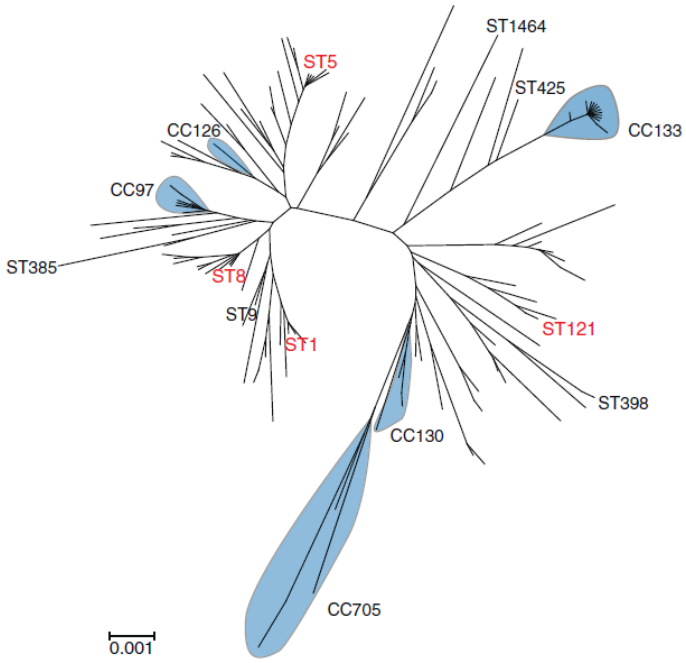

Figure 7. Neighbour-joining tree for *S. aureus*. The majority of ruminant *S. aureus* isolates belongs to the clonal complexes CC97, CC705, CC130, and CC126. CC133 predominates in small ruminants (blue branches) (Source: Guinane et al., 2010; Fitzgerald et al., 2012).

The release of the first bovine *S. aureus* genome (strain RF122) provided evidence of livestock *S. aureus* diversification in relation to human strains through a combination of the acquisition of mobile genetic elements (MGEs), gene diversification and decay (Budd et al., 2015; Guinane et al., 2010; Herron et al., 2002; Herron-Olson et al., 2007). Similar specialization was observed in small ruminants CC133 clones (Guinane et al., 2010). MGEs correspond to 15-20% of *S. aureus* genome and include bacteriophage, pathogenicity islands (SaPI), plasmids, transposons and staphylococcal chromosomal cassette (SCC) (Lindsay, 2010, 2014). However, chromosomal rearrangements were also reported in *S. aureus* isolates, which can contribute to host specialization and bovine *S. aureus* clonal diversification (Budd et al., 2015; Everitt et al., 2014). This specialization is best exemplified by the transmission of SSCmec between humans and animals. The first case of bovine mastitis associated with MRSA was reported in Belgium (Devriese et al., 1972) possibly due to the bacteria human-to-

bovine transmission intensified after domestication and globalization of the livestock industry (Aires-de-Sousa, 2017). Currently, some MRSA strains belonging to CC130, ST425, and CC1943 were described as bovine-specific (Aires-de-Sousa, 2017). Recently, isolates carrying mecC (type XI SCC*mec*), a novel mecA homolog, were firstly identified in cattle strains. Albeit they were also identified in human isolates, they currently appear to be uncommon. Beside this host specialization, some *S. aureus* clones show an interchangeable host profile as illustrated by the increase report of livestock-associated MRSA, as CC398, emerging in swine and widely transmitted to humans (Aires-de-Sousa, 2017; García-Álvarez et al., 2011; Paterson et al., 2014). Livestock-associated MRSA *S. aureus* CC398 emerged from human methicillin-susceptible *S. aureus* (MSSA) isolates that jumped to livestock and acquired methicillin and tetracycline resistance (Price et al., 2012). The major bovine complex CC97 also jumped from livestock to humans followed to host adaptation with an incorporation of methicillin resistance by selective pressure, which resulted in human-epidemic CA-MRSA spread (Spoor et al., 2013).

3.2.5.1. Virulence factors associated with ruminant mastitis

Bacterial mastitis presents different symptomatology in the host that correspond in general to the *S. aureus* strains that infect and the specific factors that they produce. When compared to human isolates, bovine strains have lost some genes encoding proteins necessary to induce a human disease, suggesting that such proteins are not required in cow MG infections (Herron-Olson et al., 2007). On the other hand, bovine strains have acquired specific phenotypic traits to adapt to their hosts, as the ability to coagulate plasma from ruminant sources (Devriese, 1984; Peton et al., 2014).

Most of bovine *S. aureus* isolates produce α and β-hemolysin and some leukocidin toxin (Haveri et al., 2007; Rainard, 2007). Human *S. aureus* isolates can produce up to five different leukotoxins, including two types of hemolysin (HlgAB and HlgCB), LukAB, LukED, and Panton-Valentine leukocidin (PVL). So far, the MGE encoding LukMF' has only been found in *S. aureus* isolates of non-human origin, being predominantly described in strains isolated from bovine mastitis (Barrio et al., 2006; Vrieling et al., 2015). All bicomponent leukocidins kill neutrophils *in vitro*; however, the cytotoxic potencies toward neutrophils can differ. LukMF' can specifically kill bovine neutrophils through recognition of chemokine receptor CCR1 expressed on its cell surface and absent in human neutrophils (Vrieling et al., 2015). This allowed confirming the host specificity that leads to distinct

evolution between human and livestock populations of *S. aureus* (Monecke et al., 2007). Other ruminant specific variants of toxins or virulence factors were also described (*e.g.* bovine variant of von Willebrand binding protein (bWbp) (Peton et al., 2014) and exfoliative toxin E (ETE) (cf. Annexes 3).

Pathogen adherence to the udder cells corresponds to the first step of MG infection and factors associated with this process are also described as abundant in bovine isolates (Budd et al., 2015). Comparing the genome of strains isolated from the different host, such as sheep, human, and bovine, the genes encoding for cell wall-associated (CWA) proteins revealed considerable variations. Considering the critical role of these proteins in bacteria-host interaction, such variation may result from the selective pressure to adapt to different host receptors (Guinane et al., 2010). *In vitro* studies showed that mutants for FnBPs reduced the adhesion by 40% in mammary epithelial cells, suggesting a significant role for the establishment of mastitis (Dziewanowska et al., 1999). Moreover, genes involved in biofilm formation, as *ica* (intercellular adhesion) and *bap* (biofilm-associated protein), also seems to be relevant factors in the pathogenesis of mastitis (Gomes et al., 2016).

3.3. Extracellular vesicles

3.3.1. Overview of extracellular vesicles

Extracellular vesicles (EVs) are nano-sized particles with a lipid bilayer that are produced during cell grown or are released in response to different conditions from budding of the cellular membranes. The production of EVs is a universal cellular process that involves organisms with relatively simple architecture, such as microorganisms, as well as more complex organisms (Lee et al., 2008, 2009). Cells are able to release into the extracellular medium different types of vesicles that differ in some particularities, including their origin and size (Whiteside, 2017). EVs have been isolated and characterized in all know pathogen classes, including viruses, bacteria, fungi, and parasites (Schorey et al., 2015). An overview of the eukaryotic and prokaryotic EVs will be presented in this section. The main differences between them are briefly summarized in table 1.

3.3.1.1. Eukaryotic EVs

In eukaryotic cells, the secreted vesicles may have an endosomal origin, being formed intracellularly by the multivesicular endosome (MVE) and then called exosomes (30-100 nm). When formed from the direct budding of the cellular plasma membrane, these vesicles are then called to microvesicles or membrane vesicles (MVs) and can range 100-1000 nm (György et al., 2011; Lee et al., 2008; Raposo and Stoorvogel, 2013). Their formation occurs at certain sites of the cell where a dilation of the membrane progresses and separates from the plasma by constriction, which enables a release of the membranous structure into the medium (Chatterjee and Chaudhuri, 2012). These two vesicles subtypes together with the apoptotic bodies (1000-5000 nm) form the most important group of EVs in eukaryotic cells (Figure 8) and their common feature is the lipid bilayer membrane that surrounds their complex cargo (Kalra et al., 2016; Konoshenko et al., 2018).

When considering eukaryotic cells, it is crucial to discriminate different EVs subpopulations. In the face of conflicting definitions, the International Society for Extracellular Vesicles (ISEV), founded in 2012, has introduced the generic term extracellular vesicles to represent heterogeneous populations of vesicles (Gould and Raposo, 2013). However, it has been shown that this eukaryotic nomenclature has some limitations, which may hide a vast subdivision of EVs subpopulations (Lässer et al., 2018).

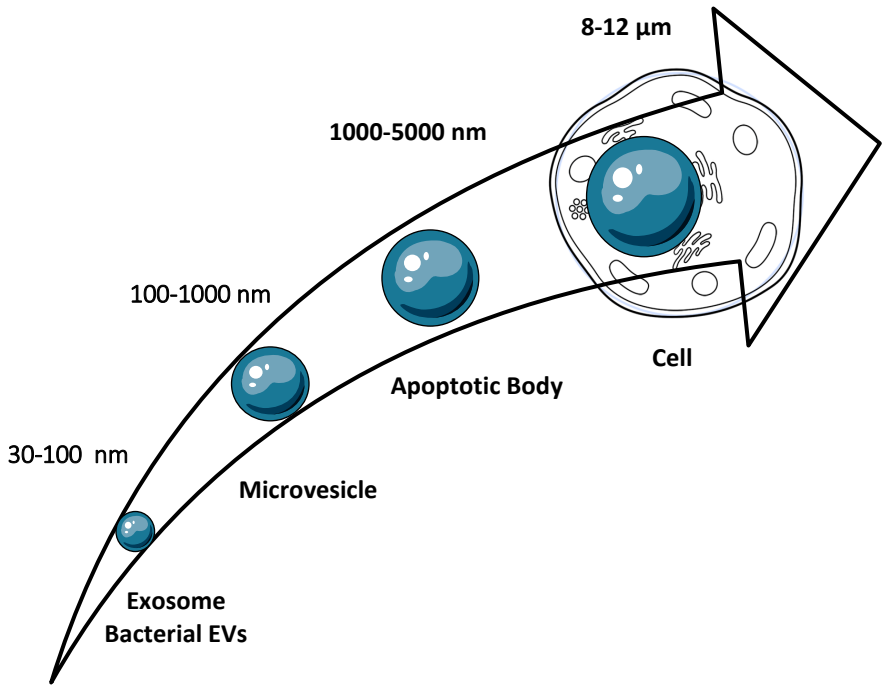

Figure 8. Representation of size range of different EVs subtypes (adapted from György et al., 2011).

The first evidence of EVs was proposed in 1964 by findings of procoagulant platelet-derived particles that were later called "platelet dust"(Wolf, 1967), although its effects had already been observed back to 1940 (Chargaff and West, 1946). Afterward, other surface-associated vesicles were identified in different biological samples. Only in 1983 the mechanism of secretion mediated by fusion of multivesicular bodies (MVBs) with the eukaryotic cell membrane was proposed. Although the release of exosomes has been described in eukaryotic cells since then (Harding et al., 1983; Pan and Johnstone, 1983), the intensification of studies occurred after the discovery that these small particles are capable of transporting small RNAs, including microRNAs (Valadi et al., 2007). This particularity classifies EVs as an important mechanism of intercellular signaling (Nazimek et al., 2015). EVs are recovered from all body fluids, suggesting certain stability in the transmission of information over long distances (Whiteside, 2017). Besides, EVs preserve some characteristics of parent cells and are more accessible in body fluids (*e.g.* blood), which attracted attention to their use as biomarkers of diseases (Duijvesz et al., 2011; Webber and Clayton, 2013). Eukaryotic EVs can also be used as mediators for drug delivery vehicles (Johnsen et al., 2014) and therapeutic applications (Ha et al., 2016). Because of these

proprieties and due to their ubiquity, the characterization of EVs biological roles has become a fascinating research field.

Table 5. Characteristic of different eukaryotic and prokaryotic EVs subtypes

	Eukaryotic cells			**Prokaryotic cells**	
	Exosomes	**Microvesicles**	**Apoptotic Bodies**	**OMVs (Gram-negative EVs)**	**EVs (Gram-positive EVs)**
Origin	Endocytic pathway	Plasma membrane	Plasma membrane	Outer membrane	Cytoplasmic membrane
Size	30 - 100 nm	100 – 1000 nm	1000 – 5000 nm	10 – 300 nm	20 – 150 nm
Function	Intercellular communication	Intercellular communication	Facilitate phagocytosis	Inter-kingdom communication	Inter-kingdom communication
Markers	Tetraspanins (CD81, CD63and CD9), Flotillin, Alix, Tsg101 Heat shock proteins	Integrins, selectins, CD40, CD63, CD81 and CD9	Histones TSP, C3b, phosphatidylserine	Variable*	Variable*
Homogeneity	Relatively uniform	Heterogeneous	Heterogeneous	Relatively uniform	Relatively uniform
Contents	Proteins Nucleic acids (mRNA, miRNA and other non-coding RNAs)	Proteins Nucleic acids (mRNA, miRNA and other non-coding RNAs)	Nuclear fractions Cell organelles	Proteins Nucleic acids (DNA, RNA) Other small molecules	Proteins Nucleic acids (DNA, RNA)

*There is no consensus regarding the markers of bacterial EVs

3.3.1.2. Bacterial EVs

The first evidences of EVs production in Gram-negative bacteria were reported in the years of 1960s (Chatterjee and Das, 1967; Knox et al., 1966; Work et al., 1966). Considering the outer membrane (OM) origin, EVs are referred to as outer membrane vesicles (OMVs) (Toyofuku et al., 2015). OMVs have a diameter ranging from 10 to 300 nm (MacDonald and Kuehn, 2012) and are constituted by lipopolysaccharides, phospholipids, periplasmic, cytosolic and outer membrane proteins, hydrophobic molecules, virulence-associated factors and recently RNA (Celluzzi and Masotti, 2016; Horstman and Kuehn, 2000; Kato et al., 2002; Keenan et al., 2000a; Mashburn and Whiteley, 2005; Wai et al., 1995, 2003; Work et al., 1966). During OMVs formation, a portion of the bacterial periplasm is taken along with other bacterial components (Schwechheimer and Kuehn, 2015). Beside the protein content, OMVs carry DNA on the surface and luminal portion, either chromosomal DNA (Dorward and Garon, 1989; Kolling and Matthews, 1999; Yaron et al., 2000), plasmid DNA (Dorward and Garon, 1989; Yaron et al., 2000) or from viral origin (Yaron et al., 2000) (Figure 9).

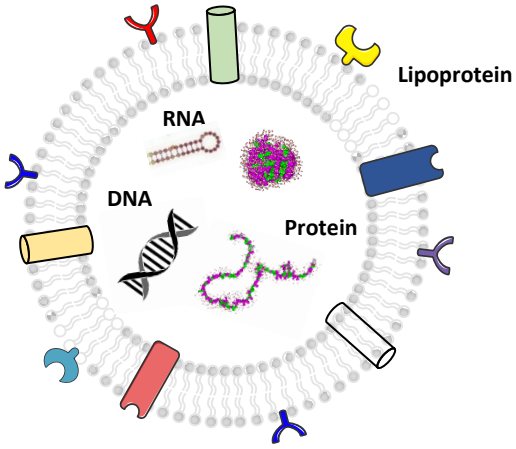

Figure 9. Extracellular vesicles (EVs) formed by a lipid bilayer and an intraluminal content (proteins, DNA, and RNA).

In Gram-positive bacteria, EVs production was firstly proposed by Dorward and Garon but with inconclusive results and EVs were not associated with a function (Dorward and Garon, 1990). Then, Lee et al. (2009) described a similar secretion of membrane vesicles by *S. aureus* despite the thick wall of peptidoglycan of this microorganism (Lee et al., 2009). It shares similarities, in density and size, with OMVs previously described in Gram-negative bacteria (Lee et al., 2009). There is no consensus regarding the nomenclature adopted for Gram-positive EVs, although much of the recent works use membrane vesicles (MVs) to

describe EVs in this class of microorganisms (Al-Nedawi et al., 2015; Jeon et al., 2016; Lee et al., 2009). In this chapter, we will use EVs as a broad term to describe these nanoparticles isolated from bacteria.

First, Gram-positive EVs have been described with a diameter that ranges from 20 to 150 nm (Gurung et al., 2011; MacDonald and Kuehn, 2012); however, size may vary in a wider range with reported diameter below 400 nm (Jiang et al., 2014). It was further shown that EVs are spherical particles surrounded by a phospholipid bilayer and secreted by pathogenic or non-pathogenic bacteria (MacDonald and Kuehn, 2012). Most of the studies in Gram-positive EVs were focused on pathogenic microorganisms, such as *S. aureus* (Gurung et al., 2011; Hong et al., 2011; Jeon et al., 2016; Kim et al., 2012; Lee et al., 2009, 2013a; Thay et al., 2013). Moreover, the production of EVs has been described in *Bacillus sp.* (Brown et al., 2014; Kim et al., 2016b; Rivera et al., 2010; Tashiro et al., 2010; Wolf et al., 2012), *Mycobacterium sp.* (Lee et al., 2015; Prados-Rosales et al., 2011, 2014a, 2014b; Rath et al., 2013; Rodriguez and Prados-Rosales, 2016; Ziegenbalg et al., 2013), *Streptococcus sp.* (Biagini et al., 2015; Haas and Grenier, 2015; Liao et al., 2014; Olaya-Abril et al., 2014; Surve et al., 2016), *Listeria monocytogenes* (Lee et al., 2013b, 2018; Vdovikova et al., 2017), *Lactobacillus sp* (Al-Nedawi et al., 2015; Li et al., 2017), *Clostridium perfringens* (Jiang et al., 2014), *Streptomyces coelicolor* (Schrempf et al., 2011) and *Bifidobacterium longum* (Kim et al., 2016a). Section 3.3.4 provides more detailed information about Gram-positive EVs.

3.3.2. Biological roles of bacterial EVs

Overall, the release of bacterial EVs was associated with inter-species (Yaron et al., 2000) and inter-kingdom communication (Yaron et al., 2000), detoxification and elimination of misfolded proteins (Kobayashi et al., 2000), threat avoidance (Manning and Kuehn, 2011), quorum sensing (Mashburn and Whiteley, 2005), elimination of competitive organisms (Li et al., 1998), biofilm formation (Im et al., 2017), transfer of genetic materials (Dorward and Garon, 1990) and favoring the acquisition of nutrients (Ellis and Kuehn, 2010; Lee et al., 2009; Park et al., 2010; Toyofuku et al., 2015). In this section, some of the bacterial biological roles of EVs will be briefly discussed.

3.3.2.1. Bacteria-host interactions

Bacterial EVs provide an interesting mechanism of cellular communication with local and long-distance activity (Jan, 2017). This represents an important advantage considering

that secreted virulence factors by pathogenic bacteria favors a great invasiveness and spread in the infectious process (Hecker et al., 2010). EVs have been then considered as an important secretory system. On one hand, the proteins that concentrate in EVs lumen include virulence factors, which enable a focused delivery to the target cells (Chantterjee and Chaudhuri, 2012). Further, factors carried by EVs can stimulate host cells and modulate their response, which constitutes an important bacterial adaptation to their environment (Jan, 2017). In fact, EVs lumen forms a protective and safe environment for biologically active components and corresponds to a relevant alternative to transport highly degradable molecules, such as nucleic acids (DNA and RNA). On the other hand, the lipid bilayer further facilitates the delivery and fusion with the target cell membrane through various components of the bacterial cell surface, such as bacterial outer membrane proteins (OMPs), lipoproteins, lipopolysaccharides (LPS) and peptidoglycan (PG) (Jan, 2017; Szatanek et al., 2017; Valadi et al., 2007). Through these surface components, EVs can stimulate and activate the host cells via a variety of pattern recognition receptors (PRR) like Toll-like receptors (TLRs) and Nod-like receptors (NLRs). TLRs play an important role in the innate immune response by activating signaling pathways under the control of the transcription factor Nuclear Factor-kappaB (NF-kB) and Mitogen-Activated Protein Kinase (MAPK), which trigger the induction of inflammatory responses (Ahmadi Badi et al., 2017; Kaparakis-Liaskos and Ferrero, 2015). However, recent works have proposed that this modulation can occur through much more complex mechanisms, such as delivery of bacterial non-coding RNAs (ncRNAs). Interestingly, intravesicular EVs ncRNAs were able to align in regions of genetic regulation on the human genome, which reinforced the theory about this complex network of inter-kingdom communication (Celluzzi and Masotti, 2016).

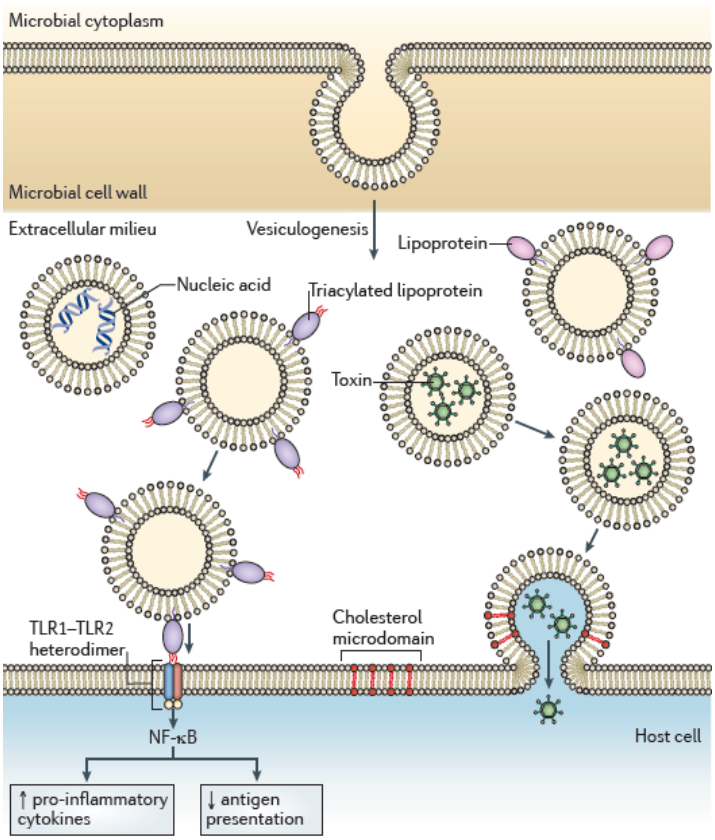

Figure 10. Schematic representation of the different cargos and function of the extracellular vesicles (EVs). Bacteria release EVs as a mechanism of transport of virulence factors that can interact with membrane receptors or of fusion with the host cells to modulate their response. EVs ligands can be recognized by Toll-like receptors (TLRs) activating signaling pathways that lead to cytokine production. However, toxin positive EVs can fusion with the host cell membrane and delivery directly their contents (adapted from Brown et al., 2015).

3.3.2.2. Elimination of harmful material

Given that most studies reported EVs enrichment with cytoplasmic and periplasmic proteins, it is reasonable to assume that one of physiological roles of EVs is to confer protection from the internal stress by eliminating unnecessary compounds. The periplasmic space of Gram-negative bacteria can become filled with misfolded envelope proteins under stressful conditions (McBroom and Kuehn, 2007), and the secretion via EVs may help the bacteria to expel this useless and harmful wastes and to avoid their intracellular accumulation. For instance, misfolded OMPs in *Pseudomonas aeruginosa* (McBroom and Kuehn, 2007; Tashiro et al., 2009) and toluene in the toluene-tolerant *Pseudomonas putida* IH-2000 (Kobayashi et al., 2000) were exported via EVs. Another evidence that corroborates the EVs role in stress response is that the absence of DegP and MucD, the major periplasmic chaperone/protease protein in *E. coli* and *P. aeruginosa*, respectively, increases the production of EVs (McBroom and Kuehn, 2007; Tashiro et al., 2009).

3.3.2.3. Biofilm formation

EVs are a common biofilm constituent (Schooling and Beveridge, 2006) and vesicles-associated biofilm contains DNA bound to their surface or packaged in their lumen (Manning and Kuehn, 2013; Schooling et al., 2009). Bacteria cells that release EVs become more hydrophobic, which favors their ability to form biofilms. This suggests a role of EVs in this type of cell organization well-known to provide resistance to harsh environment (Baumgarten et al., 2012). In the biofilm community, EVs might be associated with nutrient delivery or might constitute an adherent substrate that forms the extracellular matrix (ECM) (Manning and Kuehn, 2013). Baumgarten et al. (2012) carried out a study using different stressful conditions in *P. putida* DOT-T1E, as toxic concentrations of long-chain alcohols, NaCl, EDTA, and heat shock. These conditions led to an increase of EVs release, which consequently raised the cell surface hydrophobicity and favored the formation of biofilm (Baumgarten et al., 2012). When compared, biofilm and planktonic EVs, show differences in their content with no virulence factors in biofilm EVs (Banin et al., 2005; Schooling and Beveridge, 2006). This suggests that environmental conditions determine what type of proteins will be packaged and exported from the cell via the EVs (Schooling and Beveridge, 2006).

3.3.2.4. Nucleic acid and horizontal gene transfer

Packaging of nucleic acid and transfer of genes by EVs within or between species was demonstrated in various genera of Gram-negative bacteria (Fulsundar et al., 2014; Pérez-Cruz et al., 2013; Renelli et al., 2004; Yaron et al., 2000), and certain Gram-positive bacteria, as *Streptococcus pyogenes* (Surve et al., 2016) and *C. perfringens* (Jiang et al., 2014). The package seems to be a selective mechanism as only some genes were retrieved in EVs (Surve et al., 2016). Nucleic acids, including chromosomal DNA, plasmids, phage DNA, rRNA, tRNA, mRNA and intragenic RNA species are frequently found as cargo in EVs (Liu et al., 2018). Besides, co-incubation of OMVs containing DNA from *E. coli* O157:H7 with various hosts result in the transfer of genetic material to recipient bacteria (Yaron et al., 2000).

Streptococcus mutans can deliver extracellular DNA (eDNA) with higher concentration recovered from EVs in the early-exponential-phase cultures (2.8-fold) than those prepared from the stationary cultures (Liao et al., 2014). *C. perfringens* EVs also contained DNA components such as 16S ribosomal RNA gene (16S rRNA), the alpha-toxin gene (*plc*) and the perfringolysin O gene (*pfoA*) (Jiang et al., 2014). DNA at a concentration

of ~33 ± 5 ng per μg of EVs protein was recovered from *Streptococcus agalactiae* strain A909 EVs (Surve et al., 2016). In this work, EVs DNA was used as the template to amplify *cfb* gene (CAMP factor), a significant virulence factor of this bacterium, and the presence of intravesicular RNA was confirmed using RNA-seq and qPCR analysis (Surve et al., 2016). Interestingly, *S. pyogenes* EVs were differentially enriched with intragenic RNAs, in contrast to intergenic or tRNA species enriched in Gram-negative EVs (Ghosal et al., 2015; Resch et al., 2016). This selection of RNAs suggests a bacterial mechanism of intercellular signaling analogous to that mediated by eukaryotic exosomes (Valadi et al., 2007).

3.3.2.5. Interactions within bacterial communities

The first evidence of the EVs acting in the bacteria-bacteria interactions was based on the ability of *S. aureus* to share antibiotic resistance proteins as penicillin-binding protein (PBPs) via EVs. For instance, the clinical *S. aureus* ATCC14458 strain was described to carry biologically active BlaZ that enable the survival of other ampicillin-susceptible bacteria in the presence of this antibiotic (Lee et al., 2013a). Another noteworthy factor is that *S. aureus* EVs are able to inhibit adherence and formation of biofilm by other bacteria (Im et al., 2017). Similar microbial interactions via EVs were also observed in Gram-negative bacteria. For instance, the Gram-negative *Lysobacter* sp. XL1 secrete EVs containing endopeptidase L5 with bacteriolytic activity against Gram-positive and Gram-negative bacteria (Vasilyeva et al., 2008). *Mycobacterium tuberculosis* EVs containing mycobactin (siderophore) produced during iron limitation can deliver iron and support proliferation of iron-deficient bacteria (Prados-Rosales et al., 2014a). Gram-positive EVs mediating an increase in survival of bacteria under nutrient limiting conditions was also recovered from *Streptomyces*-derived EVs (Liu et al., 2018; Schrempf et al., 2011).

Another relevant fact is that cell communication in bacteria occurs through production and detection of small diffusible signaling molecules, which induce some shift of gene expression in the population. For example, 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas quinoline signal) (PQS), a quorum-sensing signal of *P. aeruginosa*, is packaged within EVs, which diffuse into the external environment (Tashiro et al., 2010).

3.3.2.6. Threat avoidance

EVs can be an innate bacterial defense against external stressors, which favor bacterial survival in the environment. It can act as an important factor in neutralizing environmental

agents that target the outer membrane of Gram-negative bacteria, such as antimicrobial peptides (AMP) or bacteriophages (Manning and Kuehn, 2011). Under antibiotic pressure, this increase of survival might occur due to their adsorption by LPS in the Gram-negative EVs membrane (Manning and Kuehn, 2011).

3.3.2.7. Adaptation to environmental conditions or variations

Several environmental factors influence the production of EVs, which suggests their role as a mechanism of bacterial adaptation. *S. aureus* EVs production is dependent on the bacterial growth phase with an increase in the number of EVs when the culture enters the stationary phase (Im et al., 2017), as also reported in Gram-negative bacteria (Hagemann et al., 2014). EVs production is also responsive to factors such as temperature, oxygen stress, nutrient availability, quorum sensing, and envelope-targeting antibiotics (Schwechheimer and Kuehn, 2015). For instance, an overproduction of EVs was observed in *M. tuberculosis* culture under iron deficiency, i.e. condition that mimic the infection context (Prados-Rosales et al., 2014a). Furthermore, conditions weakening the bacterial cell wall of *S. pyogenes*, such as the addition of sublethal concentrations of penicillin allowed the recovery of 20-fold higher EVs amount (Biagini et al., 2015). Genotoxic stress in the presence of DNA-damage agent mitomycin C (MMC) also induced EVs formation in *B. subtilis* (Toyofuku et al., 2017).

The food-borne pathogen *L. monocytogenes* is a Gram-positive bacterium that is also able to release EVs during *in vitro* culture. Conditions that reflect the gastrointestinal environment, such as 0.5M salt, altered the morphology, amount of production, and protein content of *L. monocytogenes* EVs (Lee et al., 2013b, 2018).

3.3.3. Biogenesis of bacterial EVs

A significant challenge in this field consists in understanding the mechanisms and environmental stimuli involved in EVs production and content selection. Furthermore, the conditions that govern the formation and release of EVs, especially in Gram-positive bacteria are only just beginning to be characterized, and no clear understanding of these processes has emerged. One of the most significant differences between Gram-negative and Gram-positive bacteria lies in the envelope structure and composition (Figure 11). The lack of an OM in Gram-positive bacteria and the physical barriers established by the thick peptidoglycan layer makes the release of EVs by these microorganisms more complicated (Brown et al., 2015).

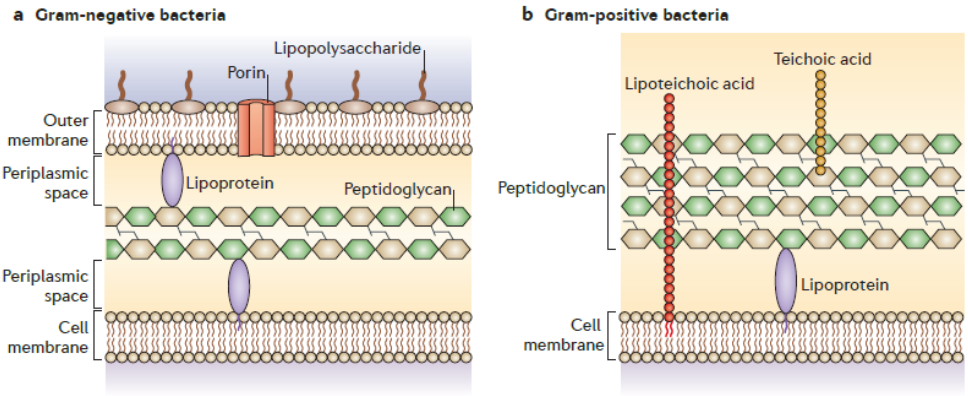

Figure 11. Differences between cell wall structure of Gram-negative and Gram-positive bacteria. a) In Gram-negative cell wall, the periplasmic space between the inner and outer lipid membrane. b) The thick cell wall in Gram-positive bacteria and lack of the outer membrane (adapted from Brown et al., 2015).

Bacterial membrane remodeling is a mechanism triggered by environmental changes that result in altered membrane composition. It was shown to be involved in microorganism survival and replication in the host (Dalebroux et al., 2015). Several models have been hypothesized to explain the vesiculogenesis process in prokaryotes. The first one concerning Gram-negative EVs generation considers that the budding and detachment results from the faster expands of the outer membrane in relation to the underlying peptidoglycan layer (Bernadac et al., 1998). In this case, the bud off constitutes a mechanism to remove envelope component that is undesirable for the cell (Schwechheimer and Kuehn, 2015). In Gram-positive, EVs may also be forced through the wall by turgor pressure after release from the plasma membrane (Figure 12) (Brown et al., 2015).

Another model considers the actions of murein hydrolases to degrade the peptidoglycan, which increases the turgor pressure in the Gram-negative periplasmic space (Lommatzsch et al., 1997). This was evidenced by the production of EVs in *Neisseria meningitides*, which depends on the peptidoglycan (PG) architecture and the low local levels of the lytic transglycosylases MltA, MltB and Slt (Lappann et al., 2013). To date, the challenge is to discover how the budding of the membrane can overcome the thick PG layer in Gram-positive bacteria. For that, the same model was proposed to explain the cellular leakage in the Gram-positive wall by the action of hydrolases (Figure 12) (Brown et al., 2015). Indeed, different murein hydrolases were identified in the intravesicular content of *S. aureus* EVs (Toyofuku et al., 2017). Recently, a mutant of the autolysin N-acetylmuramoyl-l-alanine amidase (Δ*sle1*) showed a reduction in EVs production when compared to its wild-type *S. aureus* (Wang et al., 2018). This hypothesis can also be supported by previous studies that

have demonstrated that enzymatic activities of the holin-endolysin system encoded by the prophage PBSX weaken the PG in *B. subtilis* cells, which protrude cytoplasmic membrane material through holes in the PG and these membrane blebs are then released as EVs (Toyofuku et al., 2015). Furthermore, even endolysin released from dead cells increase EVs formation in the neighboring cells (Toyofuku et al., 2015) and a putative phage-associated endolysin was specifically enriched in *Streptococcus pneumoniae* EVs (Resch et al., 2016). Indeed, vesicularization of shattered membrane fragments after explosive cell lysis induced by a cryptic prophage endolysin was also described as a mechanism for the production of bacterial EVs (Turnbull et al., 2016). In *S. aureus*, the release and size of EVs were also reduced when purified from a Δ*psmα* mutant (cf. *S. aureus* "Virulence factors" in previous section 3.2.3.3, pg. 51), which evidenced the role of these toxins by membrane-damaging activity (Ebner et al., 2017; Wang et al., 2018).

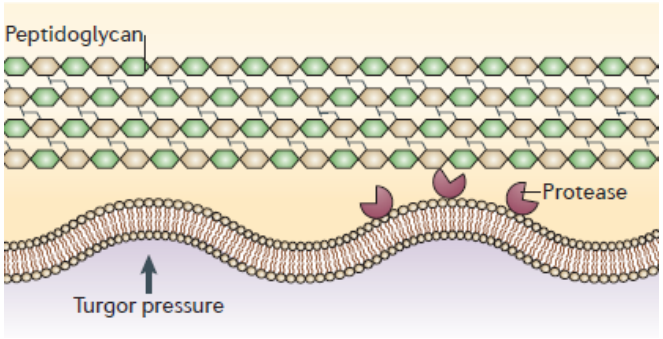

Figure 12. Hypotheses for extracellular vesicle formation. Turgor pressure caused by the release from plasma membrane that hence forces the cell wall. The action of wall-modifying enzymes as murein hydrolases in degrading the peptidoglycan wall and release of EVs (adapted from Brown et al., 2015).

Pseudomonas quinolone signal (PQS) of *P. aeruginosa* is a positive regulator of EVs production (Tashiro et al., 2010). Interestingly, it was shown to induce EVs production by Gram-negative (*e.g. Burkholderia cepacia* ATCC 25416) and Gram-positive bacteria (*e.g. B. subtilis* 168) (Tashiro et al., 2010). In Gram-negative bacteria, it was proposed that PQS strongly interacts with the LPS component lipid A and sequester divalent cations such as Mg2+ and Ca2+, a salt bridge that is critical for negative charge-charge repulsions between LPS molecules (Mashburn-Warren et al., 2008). Therefore, PQS favors the anionic charge repulsion between neighboring LPS molecules, which causes membrane blebbing (Kadurugamuwa and Beveridge, 1995; Tashiro et al., 2010). Accumulation of lipid A deacylation was also reported to impose shape modifications that result in the curvature of the outer membrane and subsequent EVs formation (Elhenawy et al., 2016).

Altogether, potential biogenesis mechanisms consist in the surface instability associated with lower levels of crosslinks between the membrane and PG. The action of hydrolases may also facilitate the release of some membranous regions. Therefore, it should be taken into account that there are different pathways that allow the EVs formation. Although there exist the hypothesis of EVs produced after cell death, it was shown that EVs were not recovered from a suspension of dead *B. anthracis* (Rivera et al., 2010) or dead *S. pneumoniae* (Olaya-Abril et al., 2014). Furthermore, cell viability was required to observe EVs production in *B. subtilis* (Brown et al., 2014) and in *S. mutans* cultures (Liao et al., 2014). Furthermore, the significant difference in ζ potential for EVs release by toxin-producing and deficient *B. anthracis* strains vehemently argued against a random assembly of phospholipids or cell membrane fragments into vesicles (Rivera et al., 2010). This hypothesis was also questioned when the lipid composition of EVs and that of membrane fractions of the producing bacteria where compared. Although an identical lipid pattern is observed in bacterial membrane and EVs from Gram-positive bacteria, the lipid proportion is different, which provides evidence for the existence of an ordered mechanism contributing to EVs biogenesis (Biagini et al., 2015; Olaya-Abril et al., 2014; Resch et al., 2016; Surve et al., 2016).

3.3.3.1. Genetic factors associated to EVs release

Peptidoglycan-associated outer membrane proteins and genes involved in membrane stability or stress response are widely engaged in Gram-negative EVs biogenesis (Wessel et al., 2013). Similarly, Gram-positive EVs production seems also to be affected by some specific genes. The laboratory *B. subtillis* strain 168, unable to produce lipopeptide antibiotic surfactin due to a mutation in *sfp*, was described to produce more recoverable EVs than environmental strain 3610 that harbor a wild-type *sfp* copy. This difference could be explained by the activity of the lipopeptide antibiotic surfactin that destabilizes EVs integrity (Brown et al., 2014). However, the large profile of genes described in Gram-negative EVs biogenesis excludes their simply formation due to membrane instability (McBroom et al., 2006).

The exact mechanism of regulation by which the bacteria bud off the membrane is still undetermined. In this context, genetic studies are underway to characterize relevant pathways that contribute to bacteria EVs production and release (Liu et al., 2018). For instance, *S. aureus* EVs production appears to be under control of the *agr* system, since a *S. aureus* JE2

mutant for AgrA (Δ*agrA*) was not able to produce EVs when compared to the wild-type strain (Im et al., 2017). In *L. monocytogenes*, the release of EVs was regulated by sigma factor σ^B, thereby EVs production of wild-type strain are nine times higher and with an intact shape when compared to those of the isogenic Δ*sigB* mutant (Lee et al., 2013b). This transcription factor also affects the selection of the intravesicular content, as confirmed by the higher levels of the Internalin B (InlB) protein (Table 1) in the wild-type strains-derived EVs (Lee et al., 2013b). In *S. pyogenes*, strains harboring a defective virulence regulator-sensor operon (*covRS*) locus also increased their EVs production (Resch et al., 2016).

3.3.4. EVs in Gram-positive bacteria

3.3.4.1. S. aureus EVs

Among the pathogenic Gram-positive bacteria producing EVs, *S. aureus* is by far the most frequently investigated, given its role in nosocomial and community infections worldwide. Most of the studies were focused on the identification of the protein content of *S. aureus* EVs, on their *in vitro* cytotoxicity and the immune response they trigger in animal models.

S. aureus-derived EVs have a complex protein composition. Cytoplasmic proteins are abundant in the luminal portion (Gurung et al., 2011), with proteins involved in cell architecture, metabolic pathways, chaperones, transport and ribosomal proteins (Lee et al., 2009). The protein pattern packaged in EVs also differs from that identified in the whole bacteria, suggesting a mechanism of proteins sorting during EVs biogenesis (Hong et al., 2011).

From a functional point of view, many of the components packaged in the EVs are virulence factors. Until now, *S. aureus*-derived EVs were indeed described as cargo for pathogenic-associated molecules such as α and γ-hemolysis, cysteine protease, superantigens (SAgs), leukocidins, FnBPs and PSMs, which induce cell perturbations and trigger the initiation of a host immune response (Hong et al., 2011; Jeon et al., 2016; Lee et al., 2009; Thay et al., 2013). A selection of toxins and virulence factors frequently reported in *S. aureus* EVs and other Gram-positive EVs is summarized in table 6.

Table 6. Virulence factors associated with Gram-positive EVs derived from different Gram-positive species.

Virulence factor	Organism	Reference
Staphylococcus		
Immunoglobulin-binding protein (SbI)	*S. aureus*	Lee et al., 2009
		Gurung et al., 2011
α-hemolysin		Thay et al., 2013
		Lee et al., 2009
γ- hemolysin		Lee et al., 2009
		Jeon et al., 2016
Protein A		Gurung et al., 2011
Staphopain A		Lee et al., 2009
Staphylococcal enterotoxin (SEQ)		Lee et al., 2009
SSaA1		Lee et al., 2009
SSaA2		Lee et al., 2009
Penicillin-binding proteins (PBPs)		Lee et al., 2009
		Jeon et al., 2016
Coagulation factors		Lee et al., 2009
✓ Staphylocoagulase		
✓ von Willebrand factor-binding proteins		
Phage protein		Jeon et al., 2016
Phenol-soluble modulins (PSMs)		Jeon et al., 2016
ESAT-6 secretion accessory factor EsaA		Jeon et al., 2016
Other Gram-positive		
Bacillus		
Protective antigen (PA)	*B. anthracis*	Rivera et al., 2010
Lethal factor (LF)		Rivera et al., 2010
Edema factor (EF)		Rivera et al., 2010
Anthrolysin (ALO)		Rivera et al., 2010
Listeria		
Internalin B (InlB)	*L. monocytogenes*	Lee et al., 2013
listeriolysin O (LLO)		Lee et al., 2013
Streptococcus		
Toxin pneumolysin (Ply)	*S. pneumoniae*	Olaya-Abril et al., 2014
Streptolysin O	*S. pyogenes*	Biagini et al., 2015
		Resch et al., 2016
Lipoproteins		Biagini et al., 2015
✓ SPy1390		
✓ SPy1882		
✓ SPy2000		

M protein		Resch et al., 2016
C5a peptidase (ScpA)		Resch et al., 2016
ECM degrading enzymes	*S. agalactiae*	Surve et al., 2016
✓ Hyaluronidase		
IgA binding beta antigen		Surve et al., 2016
Clostridium		
Beta2 toxin	*C. perfringens*	Jiang et al., 2014
Mycobacterium		
Mycolactone	*M. ulcerans*	Marsollier et al., 2007
Mycobaction	*M. tuberculosis*	Prados-Rosales et al., 2014
29-kDa antigen (CFP29)		Lee et al., 2015
ESX-1 secretion-associatedproteinEspA (EspA)		Lee et al., 2015
Lipoproteins		Lee et al., 2015
✓ LpqH, LppX, LprA, LprG, PstS1		
Heparin-binding hemagglutinin (HbhA)		Lee et al., 2015
Twin-arginine translocation TatA (TatA)		Lee et al., 2015
DNA-binding protein HU (Hup)		Lee et al., 2015
Aconitate hydratase A (Acn)		Lee et al., 2015
The antigen 85 complex (Ag85)		Lee et al., 2015
✓ FbpA, FbpB, and FbpC		
Superoxide dismutase (SodB)		Lee et al., 2015

The delivery of the EVs into the host cells was demonstrated *in vivo* and was shown to induce cytotoxic effect on host cells *in vitro* (Gurung et al., 2011; Hong et al., 2011; Kim et al., 2012). This cytotoxic activity toward host cells was closely associated with the EVs protein content (Jeon et al., 2016). The fusion and release of EVs protein content in host cells occur through interaction of EVs membrane with microdomains of the cholesterol-rich membrane (Gurung et al., 2011), as represented in figure 10. Gurung et al (2011). demonstrated that *S. aureus* EVs lose their cytotoxic effect in vitro when they are lysed prior to contact with the cell layer. This showed that membrane recognition and fusion of EVs to deliver EVs components into the host cell cytosol is a prerequisite for cytotoxicity (Gurung et al., 2011). Once the cytoplasmic membranes have fused, EVs unload their cargo and induce the cellular response of the host cell. Additionally, interaction and delivery of molecules like α-toxin and proteins A were also reported during *in vivo* infection (Gurung et al., 2011; Thay et al., 2013).

In vivo, the injection of EVs prepared from *S. aureus* clinical isolates led to the production of inflammatory mediators and caused inflammatory-like disease in mice, such as

pulmonary inflammation in the airways (Kim et al., 2012), pneumonia model (Choi et al., 2015; Gurung et al., 2011), atopic dermatitis (AD) inflammation (Hong et al., 2011; Jun et al., 2017) and systemic infection (Askarian et al., 2018). Even a low concentration (*e.g.* 1µg per animal) EVs were able to stimulate inflammatory cells in animal models (Kim et al., 2012). Since EVs are able to trigger an immune response, the use of EVs in vaccine application was investigated. Immunization with EVs was indeed shown to induce a protective immune response against staphylococcal infections *in vivo* by induction of a T-cell mediated and humoral immune responses (Choi et al., 2015; Hong et al., 2011). Interestingly, apart from the variability of virulence factors, *S. aureus* EVs protein content is also enriched with lipoproteins (*e.g.* FhuD2, ferric-hydroxamate uptake; MntC, manganese transport protein C), some of which have already been described as vaccine candidates against *S. aureus* infections (Anderson et al., 2012; Mariotti et al., 2013).

3.3.4.2. EVs in other Gram-positive pathogenic bacteria

Although the studies involving Gram-positive EVs have started using *S. aureus* as a model (Lee et al., 2009), considerable attention has been paid in the last years to this process in other Gram-positive microorganisms. The EVs released by Gram-positive bacteria present a significant heterogeneity in size, ranging from 20–80 nm (*e.g. S. pneumoniae*) to 20-400 nm diameter (*e.g*. *C. perfringens***;** *S. coelicolor*) (Jiang et al., 2014; Olaya-Abril et al., 2014; Rath et al., 2013; Rivera et al., 2010). *S. agalactiae* release two distinct subpopulations of EVs, one < 50 nm and the other in the range of 150-300 nm (Surve et al., 2016). In different Gram-positive bacteria, like *S. pneumoniae* and *S. pyogenes*, the EVs size seems variable according to the strain studied (Olaya-Abril et al., 2014; Resch et al., 2016).

Like *S. aureus* EVs, EVs recovered from different Gram-positive bacteria were also able to reduce the viability of various cells types *in vitro*. *B. anthracis* EVs contain anthrolysin and anthrax toxin, a tripartite toxin composed of protective antigen (PA), lethal factor (LF), edema factor (EF). They reduce the cellular viability of macrophages *in vitro*, suggesting a physiological role for these vesicles during anthrax disease (Table 1) (Rivera et al., 2010). Likewise, EVs purified from *M. ulcerans* positive for the toxin mycolactone also displayed cytotoxic activity *in vitro* (Marsollier et al., 2007). The relation between intravesicular content and cytotoxicity was confirmed since EVs isolated from mup045, a mutant defective in mycolactone, were not able to cause cytotoxicity on bone marrow-derived mouse macrophage cultures (Marsollier et al., 2007). A dose-dependent decrease in cellular

viability was also observed in HeLa cells after challenge with *S. agalactiae* EVs containing toxins and enzymes that degrade extracellular matrix (ECM) (Surve et al., 2016).

As previously mentioned, the lipid proportion between bacterial membrane and EVs purified from Gram-positive bacteria are different. *S. pyogenes* EVs was enriched with anionic phosphatidylglycerol (PG) (Resch et al., 2016) while in *S. pneumoniae* EVs an enrichment of short-chain saturated fatty acids such as C12 (lauric acid), C14 (myristic acid), C16 (palmitic acid) was reported (Olaya-Abril et al., 2014). Further, *S. agalactiae* membrane and purified EVs had similar lipid compositions, with palmitic acid as the major fatty acid in both cases (Surve et al., 2016).

Apart from the two medically important species of mycobacteria, *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacille Calmette-Guérin, multiple other species of mycobacteria (*M. smegmatis*, *M phlei*, *M. avium*, *M. kansasii*) were shown to be able to release EVs (Prados-Rosales et al., 2011). It appears that, likewise *S. aureus*, Gram-positive EVs are generally enriched in cytosolic and membrane proteins, particularly lipoproteins. Such profile was identified in *S. pneumoniae* (Olaya-Abril et al., 2014), *C. perfringens* (Jiang et al., 2014), *S. pyogenes* (Biagini et al., 2015; Resch et al., 2016), and *M. tuberculosis* H37Rv (Lee et al., 2015). Interestingly, sortase A (SrtA) of a non-pathogenic *S. mutans*, an enzyme involved in the anchoring of lipoproteins to the cell wall peptidoglycan, does not quantitatively affect the production of *S. mutans* EVs but rather influence their composition (Liao et al., 2014). Biagini et al. (2015) described an interesting population of *S. pyogenes* EVs with more than 72% of the predicted lipoproteins identified in the EVs content, making them the almost exclusive EVs proteinaceous component (Biagini et al., 2015). The lipoproteins identified in *M. tuberculosis* EVs are important TLR2 ligands and include LprG, LprA and LpqH proteins (Prados-Rosales et al., 2011, 2014b; Rath et al., 2013). Interestingly, pathogenic mycobacteria EVs triggered an inflammatory response in a TLR2-dependent manner higher than that observed with EVs isolated from the nonpathogenic strains (Prados-Rosales et al., 2011). This was explained by the fact that only EVs from virulent mycobacterial strains, such as *M. tuberculosis* and BCG, were enriched with TLR2 lipoprotein agonists (Prados-Rosales et al., 2011).

Many studies verified the ability of EVs to mimic clinical signs of infection using different *in vitro* and *in vivo* approaches. For instance, using the highly virulent strain *C. perfringens* CP4 EVs, macrophages are stimulated and secrete inflammatory mediators such as G-CSF, TNF-α, and IL-6 (Jiang et al., 2014). However, mice-immunization *in vivo* was not able to induce protective response when animals were challenged with *C. perfringens* despite

the high IgG titer (Jiang et al., 2014). Conversely, mice-immunization using EVs purified from *B. anthracis* (Rivera et al., 2010) and *S. pneumonia* (Olaya-Abril et al., 2014) provided protective effects against a challenge *in vivo*. Recently, Surve et al. (2016) demonstrated that *S. agalactiae* EVs can be associated with preterm birth and fetal demise observed in a pregnant woman model infected with *S. agalactiae* in the urogenital tract. In this clinical case, EVs induced a collagen degradation in the choriodecidual membranes treated *ex-vivo*, reduced the stiffness of these membranes and drove an inflammatory response *in vivo*. Furthermore, EVs showed anterograde move along the female reproductive tract, which enabled them to reach remote sites of infection (Surve et al., 2016).

3.3.4.3. Non-pathogenic bacterial EVs

The role of non-pathogenic bacterial EVs has received some attention in the last years, especially about microbiota-derived EVs. *Lactobacillus sp*. and *Bifidobacterium sp*. are the genera most commonly used as potential probiotics (Ross et al., 2005) and immunomodulation is one of the essential probiotic functionalities (Bron et al., 2011). Furthermore, EVs isolated from probiotic strains also seem to enhance host defense and to stimulate a protective innate immune response against infection by pathogenic bacteria (Li et al., 2017).

Indeed, EVs were associated with the complex network of signaling pathways that enable the interaction between gut microbiota and the host (Ahmadi Badi et al., 2017), which seems to also involve TLR2 activity (van Bergenhenegouwen et al., 2014). To date, regarding the genus *Lactobacillus*, EVs has only been purified from *L. rhamnosus* (JB-1), *L. plantarum* WCFS1 and *L. casei* BL23 (Al-Nedawi et al., 2015; Domínguez Rubio et al., 2017; Li et al., 2017).

L. plantarum WCFS1 is a probiotic strain found in the gastrointestinal tract, that can induce immunomodulatory effects in the host. Li et al. (2017) recently showed that EVs from *L. plantarum* stimulate *in vitro* the upregulation of host defense gene expression including REG3G, a potent bactericidal component that promotes spatial segregation of microbiota and host in the intestine. In this work, *L. plantarum*-derived EVs were able to prolong the survival rates of a *Caenorhabditis elegans* nematode under *Enterococcus faecium* challenge (Li et al., 2017). Another example of interaction between probiotic EVs and host is provided by the gut commensal *L. rhamnosus* (JB-1), which releases EVs that were shown to induce ex vivo a nervous signal locally, suggesting some neurobiological effect in mice. However, the bacterial

components associated with this effect in the host have not been identified (Al-Nedawi et al., 2015). *L. casei* BL23 derived EVs were also filled with cytoplasmic constituents such as DNA, RNA and proteins, including those described as mediators of probiotic effects (Domínguez Rubio et al., 2017).

A peculiar feature of the probiotic EVs is the ability to recapitulate the immune effects of the whole bacteria (Li et al., 2017), which suggests that EVs might be a supporter or even a substitute for the probiotic effects of the EVs-producing bacteria (Kim et al., 2016a). Similarly, EVs derived from *Bacterioides fragilis*, a commensal Gram-negative bacterium, alleviate colitis similarly to the parent bacteria and are directly internalized by DCs (Dendritic cells) *in vitro*. This internalization in intestinal DCs enabled the delivery of polysaccharide acapsular antigen (PSA) that is known to induce Treg cells, preventing the onset of experimental colitis (Shen et al., 2012). This delivery mode corresponds to the best bacterial example of the cell-to-cell communication system via EVs during host-bacterial mutualism (Shen et al., 2012).

DCs express pattern recognition receptors (PRR) that allow the direct recognition and activation by bacteria (van Bergenhenegouwen et al., 2014). Once exposed to EVs of *Bifidobacterium bifidum* LMG13195, DCs strongly promote differentiation of forkhead Box protein 3 (Foxp3) 1 Regulatory T cells (Treg) (López et al., 2012). EVs of another Bifidobacterium, *B. longum* KACC 91563, was shown to suppress allergic diarrhea through reduction of mast cells in the intestine, the principal effector cells of the food allergies. Furthermore, apoptosis of mast cells was more efficiently induced by *B longum* EVs than by the whole bacteria. This was attributed to the intravesicular family 5 extracellular solute-binding protein (ESBP) carried by the EVs. Once delivered, it reduces the number of mast cells and the occurrence of diarrhea *in vivo* without compromising T-cell immune response (Kim et al., 2016a).

Apart from the non-pathogenic bacteria mentioned, *B. subtilis* and *M. tuberculosis* avirulent strain H37Ra also release EVs (Brown et al., 2014; Kim et al., 2016b). Indeed, the production of EVs by *B. subtilis* was initially proposed with *S. aureus* albeit not characterized (Lee et al., 2009). Vesicles heterogeneity was also reported based on different developmental phases of this bacterium. For instance, *B. subtilis* produces EVs during vegetative and sporulation phases, with differences in cargo and abundance of proteins (Kim et al., 2016b). In EVs purified from supernatants of sporulating cultures, the proteins more abundant were associated with translation. Conversely, protein associated with metabolism were mainly found in EVs from vegetative state (Kim et al., 2016b).

3.3.5. Vaccines and applications

The high incidence of infectious diseases in the world and the critical emergence of antibiotic-resistant pathogens emphasize the necessity for new strategies and innovation in the vaccine field. Considering that EVs mimic in many aspects their producing pathogen and represent an important antigen source, they have investigated as alternative vaccine candidates in different types of infections (Girard et al., 2006).

EVs formulation may offer some advantages over conventional vaccines, such as the delivery of many packaged antigens and induction of both innate and adaptive immunity, no requirement for adjuvants, the lack of replication and possible infection, and it can be obtained easily (Liu et al., 2016). However, EVs vaccines are strain-specific formulations that should be used against clonal disease outbreaks, as the meningococcal in Norway and Cuba (Girard et al., 2006).

The most successful EVs vaccines against meningitis are summarized in table 7. Effective vaccine formulation containing EVs against meningococcal infections (VA-MENGOC-BC®) has been licensed in many countries (Sotolongo et al., 2007). In Brazil, where this Cuban vaccine has been widely administered in vaccination campaigns, it was considered of slight reactogenicity and was described as also well tolerated (Sotolongo et al., 2007). A meningococcal B:14:P1.7,16 outbreak in Normandy (France) was also controlled by using an EVs vaccine (MenBvac) developed by Norwegian Institute of Public Health (NIPH) (Caron et al., 2012). The New Zealand vaccine contains epidemic strain outer membrane proteins (B:4:P1.7b,4, NZ98/254) as the active ingredient (Jackson et al., 2009; Wong et al., 2007). Finally, the multi-component meningococcal B vaccine (4CMenB) was recently approved in Europe and Australia and contains three surface-exposed recombinant proteins (fHbp, NadA and NHBA) combined with EVs from MenB strain NZ 98/254 with PorA antigenicity (O'Ryan et al., 2014).

Table 7. EVs vaccines against Meningococcal serogroup B.

OMVs vaccines	Strain	Origin	Reference
VA-MENGO-BC®	B4:P1.19,15:L3,7,9	Cuba, Finlay Institute	Sotolongo et al., 2007
MenBvac	B:15:P1.7,16	Norwegian, Norwegian Institute of Public Health, NIPH	Caron et al., 2012
MeNZB™	B:4:P1.7b,4	New Zealand, Chiron and NIPH	Wong et al., 2007 Jackson et al., 2009
4CMenB (Bexsero® vaccine)	NZ 98/254	Switzerland Bexsero®, Novartis Vaccines and Diagnostics	O'Ryan et al., 2014

Immunization with vesicles induce host protective immunity against various pathogens, as *S. aureus* (Askarian et al., 2018; Choi et al., 2015; Kim et al., 2012), *M. tuberculosis* (Prados-Rosales et al., 2011, 2014b), *B. anthracis* (Rivera et al., 2010), *N. meningitidis* (Gonzalez et al., 2006), *E. coli* (Kim et al., 2013b), *H. pylori* (Keenan et al., 2000b), *Vibrio cholerae* (Schild et al., 2008), *S. pneumoniae* (Olaya-Abril et al., 2014), *Salmonella typhimurium* (Alaniz et al., 2007), and *P. aeruginosa* (Zhao et al., 2013).

Since *M. tuberculosis* EVs were also able to boost BCG vaccine efficacy, new formulations were designed using EVs to improve the BCG protective effects (Prados-Rosales et al., 2011, 2014b). However, although Gram-positive EVs were described as promising vaccine candidates, there are still some practical aspects that need to be verified, such as the variable composition. Engineering EVs with exogenous and uniforms content may represent a valuable source of a therapeutic formulation but still requires research and development (Gerritzen et al., 2017).

The possibility of applying bacterial EVs in other areas of biotechnology remains mostly unexplored. In biotechnology, EVs can be used as genetic engineering tools (*e.g.* delivering CRISPR/Cas cassettes) for strains optimization (Liu et al., 2018). EVs can also be used as (1) delivery vehicles for nutritional compounds to the host (*e.g.* vitamin K2); (2) vectors for natural enrichment of membrane-associated compounds in fermented foods or food supplements (*e.g.* hydrophobic aromatic compounds); (3) Agents for shaping starter culture communities by suppressing dominance of one single strain with inhibitory effects and (4) orally administered vaccines since they can merge with intestinal epithelial cells and interact with the host immune system (Liu et al., 2018).

3.4. Extracellular vesicle isolation and characterization techniques

3.4.1. Isolation methods

Despite the huge interest in EVs of eukaryotic and prokaryotic origin, their correct isolation and purification remains a significant technical challenge. Current EVs methods are generally complex, expensive and with low-yield, which could limit research advances and large-scale medical applications (Sáenz-Cuesta et al., 2015). EVs can be recovered from *in vitro* culture supernatants from all kinds of cell studied so far, such as all eukaryotic body fluids (Konoshenko et al., 2018). Further, purified EVs without contaminants is essential for real characterization of these vesicles; however, no single method was identified to completely remove cell lysates and other non-vesicular particles (Bauman and Kuehn, 2006; Chatterjee and Chaudhuri, 2012).

Purification techniques involve filtration steps of the culture supernatants followed by ultracentrifugation and density gradient centrifugation (Bauman and Kuehn, 2006). Indeed; the incorporation of the density gradient step into the EVs isolation protocol supposedly eliminates the sample contamination with large proteins and/or proteins that are non-specifically associated with EVs (Szatanek et al., 2015). Furthermore, to overcome the lack of standardization with regard to purification and characterization methods, ISEV has outlined methodological guidelines involving the minimal experimental requirements for EVs field (Coumans et al., 2017; Lötvall et al., 2014). This guideline includes a series of criteria as also suggest controls in different samples to aid in the experimental design and report of results (Lötvall et al., 2014). However, the most widely used for collecting EVs is differential centrifugation (Gould and Raposo, 2013), while highly purified EVs may be obtained by gel filtration chromatography (Post et al., 2005; Chatterjee and Chaudhuri, 2012).

3.4.2. Current methods for EVs analysis

The most commonly used techniques for EVs characterization are nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS), dynamic light-scattering (DLS), atomic force microscopy (AFM), high-resolution flow cytometry (hFC), as well as transmission and scanning electron microscopy (TEM, SEM) (Konoshenko et al., 2018; Sitar et al., 2015). Unfortunately, quantification of EVs still represents a challenge, since the small size, low refractive index, and heterogeneity of the samples make them technically difficult to characterize (Maas et al., 2015; Sitar et al., 2015). Besides, analysis using different methods can significantly influence or even bias the corresponding results (Konoshenko et al., 2018;

Maas et al., 2015), which evidences the need for standardization in the EVs field. The main techniques used to characterize EVs and their disadvantages are summarized in the table 8.

Table 8. Main EVs characterization techniques.

Method	Suggested for	Disadvantages	Reference
TEM	Visualization of morphology and size Immune labelling techniques (markers);	Sample preparation artifacts User training and experience	Coumans et al., 2017
NTA	Determination of EVs size and concentration	Less acurate in the detection of size based subpopulation User training and experience	Maas et al., 2015 Sitar et al., 2015
TRPS	Determination of EVs size and concentration	Underestimation of the concentration.	Maas et al., 2015
DLS	Determination of EVs size	Not optimal for polydisperse samples	Hoo et al., 2008
AFM	Visualization of morphology and size	User training and experience	Hoo et al., 2008
hFC	Individual characterization of EVs molecular markers	User training and experience	Maas et al., 2015; Nolte-'t Hoen et al., 2012

To gain insights and help in the exploration of high-throughput datasets from prokaryotic and eukaryotic EVs, the analytical tool EVpedia was developed (D.-K. Kim et al. 2013). It constitutes an integrated database that might help in comparative analyses using vesicular proteome, transcriptome, and lipidome.

4. Context and aim of the Ph.D. project

4.1. Context of the thesis project

Mastitis is an inflammation of the mammary gland in small and large ruminants that results in widespread damages on animal health and represents economic losses to the world dairy market. *S. aureus* is one of the most important etiological agent of mastitis worldwide, and, to date, the mechanisms involved in its pathogenesis is not fully understood. Indeed, *S. aureus* is an opportunistic pathogen associated with various types of infections in human and animals. This bacterium can breach host cell barriers and stimulate the production of pro-inflammatory mediators, which facilitate the damage and spread in the host tissue. Moreover, *S. aureus* can trigger different clinical manifestations on their hosts, some of which result from the action of specific virulence factors.

The secretion of proteins, in particular virulence factors, is a crucial step in the infectious process of pathogenic bacteria and, nowadays, the production and release of bacterial extracellular vesicles (EVs) have been broadly embraced as one of the important secretion systems. It has been shown that clinical *S. aureus* strains interact with host cells *in vitro* and modulate an immune response *in vivo*. *S. aureus* EVs are considered as cargo for the delivery of various bacterial compounds in the surrounding environment. Purified *S. aureus* EVs have been shown to interact with host cells and they are therefore increasingly studied. Indeed, the identification of new factors that may help to elucidate the pathogenic mechanisms associated with *S. aureus* mastitis represents an alternative in the fight and control of this disease. *S. aureus* EVs might also be a relevant option for the development of new strategies of prevention and control of *S. aureus* infections, including mastitis.

Here, we hypothesized that EVs derived from bovine and ovine isolates contribute to mastitis pathogenesis.

4.2. Aim of the Ph.D. project

This thesis project aimed to evaluate the ability of *S. aureus* strains isolated from animals to produce and secrete EVs and to determine their contribution to mastitis pathogenesis.

EVs – host communication

In order to achieve this aim, EVs purified from the archetypal bovine isolate *S. aureus* Newbould 305 (*S. aureus* N305) were physically and chemically characterized. The ability of *S. aureus* N305-secreted EVs to induce an immunostimulatory response and cytotoxic effect in bovine mammary epithelial cells (bMEC) was determined. Then, *S. aureus* N305-secreted EVs were evaluated for their immunomodulatory properties *in vivo*, using a murine model of mastitis

Comparative proteomics

The ability of six *S. aureus* strains to produce and secrete EVs was evaluated. The strains were selected according to their origin (bovine, ovine and human) and their infection properties.

By using a proteomic approach, this work aimed to evaluate a proteomic profile shared between EVs isolates that might enable to infer about function, mechanism of production and release of bacterial vesicles. We also wanted to check whether EVs had protein profiles that could somehow reflect the host-specificity of the producing strains.

5. Chapter 2. Immunomodulatory effects induced by *S. aureus* extracellular vesicles

Pathogenic microorganisms have developed a vast arsenal of mechanisms to subvert and control physiological processes in the host cells in order to colonize and multiply in host tissues. Production and release of extracellular vesicles (EVs) can be classified as one of these mechanisms. EVs production is a conserved process in all branches of life: eukaryotes, archaea, and bacteria. It enables the delivery of factors to distant target cells. EVs production has recently been described in Gram-positive bacteria. Since then, they have been purified from several clinical isolates of *S. aureus*, usually highly virulent strains involved in nosocomial infections.

In this chapter, we evaluated the ability of the bovine strain *S. aureus* Newbould 305 (ATCC 29740) isolated in 1958 in Orangeville (Ontario, Canada) to produce and release EVs *in vitro*. This strain reportedly induces moderate and chronic mastitis in cows. Although it has been used as a model strain in experimental mastitis, its genome was sequenced and characterized only on the early 2010s in our group (Bouchard et al., 2012; (Peton et al., 2014).

In this work, EVs were purified from *S. aureus* N305 supernatants and characterized through a proteomic approach. Furthermore, *S. aureus* N305-derived EVs were evaluated with regard to their cytotoxicity and immunomodulatory properties *in vitro* using bovine mammary epithelial cells (bMEC) and *in vivo* in murine model of mastitis. Although *S. aureus* N305 EVs did not induce cytotoxic effects in bMEC, they were able to stimulate an immune response *in vivo* mainly associated with neutrophils recruitment. These results show that EVs released by *S. aureus* N305 contain virulence factors and that they can play a role in the inflammatory process and, more generally, in the *S. aureus* pathogenesis in a mastitis context.

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Tartaglia, Natayme R., et al. *Staphylococcus aureus* Extracellular Vesicles Elicit an Immune Response in Vivo on the Murine Mammary Gland

Staphylococcus aureus extracellular vesicles elicit an immunostimulatory response in vivo on the murine mammary gland

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ABSTRACT

Staphylococcus aureus is a major pathogen responsible for bovine mastitis, the most common and costly disease affecting dairy cattle. *S. aureus* naturally releases extracellular vesicles (EVs) during its growth. EVs play an important role in the bacteria-bacteria and bacteria-host interactions and are notably considered as nanocarriers that deliver virulence factors to the host tissues. Whether EVs play a role in a mastitis context is still unknown. In this work, we showed that *S. aureus* Newbould 305 (N305), a bovine mastitis isolate, has the ability to generate EVs *in vitro* with a designated protein content. Purified *S. aureus* N305-secreted EVs were not cytotoxic when tested *in vitro* on MAC-T and PS, two bovine mammary epithelial cell lines. However, they induced the gene expression of inflammatory cytokines at levels similar to those induced by live *S. aureus* N305. The *in vivo* immune response to purified *S. aureus* N305-secreted EVs was tested in a mouse model for bovine mastitis and their immunogenic effect was compared to that of live *S. aureus* N305, heat-killed *S. aureus* N305 and to *S. aureus* lipoteichoic acid (LTA). Clinical and histopathological signs were evaluated and pro-inflammatory and chemotactic cytokine levels were measured in the mammary gland 24 hour post-inoculation. Live *S. aureus* induced a significantly stronger inflammatory response than that of any other condition tested. Nevertheless, *S. aureus* N305-secreted EVs induced a dose-dependent neutrophil recruitment and the production of a selected set of pro-inflammatory mediators as well as chemokines. This immune response elicited by intramammary *S. aureus* N305-secreted EVs was comparable to that of heat-killed *S. aureus* N305 and, partly, by LTA. These results demonstrated that *S. aureus* N305-secreted EVs induce a mild inflammatory response distinct from the live pathogen after intramammary injection. Overall, our combined *in vitro* and *in vivo* data suggest that these EV play a significant role in the pathogenesis of bovine *S. aureus* mastitis.

INTRODUCTION

Mastitis is an inflammatory response of the mammary gland that often results from a bacterial infection and that induces local to systemic symptoms in small and large ruminants (Bradley, 2002; Le Maréchal et al., 2011a). In dairy farms, mastitis severely impacts both the animal health and the quality of milk, causing important economic losses in the dairy industry (Le Maréchal et al., 2011a; Peton and Le Loir, 2014). The Gram-positive pathogen *Staphylococcus aureus* is one of the most important etiological agent of mastitis worldwide (Bradley, 2002; Le Maréchal et al., 2011a). Signs of *S. aureus* mastitis range from subclinical to gangrenous infection in ruminants and rely on strain-specific features such as the production and secretion of specific virulence factors that increase invasiveness or enable the mammary epithelial colonization of *S. aureus* (Le Maréchal et al., 2011b, 2011c; Peton and Le Loir, 2014). Clearance of *S. aureus* from the infected udder is impaired as the pathogen is able to adhere, internalize, survive and multiply into the mammary epithelium (Alekseeva et al., 2013; Bouchard et al., 2013; Peton et al., 2014). This ability of *S. aureus* to induce chronic infections negatively affects animals and notably, *S. aureus* mastitis are reportedly difficult to cure and show a high recurrence rate (Conlon, 2014; Peton and Le Loir, 2014; Peton et al., 2014).

The bovine strain *S. aureus* Newbould 305 (Bouchard et al., 2012; Prasad and Newbould, 1968), hereafter referred to as *S. aureus* N305, has been used as a model strain for *S. aureus* mastitis in numerous studies including several from our group (Bouchard et al., 2013; Breyne et al., 2014, 2017a, 2017b; Peton et al., 2016). Despite many efforts dedicated to understand the pathogenesis of *S. aureus* mastitis, the infectious process is still poorly understood and a better knowledge on host-pathogen interactions is required to allow the development of effective preventive or curative strategies. *S. aureus* secretes many virulence factors as well as exports both envelope-associated proteins through classical Sec-dependent pathways and cytoplasmic proteins through non-classical secretion mechanisms (Bendtsen et al., 2005; Hecker et al., 2010). One of these latter mechanisms is the release of extracellular vesicles (EVs) which has been extensively described in eukaryotes (van der Pol et al., 2012; Roy et al., 2018). These EVs are spherical nano-sized particles with a lipid bilayer secreted naturally by pathogenic and non-pathogenic bacteria from budding of the cellular membranes (Al-Nedawi et al., 2015; Deatherage and Cookson, 2012; Prados-Rosales et al., 2011). Donor cells use EVs to transport various proteins which can be delivered to local or distant cellular targets to interact with and modify them. The first evidence of this novel secretion process was obtained in Gram-negative bacteria already in the 1960s (Chatterjee and Das, 1967; Knox et al., 1966;

Work et al., 1966). EVs are now recognized as important vehicles of intra- and inter-species cellular communication across all three kingdoms of life (Celluzzi and Masotti, 2016; Deatherage and Cookson, 2012). The protein content of bacterial vesicles includes factors involved in virulence, biofilm formation, modulation of the host immune response, resistance to antibiotics, bacterial survival and intra- and interspecies communication and cooperation (Brown et al., 2015; Kim et al., 2015; MacDonald and Kuehn, 2012). Most studies have been conducted on Gram-negative bacteria (Ellis and Kuehn, 2010; Horstman and Kuehn, 2000; José Fábrega et al., 2016). Consequently, our knowledge regarding Gram-positive EVs still remains limited (Brown et al., 2015). Since 2009, a few works reported the production and secretion of EVs by *S. aureus*, with particular emphasis on their protein content characterization and their impact on host cells (Lee et al., 2009; Hong et al., 2011; Gurung et al., 2011; Kim et al., 2012; Lee et al., 2013a; Thay et al., 2013; Choi et al., 2015; Jeon et al., 2016; Bae et al., 2017; He et al., 2017; Im et al., 2017; Jun et al., 2017; Askarian et al., 2018). In analogy with Gram-negative bacteria *S. aureus* EVs harbor, inter alia, numerous virulence factors, can have cytotoxic effects on host cells *in vitro* (Gurung et al., 2011; Jeon et al., 2016) and trigger a pro-inflammatory response both *in vitro* and *in vivo* (Hong et al., 2011; Kim et al., 2012).

However, the potential contribution of *S. aureus* EVs to bacterial pathogenesis has only been explored for human isolates. Therefore, in the present report we aimed to at first characterize EVs produced by the bovine mastitis strain *S. aureus* N305 to investigate their role in the context of mastitis. To obtain this aim, we evaluated whether these purified EVs are capable to induce a stimulation of the host immune response comparable to either live or heat-killed *S. aureus* N305 and LTA. Our data suggest a role of *S. aureus* N305-secreted EVs both *in vitro* and *in vivo* to the immunopathogenesis of bovine Gram-positive mastitis.

MATERIALS AND METHODS

Bacterial strain and growth conditions

S. aureus N305 (ATCC 29740) was grown in Brain Heart Infusion (BHI) (Difco, pH 7.4) broth at 37°C under vigorous shaking (150 rpm/min). The phases of bacterial growth were determined by measurement of optical density at 600nm (OD_{600}) and routinely the colony forming units (CFU) were counted on BHI agar using the micromethod (Baron et al., 2006).

Mammary epithelial cell lines and culture conditions

The bovine mammary epithelial cell line MAC-T (Nexia Biotechnologies, Quebec, Canada) was cultured in Dulbecco's modified eagle medium (DMEM) (D. Dutscher) supplemented with 10% heat-inactivated fetal calf serum, 40 U/mL penicillin, 40 µg/mL streptomycin (LONZA), and 5 µg/ml insulin (Sigma-Aldrich). The bovine mammary epithelial cell line PS (INRA, Tours, France) (Roussel et al., 2015) was cultured in mammary epithelial cells growth medium (GM) which contain Advanced DMEM/F12 (Gibco) supplemented with 20 mM HEPES buffer (Fisher Scientific), 2 mM L-glutamine (Gibco), 1 µg/mL hydrocortisone (Sigma-Aldrich), 10 ng/mL insulin-like growth factor 1 (Preprotech), 5 ng/mL fibroblast growth factor (Preprotech), 5 ng/mL epidermal growth factor (Sigma-Aldrich) (Roussel et al., 2015). Infections of PS cells were performed with stimulation medium (SM) without growth factors (Roussel et al., 2015). MAC-T and PS cells were incubated at 37°C in humidified incubator with 5% CO_2. They were cultured to a confluent monolayer (80%), treated with 0.05% trypsin (PAN-Biotech) and suspended in fresh medium.

Purification of *S. aureus* N305-secreted EVs from culture supernatants

EVs were purified from *S. aureus* N305 culture supernatants using a method adapted from (Gurung et al., 2011). Sub-cultured cells at the end of exponential phase were diluted 1:1000 in 1L of fresh BHI medium and were grown until the stationary phase. After the cells were pelleted at 6 000 *g* for 15 min, the supernatant fraction was filtered through a 0.22 µm vacuum filter (PES) and the filtrate was concentrated around 100-fold using Amicon ultrafiltration system (Millipore) with 100 kDa filter. The resulting filtrate was subjected to ultracentrifugation at 150 000 *g* for 120 min at 4°C and were applied to a discontinuous sucrose density gradient (8% - 68%). After centrifugation at 100 000 *g* for 150 min at 4°C, each fraction of the gradient was collected. The fractions with density around 1.08 - 1.13 g/cm^3 were then recovered by sedimentation at 150,000 *g* for 120 min and suspended in Tris-Buffered Saline (TBS) (150 mM NaCl; 50 mM Tris-Cl, pH 7.5). Purified EVs were checked

for absence of bacterial contamination and stored at -20°C before use. The EVs amount were measured based on protein concentration using the Bradford reagent (Bio-Rad) and visualized by SDS-PAGE. Hereafter, the *S. aureus*-secreted vesicle dose correspond to the quantity of *S. aureus*-secreted vesicle proteins.

Negative staining electron microscopy (EM)

Negative staining electron microscopy was performed at the Microscopy Rennes Imaging Center platform (MRic TEM) (University of Rennes 1, Rennes, France). Purified EVs were applied to copper grids and were negatively stained with 2% uranyl acetate as previously described (Gurung et al., 2011). The samples were visualized on a transmission electron microscope Jeol 1400 TEM (Jeol, Tokyo, Japan) operating at 120 kv accelerating voltage.

Cryo-electron tomography (Cryo-ET)

Vitrification of purified EVs was performed using an automatic plunge freezer (EM GP, Leica) under controlled humidity and temperature (Dubochet and McDowall, 1981). Mix-capped gold nanoparticles of 10 nm in diameter (Duchesne et al., 2008) were added to the sample at a final concentration of 80 nM to be used as fiducial markers. The samples were deposited to glow-discharged electron microscope grids followed by blotting and vitrification by rapid freezing into liquid ethane. Grids were transferred to a single-axis cryo-holder (model 626, Gatan) and were observed using a 200 kV electron microscope (Tecnai G^2 T20 Sphera, FEI) equipped with a 4kx4k CCD camera (model USC4000, Gatan). Single-axis tilt series, typically in the angular range ±60°, were acquired under low electron doses (~0.3 $e^-/Å^2$) using the camera in binning mode 2 and at a nominal magnifications of 29,000x. Tomograms were reconstructed using the graphical user interface eTomo from the IMOD software package (Mastronarde, 1997). Slices through the tomograms were extracted using the graphical user interface 3dmod of the IMOD package. Measurements were performed using the measuring tools available in the slicer panel of 3dmod.

Size distribution of *S. aureus* N305-secreted EVs

The size distribution of EVs was estimated by three different methods: nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS) and cryo-EM. NTA analysis was carried out using a NanoSight NS300 (Malvern Instruments, United Kingdom). EVs were thawed and diluted in TBS at 1:10 000 until an optimum visualization of a maximum number of vesicles. Data was analyzed by NTA 3.0 software (Malvern Instruments). All

measurements were performed at 22°C. TRPS analysis was carried out using the IZON qNano system (Izon Science). EVs were diluted 1:100 and applied to qNano instrument (Izon Science) at 22°C using a nanopore NP140 after the calibration of the system with 70 nm standard carboxylated polystyrene particles (CPC70). Finally, the diameter of vesicles was measured using the images obtained by Cryo-EM from 90 round vesicles using the measuring tools available in the slicer panel of 3dmod (IMOD package).

In-solution digestion and identification of proteins in *S. aureus* N305-secreted EVs

Three independent biological replicates of EVs, purified as described above, were digested for NanoLC-ESI-MS/MS analysis. Purified EVs (approximately 50µg) were pelleted at 150 000 *g* for 2 h at 4°C and suspended with the solution of 6 M Guanidine-HCl (Sigma-Aldrich), 50 mM Tris-HCl (pH 8.0) (VWR C) and 2 mM DTT (Sigma-Aldrich). EVs were heated at 95ºC for 20 min and cooled in 50 mM NH_4HCO_3 (pH 7.8) (Sigma-Aldrich). Then, samples were digested in solution using sequencing grade-modified trypsin (Promega) with the ratio 1:50 of enzyme:protein for 15 h at 37°C, as previously described by Lee et al. (2009). After digestion, the peptides were stored at -20°C until further analysis. Nano-LC experiments were performed as previously reported (Le Maréchal et al., 2011c), with minor modifications. Briefly, the peptide mixture was loaded using a Dionex U3000-RSLC nanoLC system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, USA) equipped with a nano-electrospray ion source (ESI) (Proxeon Biosystems A/S). Samples were first concentrated on a PepMap 100 reverse-phase column (C18, 5 µm, 300 µm inner diameter (i.d.) by 5 mm length) (Dionex). Peptides were then separated on a reverse phase PepMap column (C18, 3 µm, 75 µm i.d. by 250 mm length) (Dionex) using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water). A linear gradient from 5 to 85% of solvent B was applied for the elution at a flow rate of 0.3 µL/min. MS data was acquired in positive mode and the spectra were collected in the selected mass range 250 to 2 000 m/z at a resolution of 70 000 for MS and at a resolution of 17 500 for MS/MS spectra. The peptides were identified from the MS/MS spectra using the X! Tandem pipeline software (Langella et al., 2017), matched against the genome sequence of the *S. aureus* N305 and *S. aureus* RF122, a bovine strain associated with severe symptoms in the host (Herron-Olson et al., 2007). A minimum of two peptides per protein was imposed with a false discovery rate (FDR) of < 0.1% at the peptide level.

Bioinformatics

The biological functions and distribution of *S. aureus* N305 EVs proteins were categorized according to the Clusters of Orthologous Groups of proteins (COGs) (Tatusov et al., 2000). The proteins identified in this study were searched against the UniProt (http://www.uniprot.org/) and NCBI (https://www.ncbi.nlm.nih.gov/) databases. Their subcellular locations were analyzed using PsortB (http://www.psort.org/psortb/) and the cleavage of the signal peptide was inferred through SignalP version 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen, 2017). The prediction of lipoproteins was performed using LipoP version 1.0 (http://www.cbs.dtu.dk/services/LipoP/) (Rahman et al., 2008) and TMHMM version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to inferred the transmembrane helices in proteins. The moonlight proteins were identified using MoonProt database (Mani et al., 2015).

Protein extraction and SDS-PAGE electrophoresis

Proteins samples for total bacterial lysates and supernatants were extracted as previously described (Le Maréchal et al., 2009). For the extraction of proteins from the supernatant (SP), bacterial cultures were centrifuged at 7 000 *g* for 20 min at 4°C and the supernatants were filtered through a 0.45 µm filter. Then, the proteins were precipitated with 10% TCA at 4°C for 16 h and were centrifuged at 7 000 *g* for 90 min at 4°C. Protein pellets were washed with ethanol 96% and the samples were stored at -20°C. For total protein extracts (WC), cells were lysed with 200 µg/ml lysostaphin (Invitrogen) for 1 h at 37°C in Tris-EDTA buffer (Sigma-Aldrich). 10 µg of each extract (WC, SP and intact EVs) were treated for 10 min at 100°C in Laemmli buffer and separated by 12% SDS-PAGE (Laemmli, 1970) and the gel was subsequently stained with Bio-Safe Coomassie (Biorad).

Eukaryotic cell viability assay

The viability of eukaryotic cells was evaluated as previously described with slight modifications (Peton et al., 2014). Briefly, MAC-T and PS cell lines were seeded in 96-well plates at densities of 10^4 cells per well, cultured to 80% confluence and incubated for 24h with DMEM alone (mock control) and DMEM containing triton X-100 (0.01%) (positive control) or various quantities of *S. aureus* N305 EVs (0.01, 0.1, 1 and 10 µg per well). The cell viability was evaluated using 0.5 mg/mL Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich) according to manufacturer's protocol. The absorbance was evaluated at 570 nm and viability was expressed using 100% viability as mock control condition.

qRT-PCR gene expression

Confluent monolayers of PS cells were seeded in a 12-well cell culture plate at densities of 2.0 x 10^5 cells per well. Briefly, cells were washed twice with *Hank's* Balanced Salt Solution (HBSS) (D. Dutscher) and incubated for 3 h with DMEM (mock control), and DMEM containing living (N305) and heat-killed *S. aureus* N305 ($N305_{HK}$) cells at a multiplicity of infection (MOI) of 100 bacteria per cell, 10 µg of purified staphylococcal lipoteichoic acid (LTA) (InvivoGen, USA), and 10 µg and 20 µg of N305 EVs. Note that 20 µg of *S. aureus* N305 EVs corresponded to the relation of 1 µg per 10^4 cells in the viability assays. Once heat-treated for 30 min at 80°C, the samples were re-plating to ensure that all bacteria had been inactivated. After the incubation period, total RNA was extracted using RNeasy mini-kit (Qiagen) and treated with DNAse-free DNA Removal Kit (ThermoFisher Scientific) to remove residual genomic DNA according to manufacturer's instructions. RT-qPCR was carried out using first-strand cDNA synthetized from 500 ng of total RNA samples by qScript cDNA Synthesis kit (Quantabio). The PPIA (peptidyl-prolyl *cis-trans* isomerase A), RPL19 (ribosomal protein 19) and YWHA (14-3-3 phospho-serine/phospho-threonine binding protein) housekeeping genes were used as reference genes for normalization. Amplification was performed on a CFX96 real-time system (Bio-Rad, France) and the primers used in this study are listed in Table S1. The samples setups included biological triplicates and experimental triplicates. Genes considered significantly differentially expressed corresponded to those with a *P*-value < 0.05 (student's t-test) when compared to the mock control.

Intraductal inoculation of *S. aureus* N305-secreted EVs in the mouse mammary gland

The *in vivo* experimental mastitis study was conducted with a comparable protocol as previously described (Brouillette et al., 2004; Le Maréchal et al., 2011c; Peton et al., 2016). It was performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals under approval the Ethical Committee of the Faculty of Veterinary Medicine in the University of Ghent, Belgium (no. EC2015_127). Forty Hsd:ICR (CD-1) outbred lactating female mice (Envigo, The Netherlands) were mated with male mice and were used 12 days after birth of the offspring. The pups were separated 2h before of the intraductal inoculation in the fourth mammary gland pair. A mixture of oxygen and isoflurane (2–3%) was used for inhalational anesthesia of the mice and a bolus of PBS-diluted Vetergesic (i.e., buprenorphine 10 µg/kg, Val d'Hony Verdifarm NV, Belgium) was administered intraperitoneally (i.p.) as analgesic prior to any surgical intervention. The mammary gland duct was exposed through a small cut at the teat tip and each sample was

slowly intraductally injected at a volume of 100 µl with a 32 gauge blunted needle. Six groups of mice were simultaneously inoculated: two groups each received *S. aureus* N305-secreted EVs (at concentrations of 1 µg and 10 µg in phosphate-buffered saline or PBS, both n=7) and compared to a negative control group (sham) receiving PBS only (n=7). Three independent positive control groups were included in the study set-up for comparative purposes, a first one receiving 117 CFU of viable *S. aureus* N305 in PBS (N305, n=7), a second one receiving 100 CFU of heat-killed *S. aureus* N305 in PBS ($N305_{HK}$, n=6), and a third one receiving 10 µg of lipoteichoic acid (LTA) in PBS (InvivoGen, USA) (n=6). Twenty-four hours post-infection (p.i.), mice were sedated by an intraperitoneal administered mixture of ketamine (100 mg/kg Anesketin, Eurovet Animal Health BV, Bladel, The Netherlands) and xylazine (10 mg/kg; Xylazini Hydrochloridum, Val d'Hony-Verdifarm, Belgium) and, subsequently, euthanized through cervical dislocation.

Bacterial load, cytokine profiling and histology

Upon necropsy, all mammary glands were isolated and mechanically homogenized. A serial dilution derived from 20 µL of homogenate was plated on Tryptic Soy Agar to obtain a number of CFU per amount of tissue (g). To another 100 µL-aliquot of the homogenate 400 µL of lysis buffer supplied with protease inhibitors (200 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM oxidized L-glutathion, 100 µM PMSF, 2.1 µM leupeptin and 0.15 µM aprotinin) was added for later extraction of proteins. Mammary gland lysates were frozen overnight and centrifuged the following day at 12 250 *g* for 1 h. After recovering of the supernatant, the sample was quantified through Bio-Rad protein staining followed by spectrophotometry at 595 nm (Genesys 10S). All the samples were then adjusted to reach the same protein concentration (5µg/µL). Selected cytokine profiling was done using a bead-based multiplex immunoassay (ProcartaPlex, Thermo Fisher Scientific) for the simultaneous quantification of IL-1α, IL-1β, -6, TNF-α, MCP-1, CXCL2 (MIP-2), RANTES and BAFF and specific simplex immunoassays (ProcartaPlex) for mouse CXCL1 (KC) and IL-17A. All assays were performed in accordance with the manufacturer's instructions after in house validation for mammary gland matrix. Isolated mammary glands (n= 2 per condition) were fixed in 3.5% buffered formaldehyde, embedded in paraffin and sections were deparaffinized and stained with hematoxylin and eosin (H&E). Mammary gland tissues were visualized at x200 and x400 magnification.

Statistical analysis

The data were presented as mean concentration ± standard error. The differences between the animal groups were assessed using one-way analysis of variance, followed by Tukey's range test. The statistical program Prism 5 (GraphPad) was used considering significant a P-value lower than 0.05.

RESULTS

The bovine mastitis-associated *S. aureus* strain N305 produces EVs *in vitro*

EVs secreted by *S. aureus* N305 were isolated from the cell-free supernatants of stationary phase cultures. For that purpose, we used centrifugation, filtration and density gradient ultracentrifugation, the standard method for the isolation and purification of membrane vesicles with higher purity (Dauros Singorenko et al., 2017; Yamada et al., 2012). Homogeneity and integrity of vesicles were evaluated by both negative staining electron microscopy and cryo-electron tomography (cryo-ET). Electron micrographs of purified EVs revealed nano-sized vesicular structures with a typical cup-shape (Raposo and Stoorvogel, 2013) (Fig. 1A). Cryo-ET analysis showed homogeneously shaped spherical particles (Fig. 1B). The size distribution of EVs was estimated by three different methods: nanoparticle tracking analysis (NTA), tunable resistive pulse sensing (TRPS) and cryo-ET. Average EV sizes were 67 ± 13 nm (mean and standard deviation) for TRPS, 91 ± 23 nm for cryo-ET and 126 ± 2 nm for NTA (Fig. 1C). Although average size of EVs may vary according to the analytical method due to the limitations of each methodology (Maas et al., 2015; Van der Pol et al., 2014; Sitar et al., 2015; Van Der Pol et al., 2010), these complementary approaches highlighted the monodisperse size distribution of *S. aureus* N305 EVs. In addition, the total particle count evaluated by TRPS and NTA was similar and close to 4×10^9 particles per mL of treated supernatant. These results demonstrated that the bovine mastitis-associated *S. aureus* strain N305 released a high number of EVs homogenous in size and shape under laboratory culture conditions.

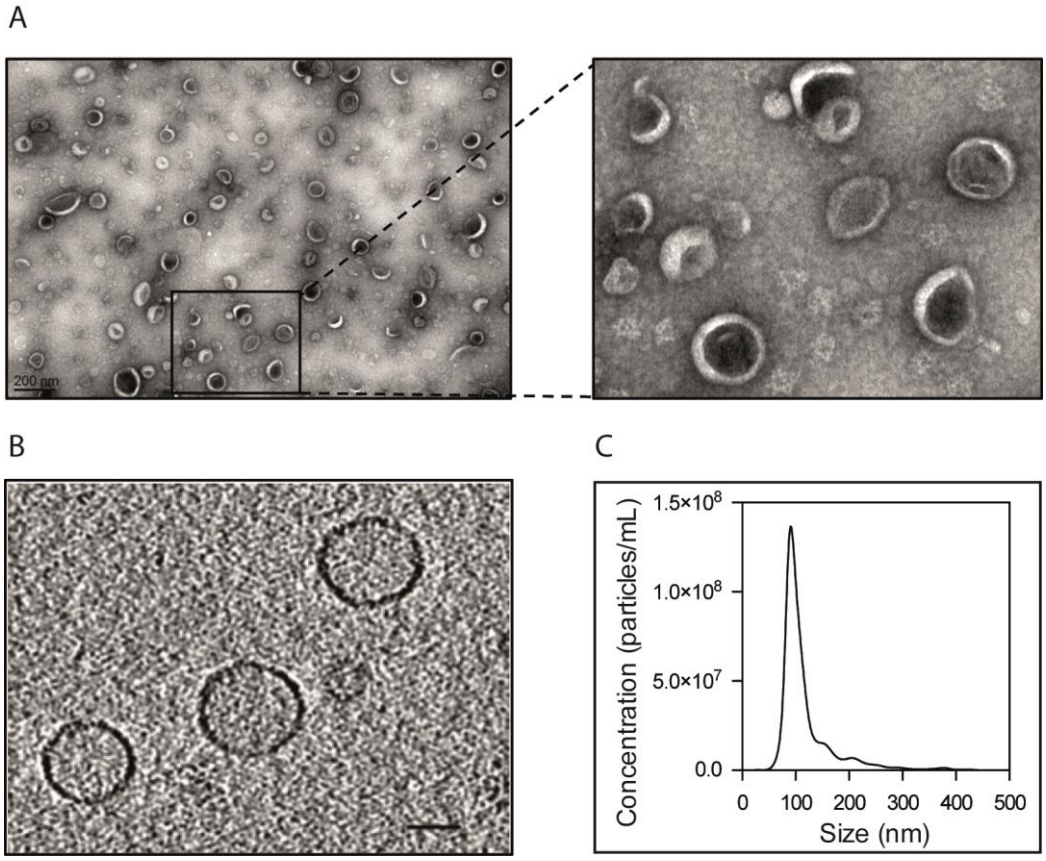

Figure 1. **Bovine *S. aureus* Newbould 305 (N305) releases EVs in vitro**. TEM of *S. aureus* Newbould 305 (N305) purified EVs after negative staining (A) and selected EVs. (B) Slice through a cryo-electron tomogram obtained from *S. aureus* N305 EVs. (C) Representative graph of size distribution of *S. aureus* N305 EVs measured with nanoparticle tracking analysis (NTA).

Bovine *S. aureus* N305-secreted EVs carry virulence factors

In addition to their structural characterization, cargo proteins of *S. aureus* N305-secreted EVs were determined through LC-MS/MS analysis of their proteomic profiles on three independent purified samples. The pattern of proteins associated with purified *S. aureus* N305-secreted EVs differed from that of the other bacterial cell fractions (Fig. 2A). A total of 222 proteins were consistently identified (Table S2), with the majority (n=160) predicted to be either cytoplasmic (n=89) or putatively membrane-associated (n=71) (Fig. 2B), showing that shedding of EVs appears to be also a pathway for protein secretion in *S. aureus* N305. The latter were overrepresented in EVs when compared to the predicted whole membrane proteome (32% versus 26%). More than half (34/58) of the number of predicted lipoproteins from the whole proteome (i.e. proteins with a signal peptidase II cleavage site) were identified, indicative for their relative enrichment in EVs (Fig. 2C).

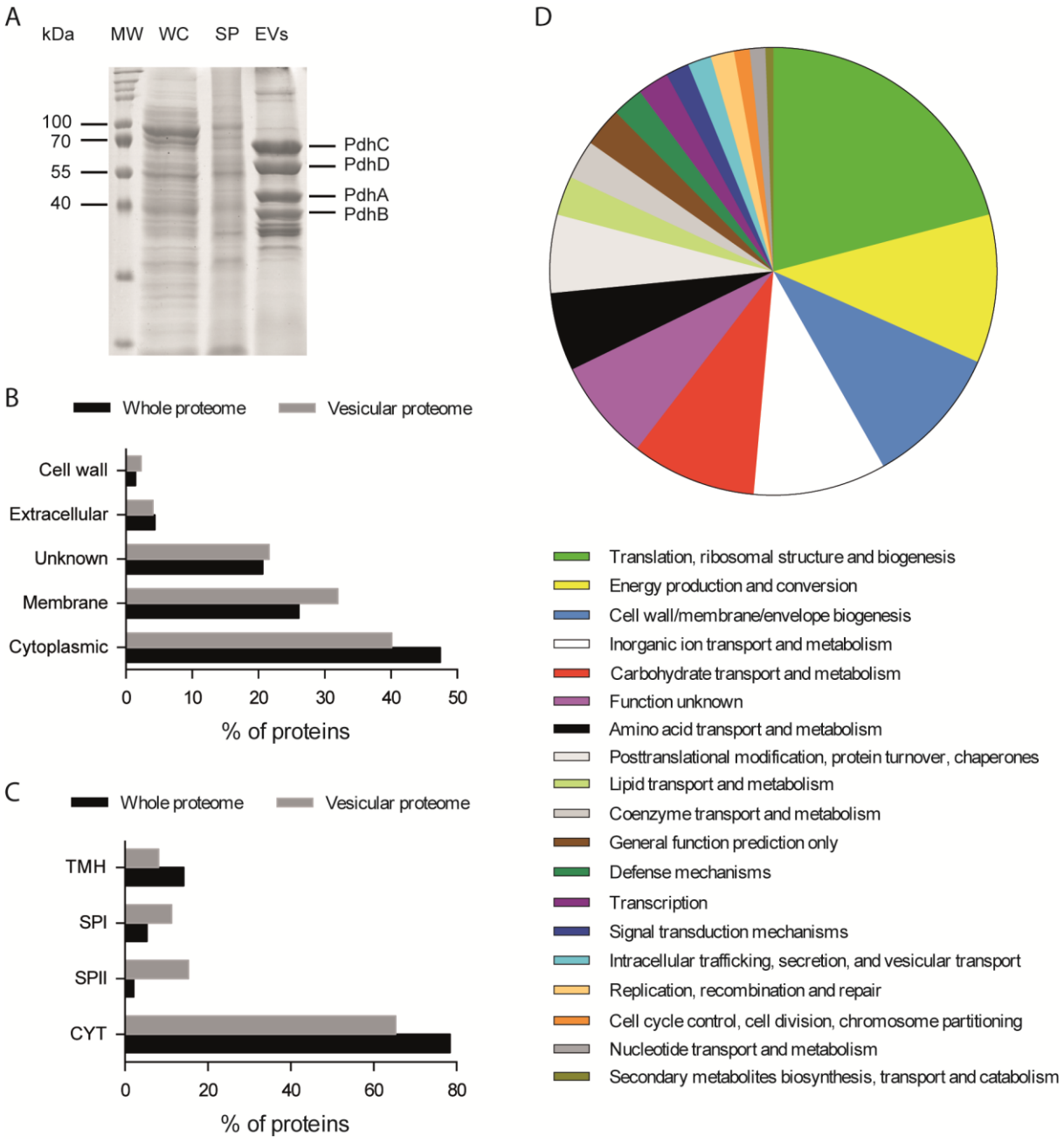

Figure 2. Identification and distribution of proteins associated with *S. aureus* N305-secreted EVs. (A) SDS-PAGE (12%) protein separation. Lanes: MW, Molecular weight standards are indicated on the left (kDa); WC, whole-cell lysates; SP, supernatant; EVs, *S. aureus* N305 EVs. (B) Protein distribution compared to whole bacterial proteome based on their localization (PsortB). (C) Specific protein distribution based on their localization (LipoP). TMH: N-terminal transmembrane helices; SPI and II: signal peptidase I or II; CYT: cytoplasmic proteins. (D) Protein distribution based on their COG annotation (IMG source).

These identified proteins were involved in various bacterial processes (Fig. 2D). In comparison with the whole *S. aureus* N305 proteome, some COGs were overrepresented in *S. aureus* N305-secreted EVs related to translation, ribosomal structure and biogenesis (19.0% versus 9.0%), energy production and conversion (10.4% versus 5%), cell wall (9.4% versus

5.7%), membrane and envelope biogenesis (9.4% versus 5.7%) and defense mechanisms (5.8% versus 2.8%). Furthermore, proteins with moonlighting abilities, such as autolysin, enolase, GAPDH and elongation factor Tu were identified. Most importantly, EVs contained numerous virulence factors (Table 1) including the immunoglobulin G-binding protein (Sbi) (Burman et al., 2008; Jeon et al., 2016; Lee et al., 2009), penicillin-binding protein (PBPs) (Jeon et al., 2016; Lee et al., 2009; Lowy, 2003), elastin binding protein (EbpS) (Park et al., 1996), the autolysin (Atl) (Hirschhausen et al., 2010; Jeon et al., 2016; Lee et al., 2009), the phenol soluble modulins (PSMs) (Cheung et al., 2014; Jeon et al., 2016) suggesting that *S. aureus* N305 EVs contribute to pathogenesis.

Table 1. Potentially associated virulence factors identified in *S. aureus* Newbould 305-secreted EVs.

Gene ID	Description	Function	Reference
ADHESION AND TISSUE DAMAGE			
Adhesion and internalization			
Newbould305_1791	Fibrinogen-binding protein (FnBP)	Binds to host fibrinogen	(Rivera et al., 2007)
Newbould305_2258	Elastin binding protein (ebpS)	Promotes binding of soluble elastin peptides and tropoelastin to *S. aureus* cells	(Park et al., 1996)
Evasion of host immune system			
Newbould305_2589	Immunoglobulin G-binding protein (Sbi)	Interacting selectively and non-covalently with an immunoglobulin	(Burman et al., 2008)
Toxins			
Newbould305_2342	Delta-hemolysin (Hld)	Lyses erythrocytes and many other mammalian cells	
PSMA1_STAAB	Alpha-class phenol-soluble modulin (PSMα1)	Pathogenesis	
PSMA2_STAAB	Alpha-class phenol-soluble modulin alpha 2 (PSMα2)	Pathogenesis	
PSMA4_STAAB	Alpha-class phenol-soluble modulin alpha 4 (PSMα4)	Pathogenesis	(Vandenesch et al., 2012)
Newbould305_1816	Beta-class phenol-soluble modulin (PSMβ1)	Pathogenesis	
Newbould305_1817	Beta-class phenol-soluble modulin (PSMβ2)	Pathogenesis	
Newbould305_2380	Uncharacterized leukocidin-like protein 2	Cytolysis in other organism; Pathogenesis	
Regulatory system			

Newbould305_2136	Peptide methionine sulfoxide reductase regulator MsrR	Involved in SarA attenuation. Role in resistance to oxacillin and teicoplanin, as well as the synthesis of virulence factors	(Rossi et al., 2003)
Poorly characterized			
Newbould305_1662	Staphylococcal secretory antigen ssaA2	Immunogenic protein of unknown function	(Dubrac and Msadek, 2004; Lang et al., 2000)
Newbould305_1498	Putative transcriptional regulator LytR	Cell wall organization	(Sharma-Kuinkel et al., 2009)
Newbould305_1676	Putative transcriptional regulator LytR	Cell wall organization	
CELL WALL, MEMBRANE AND ENVELOPE BIOGENESIS			
Resistance			
Newbould305_2227	Penicillin-binding protein 2 (PBP2)	Response to antibiotics	(Lowy, 2003)
Newbould305_0327	Penicillin-binding protein 3 (PBP3)	Response to antibiotics	
Newbould305_1169	Penicillin binding protein 4 (PBP4)	Response to antibiotics	
Newbould305_1499	Protein FmtA	Affects the methicillin resistance level and autolysis	(Komatsuzawa et al., 1999)
Newbould305_1724	Membrane-associated protein TcaA	Response to antibiotics	(Maki et al., 2004)
Envelope biogenesis			
Newbould305_0797	Teichoic acid biosynthesis protein F	Cell wall organization; Teichoic acid biosynthetic process	(Fitzgerald and Foster, 2000)
Newbould305_1248	Lipoteichoic acid synthase (LTA synthase)	Catalyzes the polymerization of lipoteichoic acid (LTA) polyglycerol phosphate	(Karatsa-Dodgson et al., 2010)
INFORMATION STORAGE AND PROCESSING			
Newbould305_1067	DNA-directed RNA polymerase subunit beta	DNA-directed 5'-3' RNA polymerase activity	(Wichelhaus et al., 1999)
METABOLISM			
Newbould305_1866	Serine/threonine-protein kinase PrkC	Cellular response to peptidoglycan; Spore germination	(Debarbouille et al., 2009)
MOONLIGHTING PROTEINS			
Newbould305_0110	Peptidoglycan endo-beta-N-acetylglucosaminidase	Hydrolase activity	(Heilmann et al., 2003, 2005)
Newbould305_1307	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	(Modun and Williams, 1999)
Newbould305_1311	Enolase	Catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate	(Antikainen et al., 2007)

S. aureus N305-secreted EVs are not cytotoxic against bMEC *in vitro*

We first evaluated whether *S. aureus* N305 EVs could affect the viability of eukaryotic cells. For that purpose, two bovine mammary epithelial cell (bMEC) lines, MAC-T and PS were treated for 24 h with growing EVs doses: 0.01, 0.1, 1 and 10 µg per well. The analysis of viability by MTT analysis did not reveal any differences between the MAC-T and PS control cells and the cells exposed to EVs (Fig. 3). These results suggest that *S. aureus* N305 EVs did not induce the cytotoxic effect in both MAC-T and PS cells in the tested conditions (Fig.3).

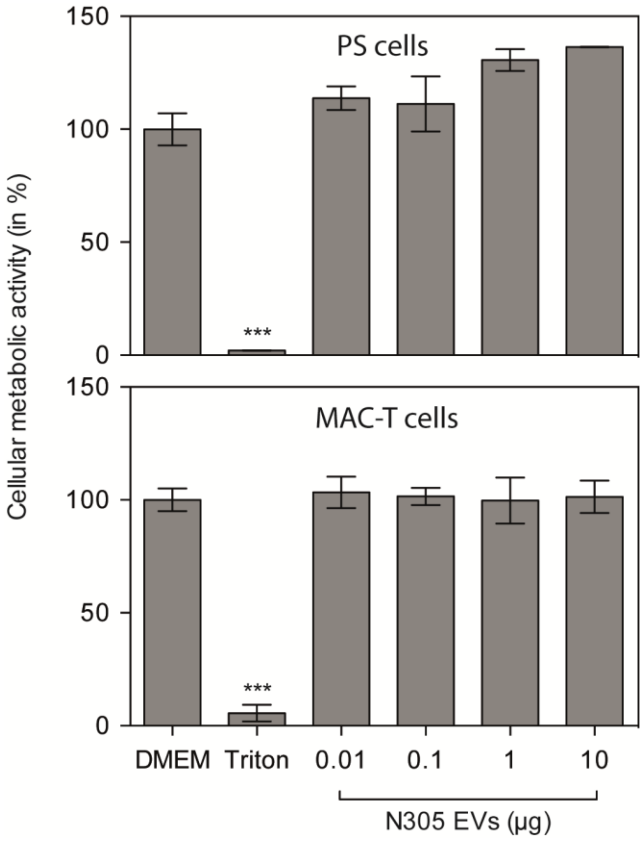

Figure 3. *S. aureus* N305-secreted EVs are not cytotoxic in vitro on MAC-T and PS bovine mammary epithelial cells. Either MAC-T or PS cells were treated with different EVs doses: 0.01µg, 0.1µg, 1µg and 10µg for 24 h. DMEM alone was used as mock control. Cellular metabolic activity was evaluated by MTT. The results are shown as the percentage of the control. Data are presented as mean ± SD. Each experiment was done in triplicate. The differences among the groups were assessed by ANOVA. Tukey's Honestly Significant Difference test was applied for comparison of means. No cytotoxic effect of EVs in MAC-T or PS cells was observed after 24h of treatment.

S. aureus N305-secreted EVs induce an immunostimulatory response in bMEC _in vitro_

To examine whether *S. aureus* N305-secreted EVs could induce the host's immunity *in vitro*, particularly the innate defense, the PS cell line was then treated with *S. aureus* N305-secreted EVs (10 and 20 µg per well). Live and heat-killed *S. aureus* N305 (25 µg) and LTA, a pro-inflammatory component of the *S. aureus* envelope (von Aulock et al., 2003) were used as complementary positive controls. The expression levels of host genes coding for key pro-inflammatory cytokines (IL-1β, IL-8 and TNF-α) and for the antimicrobial peptides β defensing-1 (DEFβ1) were compared to those of untreated PS cells (Fig. 4). A significant induction of all genes was observed after treatment with live *S. aureus* compared to untreated PS cells. In contrast, no differences in IL-1, TNF-α and DEFβ1 expression were observed following treatment with heat-killed bacteria, while the IL-8 expression was slightly (fold-change = 1.7) but significantly increased compared to untreated PS cells., Treatment with the positive control LTA increased the expression level of tested genes. Finally, we observed a significant and dose dependent increase of IL-8, IL1-β, TNF-α and DEFβ1 expression level in presence of *S. aureus* N305 EVs (Fig. 3, EV_{10} and EV_{20}), either to a similar (IL-1β, DEFβ1) or slightly lower (IL-8, TNF-α) level than those following treatment of PS cells with live *S. aureus* N305. These results demonstrated the ability of *S. aureus* N305-secreted EVs to stimulate bMEC *in vitro* in a way similar to that of live bacterial cells.

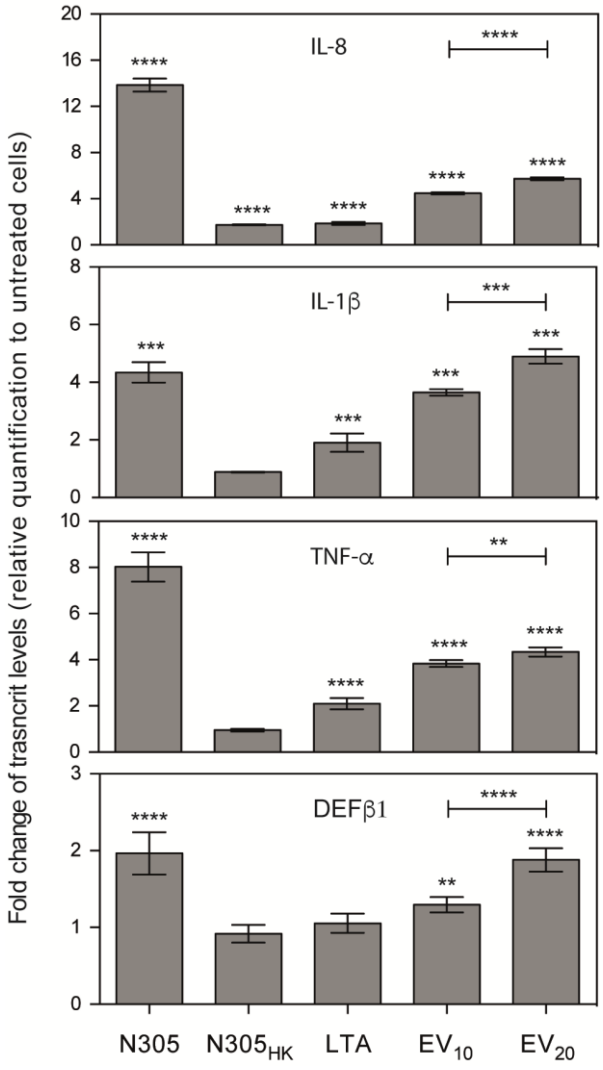

Figure 4. ***S. aureus*** **N305-secreted EVs induce an immunostimulatory response in vitro on PS bovine mammary epithelial cells**. Expression of IL-1β, IL-8, TNF-α and DEFβ1 by bovine mammary epithelial PS cells shown as fold changes after 3 h post stimulation with either living *S. aureus* N305 cells (N305), heat-killed *S. aureus* N305 cells ($N305_{HK}$), 10 µg of purified staphylococcal lipoteichoic acid (LTA), 10 µg and 20 µg of N305 EVs (EV_{10}, EV_{20}). Values were calculated as the mean ± SD obtained from three independent experiments after normalization to mock control DMEM. Asterisks indicate statistical significance as evaluated by one-way analysis of variance (ANOVA). ****, $P < 0.0001$; ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$.

S. aureus **N305-secreted EVs induce inflammatory and a local host innate immune response *in vivo***

To evaluate the *in vivo* modulation of *S. aureus* N305-secreted EVs on mammary gland inflammation, a well-defined experimental model of bovine *S. aureus*-induced mouse mastitis was used (Peton et al., 2016). Six groups of mice were inoculated with either PBS (negative control), live *S. aureus* N305 (first positive control), heat-killed *S. aureus* N305 (*S. aureus* $N305_{HK}$, second positive control), LTA (third positive control), *S. aureus* N305-secreted EVs

(1 µg, EV_1 or 10 µg, EV_{10}). At 24 h p.i., macroscopic signs of inflammation were observed in the glands that received live *S. aureus* N305, LTA and EV_{10} and in a much lesser extent in the glands that received *S. aureus* $N305_{HK}$ and EV_1. The mammary gland inoculated with *S. aureus* N305 had an average bacterial load of 8.94 ± 0.25 x log10 (CFU/g) at 24h p.i. and showed a profound edema and hemorrhage. This severe clinical response was attenuated in the 2 other positive control groups (*S. aureus* $N305_{HK}$ and LTA), and also in the EV_1 and EV_{10} (Fig. 5). Upon microscopical evaluation, a comparable influx of immune cells was observed in the alveoli of all treated mammary glands except for the PBS-inoculation (Fig. 5). Of note, mammary glands treated with EV_1 had less immune cells in their alveoli compared to EV_{10}-inoculated mice again indicating a stronger inflammatory response for the higher dose.

The local levels of cytokines IL-1α, IL-1β, IL-6, MCP-1 (CCL2), IL-17A, RANTES (CCL5), BAAF, MIP-2 and KC (CXCL1) were significantly higher in the mammary glands inoculated with live *S. aureus* N305 compared to PBS (Fig. 6). Inoculation with *S. aureus* $N305_{HK}$ and LTA also induced some of these cytokines (i.e., BAFF and KC) but this increase was much more modest (LTA: BAFF and KC $P < 0.0005$; *S. aureus* $N305_{HK}$: BAFF $P < 0.0005$ and KC $P < 0.05$). *S. aureus* N305-secreted EVs significantly induced several local cytokine levels i.e. MCP-1 (CCL2), RANTES (CCL5), KC, MIP-2 and BAFF compared to PBS. In addition, the increase of MCP-1, BAFF, MIP-2 and KC appeared to be dose-dependent. The local IL-1β level showed a modest and also a dose-dependent increase, albeit non-significant compared to the PBS control. In terms of chemokines, *S. aureus* N305-secreted EVs elicited even a stronger local response than both the *S. aureus* $N305_{HK}$ and LTA positive controls: for EV_{10} the average BAFF level was only slightly lower to that in live *S. aureus* N305-injected glands (91 ± 12 pg/mL versus 72 ± 25 pg/mL), while average KC levels (328 ± 110 pg/mL versus 205 ± 75 pg/mL) were even higher. These *in vivo* results demonstrated that *S. aureus* N305-secreted EVs induce a predominantly chemotactic local immunostimulatory response.

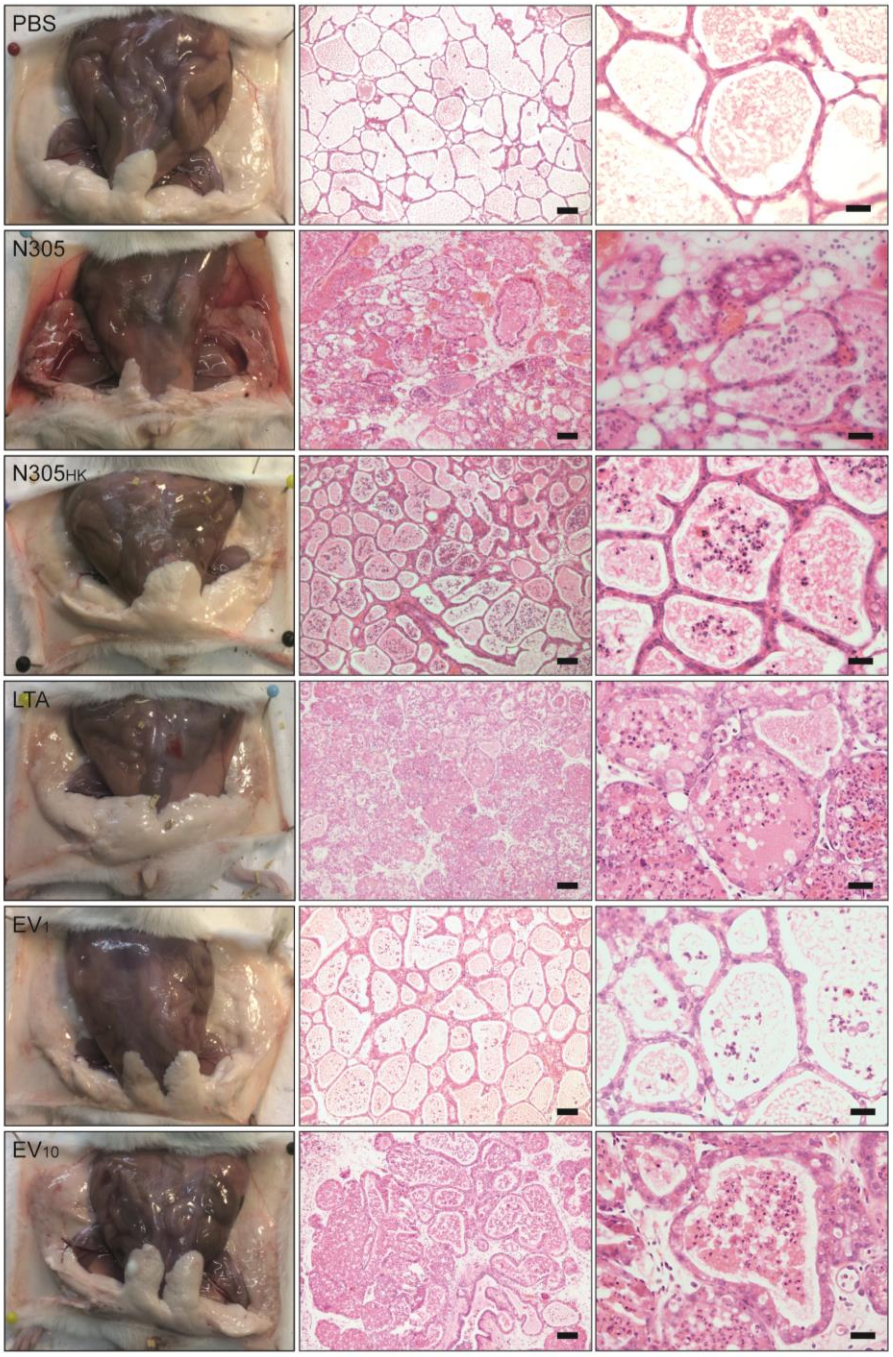

Figure 5. Histological consequences of the injection of N305 EVs in murine mammary glands. Right panel: Gross pathology of mammary glands. Representative photographs from dissected mice are shown. Conditions are PBS treatment (PBS) (negative control group), living *S. aureus* N305 cells (N305) (positive control group), heat-killed *S. aureus* N305 cells ($N305_{HK}$) (positive control group), 10 µg of purified staphylococcal lipoteichoic acid (LTA) (positive control group), 1 µg of EVs (EV_1) (test group) and 10 µg of EVs (EV_{10}) (test group). Macroscopic differences resulting from the different treatments of the mammary glands are clearly visible (e.g., prominent redness and inflammation in the *S. aureus* N305, LTA and EV_{10} groups). Middle and left panels: Representative H&E stained tissue sections from each group acquired at two magnifications are shown; middle panel: 20x, scale bar = 50 µm; left panel: 40x, scale bar = 20 µm. At 24 h p.i. the PBS group did not show any immune cell influx in the alveolar space, the *S. aureus* N305 group alveolar lumen had a profound hemorrhage and a stronger immune cell influx compared to the *S. aureus* $N305_{HK}$ and LTA groups. The EV_1 and EV_{10} groups had a dose-dependent recruitment of immune cells with an influx for EV_{10} similar to that observed in the LTA group.

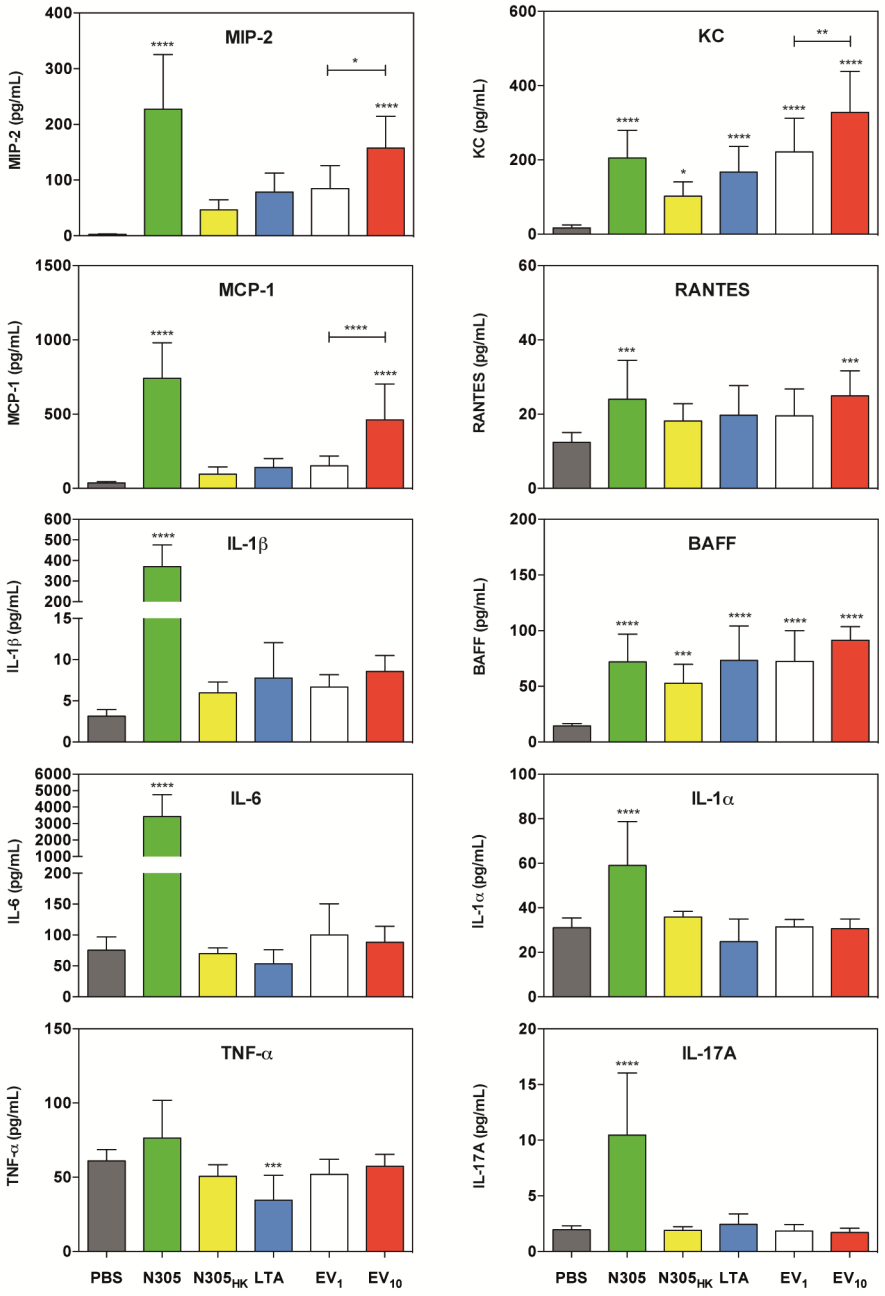

Figure 6. Immunological consequences of inoculation of *S. aureus* N305-secreted EVs in murine mammary glands. Cytokines were quantified from mammary gland lysates using multiplex immunoassay. Conditions are PBS treatment (negative control group), live *S. aureus* N305 (positive control group), heat-killed *S. aureus* N305 ($N305_{HK}$), positive control group), purified staphylococcal lipoteichoic acid (LTA), positive control group), 1 µg of *S. aureus* N305-secreted EVs (EV_1) and 10 µg of EVs (EV_{10}). EVs induced significantly the secretion of MIP-2, MCP-1, KC, RANTES and BAFF. The induction of MIP-2, KC and MCP-1 secretion was dose-dependent. The secretion of the cytokines IL-1α, IL-1β, IL-6 and IL-17A was only induced by *S. aureus* N305. The secretion of TNF-α was only induced by LTA. Asterisks indicate statistical significance compared to the negative control (PBS) as evaluated by one-way analysis of variance (ANOVA). ***, $P < 0.0005$; **, $P < 0.005$; *, $P < 0.05$.

DISCUSSION

The Gram-positive pathogen *S. aureus* infects a wide range of tissues and is one of the most important bacteria in bovine mastitis negatively affecting milk production worldwide (Peton and Le Loir, 2014). The different degrees of clinical manifestations can be correlated to inter-strains variations in terms of specific virulence factors (Le Maréchal et al., 2011a). Furthermore, our knowledge of the host-pathogen interactions, as well as the molecular basis associated with persistence of *S. aureus* infections remains to be fully elucidated. Although EVs have been associated with multiple *S. aureus* infectious processes this is not yet the case in veterinary medicine (Hong et al., 2011; Kim et al., 2012). Thus, our objective was to investigate if EVs are secreted by the bovine udder isolate *S. aureus* N305 and their role in the context of mastitis.

S. aureus N305 secreted EVs that displayed the basic features of extracellular prokaryotic membrane vesicles, i.e. a nanometric size range and a cup-shaped morphology and spherical structure (Raposo and Stoorvogel, 2013). In terms of their protein cargo, they appeared to be enriched with lipoprotein and membrane protein classes as also shown for human *S. aureus* strains and other bacterial species (Askarian et al., 2018; Brown et al., 2014; Deatherage and Cookson, 2012; Rath et al., 2013). Furthermore, *S. aureus* N305-secreted EVs share several proteins in common with those of *S. aureus* strains isolated from human clinical sources (Jeon et al., 2016; Lee et al., 2009) (Supplementary data), supporting the hypothesis that conserved regulatory mechanisms for cargo sorting may exist. A remarkable feature of *S. aureus* N305-secreted EVs was the predominance of virulence factors that accounted for approximately 10% of their vesicular proteome. Similar observations exist for other pathogenic Gram-positive bacteria, such as *Mycobacterium tuberculosis* (Lee et al., 2015), *Bacillus anthracis* (Rivera et al., 2010), *Streptococcus pneumoniae* (Olaya-Abril et al., 2014), *Listeria monocytogenes* (Lee et al., 2013b) and *Clostridium perfringens* (Jiang et al., 2014). This feature suggests that virulent protein delivery via EVs represents an important common mechanism in the development or progression of infections. Consistent with this, in the current study proteins involved in key steps of mastitis pathogenesis such as adherence to host tissues, development of lesions and tissue damage, and evasion from the host immune system were observed. *S. aureus* N305-secreted EVs also contained numerous proteins associated with metal ion acquisition, a mechanism essential for local bacterial proliferation and for circumventing nutritional immunity, as well as proteins involved in resistance to antimicrobial agents. Additionally, several moonlighting proteins with secondary roles closely related with pathogenesis (*e.g.* enolase, GAPDH, autolysin, Tuf) (Antikainen et al., 2007; Heilmann et al.,

2005; Modun and Williams, 1999; Widjaja et al., 2017) and lipoproteins involved in *S. aureus* Toll-like receptor 2 (TLR-2) activation and pathogenicity (Shahmirzadi et al., 2016) were identified. Collectively, our proteomic data provide strong indications for a role of EVs in *S. aureus* N305 pathogenesis.

Bovine mammary epithelial cells (bMECs) play an important role as the first line of defense against intramammary infections through the recognition of pathogens and the secretion of chemokines, cytokines and antimicrobial peptides that lead to neutrophil recruitment (Gray et al., 2005; Rainard and Riollet, 2006). Our *in vitro* data showed that bMECs exposure to *S. aureus* N305-secreted EVs led to a significant and dose-dependent increased expression of two pro-inflammatory cytokines (IL-1β, TNF-α), one chemokine (IL-8) and one bactericidal peptide (DEFβ1). Both cytokines are key elements of the early innate immune response in the mastitic mammary gland, comprising also the chemokine which is responsible for neutrophil recruitment and activation (Lahouassa et al., 2007), and the bactericidal peptide which is involved at the level of the oxygen-independent antimicrobial processes (Gurao et al., 2017). The ability to modulate the epithelial immune response has been described for EVs originated from both Gram-negative and -positive bacteria, including human *S. aureus* strains (Bauman and Kuehn, 2006; Bomberger et al., 2009; Ellis and Kuehn, 2010; Ismail et al., 2003; Jun et al., 2017; Kaparakis et al., 2010; Parker et al., 2010). Although the current study did not aim to unravel the molecular mechanism behind the response elicited by the *S. aureus* N305-secreted EVs, it observed no cytotoxicity on bMECs after 24h of incubation. At first sight, this finding may seem unexpected given the abundance of virulence factors within these EVs. However, although they all harbor an arsenal of virulence factors, cytotoxic activity is not shared by all *S. aureus*-secreted EVs (Gurung et al., 2011; Jeon et al., 2016; Jun et al., 2017; Thay et al., 2013). The presence or absence of cytotoxicity may result from proteome differences between *S. aureus*-secreted EVs (Jeon et al., 2016). EVs produced by *M. tuberculosis* induce a TLR2-dependent pro-inflammatory response via their lipoprotein cargo in interacting directly with the plasma membrane receptor that stimulates intracellular signaling cascades (Prados-Rosales et al., 2011). A plausible hypothesis states that *S. aureus* N305-secreted EVs stimulate bMECs in a similar way, since they are also enriched in lipoproteins that are TLR2 ligands (Shahmirzadi et al., 2016). However, a variety of other mechanisms of action may exist. For example, *Helicobacter pylori*-secreted EVs exhibit NF-κB-dependent pro-inflammatory activities via inflammasome-dependent signaling through the cytosolic NOD1 receptor after their fusion with the epithelial plasma membrane and delivery of their cargo into the cytosol (Kaparakis et al., 2010). Another example is the pore-forming

toxin cytolysin A delivered by *Escherichia coli* outer membrane vesicles which induces an epithelial proinflammatory response via alteration of the cellular Ca^{2+} homeostasis (Söderblom et al., 2005; Uhlén et al., 2000). Interestingly, *S. aureus* N305-secreted EVs also harbors toxins (PSMs, leukocidin) that are able to trigger Ca^{2+}-mediated host cell activation (Barrio et al., 2006; Forsman et al., 2012). Whether these EVs act extracellularly through ligand-receptor interactions, intracellularly after their internalization, or by inducing subtle perturbations such as on the cellular Ca^{2+} homeostasis to modulate the epithelial immune and inflammatory response remains to be investigated. It will be of high interest to examine more closely the role of lipoproteins and toxins in this modulation.

Consistent with our *in vitro* results, intramammary inoculations with *S. aureus* N305-secreted EVs elicited a local response with a dose-dependent immune cell recruitment and the induction of a pro-inflammatory cytokine profile. LTA is a major immunostimulatory of Gram-positive bacteria and can induce secretion of cytokines *in vivo* (Fournier and Philpott, 2005; Rainard and Riollet, 2006). Of relevance, these EVs were able to induce a higher *in vivo* response than LTA and *S. aureus* $N305_{HK}$ both at the histological and cytokine levels, which suggests their role in *S. aureus* N305 pathogenesis as immunostimulatory factors. The influx of inflammatory cells at inflammation sites is generally associated with elevated levels of CXC chemokines (Zlotnik and Yoshie, 2000). Accordingly, we detected an induction of the murine IL-8-like chemokines KC (CXCL1) and MIP-2 (CXCL2) reportedly involved in neutrophilic recruitment at inflammation sites (De Filippo et al., 2008; Leemans et al., 2003; Rollins, 1997). In addition, the levels of MCP-1, a monocyte chemoattractant (Rollins, 1997), RANTES, a monocytes, T cells, basophils and eosinophils chemoattractant (Arango Duque and Descoteaux, 2014) and BAFF, the B-cell-activating factor increased. The immune response induced by EVs was comparable to that observed with live *S. aureus* N305 although attenuated and not restricted to chemokine induction. Notably, the induction of IL-17, a critical cytokine for immune response and clearance of the pathogens at epithelial surfaces (Marks and Craft, 2009), was detected only with live *S. aureus* N305. These results showed that the immune response induced by *S. aureus* N305-secreted EVs might not be associated with IL-17-dependent T cell signaling. In addition live *S. aureus* N305 induced an increase production of several pro-inflammatory cytokines (i.e. IL-1α, IL-1β, IL-6), as previously reported (Breyne et al., 2014; Peton et al., 2016). These EVs appeared to mainly induce a chemotaxis related migratory response *in vivo* when compared to the responses induced by live *S. aureus* N305. This raises the question of the role of EVs and the biological significance of their pro-chemotactic effects during the *S. aureus* infectious process. In the infected udder,

colonization and invasion of the mammary gland by bacteria is followed by a recruitment of polymorphonuclear neutrophilic granulocytes, which are responsible for clinical symptoms and determine the course of infection. *S. aureus* cells are able to survive within a variety of host cells including professional phagocytes such as neutrophils (Voyich et al., 2005) and monocyte-derived macrophages (Kubica et al., 2008) that may serve as a vehicle for persistence and dissemination of the infection (Garzoni and Kelley, 2009). One could view the chemotactic activity of *S. aureus* N305 EV as a strategy to recruit phagocytic cells to allow the internalization of the bacterium and therefore its survival. This hypothetic strategy may also explain the persistence observed in *S. aureus* N305 infections. Additional studies are needed to better understand the role exerted by EVs in *S. aureus* pathogenesis, particularly with regard to strain-dependent clinical manifestations of mastitis and their involvement in chronic infection.

In summary, our study demonstrated at first that EVs are produced by the mastitis strain *S. aureus* N305 and that they induce an immunostimulatory response, both in bMEC *in vitro* and in a preclinical model of bovine mastitis. Furthermore, it provides evidence that *S. aureus* N305-secreted EVs principally modulate the chemotaxis of innate immune cells. These findings provide both novel insights in *S. aureus* mastitis pathogenesis and innovative avenues to control mastitis in which EVs are proposed as potential candidates for the development of vaccines as these are currently lacking in the treatment of Gram-positive udder infection.

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6. Chapter 3. Comparative proteomics of EVs released by human, bovine and ovine *S. aureus* strains

EVs have attracted attention in recent years due to their wide applicability in diagnosis and treatment of diseases (Fuhrmann, Neuer, et Herrmann 2017). One of the most promising applications involves the use of these nanoparticles as a tool for disease prevention (Van der Pol, Stork, et van der Ley 2015). In order to achieve this long-term perspective, new insights must be gained in terms of EVs formation, targeting capability and immunogenicity. The field of Gram-positive EVs suffers from a lack of knowledge when compared to Gram-negative OMVs. In *S. aureus*, the studies performed so far were focused on human clinical isolates.

In this chapter, we evaluated the ability of six *S. aureus* strains to produce EVs. The strains were selected according to their host and to the clinical manifestations they triggered during infection. The two bovine strains used in this project were *S. aureus* Newbould 305 (N305; Chapter 2) and *S. aureus* RF122. As previously mentioned (Chapter 2), *S. aureus* N305 was shown to induce moderate symptoms in cow mastitis (Bouchard et al. 2012; Prasad et Newbould 1968) and has been used for experimental challenges due to its ability to induce chronic mastitis (Bannerman et al. 2004; Hensen et al. 2000; Kozytska et al. 2010). *S. aureus* RF122 (ET3-1) was isolated in 1993 and corresponds to a widespread clone associated with severe mastitis in bovine. Furthermore, it was the first genome of animal *S. aureus* to be sequenced (Herron-Olson et al. 2007). The two selected ovine strains were shown to induce divergent degrees of virulence although they are closely related at the phylogenetic level. *S. aureus* O11 was isolated from a gangrenous mastitis and reproducibly induced severe symptoms in experimental ovine mastitis, while *S. aureus* O46 was isolated from a subclinical mastitis and induced a milder symptoms in experimental mastitis (Le Maréchal et al. 2011; Vautor et al. 2009). Among the human strains, we selected a hospital acquired methicillin-resistant and vancomycin-resistant *S. aureus* (HA-MRSA, VRSA) Mu50 (Kuroda et al. 2001) and the highly virulent community-associated MRSA (CA-MRSA) *S. aureus* MW2 (Baba et al. 2002).

Nowadays, EVs are regarded as a nonconventional means of protein secretion, with a yet-unknown mechanism for proteins selection and incorporation in intravesicular cargo. We evaluated the EVs protein cargo of the six *S. aureus* isolates in order to better understand the formation of these nanoparticles. A total of 261 proteins were identified and 44 of these proteins were conservatively released by EVs from all isolates. Most of them belonged to evolutionary conserved processes, such as the GAPDH and EF-Tu proteins.

This set of data did not allow correlating the EVs protein cargo with host specificity or type of infection; however, a certain level of organization and the enrichment of potential targets open up perspectives for further studies.

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Proteomic analysis of extracellular vesicles produced by Staphylococcus aureus strains isolated from human, ovine, and bovine hosts.

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen associated with both human and veterinary disease and is a common cause of mastitis. It is described as versatile and specialized bacterium that share with other Gram-positive bacteria, as well as Gram-negative, the ability to produce and secrete extracellular vesicles (EVs). The release of EVs constitutes a mechanism of cellular communication that enables inter-kingdom crosstalk, delivery of virulence factors and stimulation of the host immune response. However, the mechanisms by which Gram-positive bacteria release these nanoparticles are still unknown. Besides, packaging of proteins within EVs is responsive to several environmental conditions; however, cargo specificity in terms of the host has not been elucidated. To gain insight into the proteins that may contribute to the budding of the cell membrane, we characterize *S. aureus* EVs from six isolates (bovine, ovine and human) using a proteomic approach. Together, we identified 261 proteins, although the EVs protein cargo was strain-dependent. The major components in EVs were cytoplasmic or membrane-associated proteins and 44 of them were shared between all samples. This set constitutes a core proteome vesicular mainly formed by proteins evolutionary conserved, including those with moonlight activities. Among them are the fusogenic GAPDH and the adhesive elongation factor Tu (Ef-Tu). Lipoproteins and virulence factors were also shared between EVs purified from all isolates. This proteomic repertoire fortified the theory of cargo selection involving bacterial EVs. Furthermore, the concentration of proteins with targeting capability and antigen immunogenicity highlight the applicability of EVs for therapeutic purposes. The results revealed some factors that can be exploited in the future studies and can be useful in the development of diagnostic tools and formulations against *S. aureus* mastitis.

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive opportunistic pathogen that causes a wide spectrum of infections in humans and animals (Peton and Le Loir, 2014; Thomer et al., 2016). In humans, *S. aureus* diseases range from superficial skin and soft tissue infections to life-threatening diseases, requiring hospitalization and extensive medical support (Olaniyi et al., 2017; Thomer et al., 2016). In animals, *S. aureus* is notably responsible for ruminant mastitis, an inflammation of the mammary gland that dramatically affects animal health and welfare, is the main cause of antibiotic use in dairy herds, and induces huge economic losses in the milk production chain (Hata et al., 2008). *S. aureus* possesses a large arsenal of virulence factors, which include structural components and extracellular factors, such as enzymes and toxins (Magro et al., 2017). Despite huge research efforts, *S. aureus* colonization and pathogenesis in the various clinical manifestations of the infection are not fully understood yet (Mulcahy and McLoughlin, 2016).

Recent works have shown that *S. aureus* secretes extracellular vesicles (EVs) that interact with host cells *in vitro* and *in vivo* (Askarian et al., 2018; Gurung et al., 2011; Hong et al., 2011; Lee et al., 2009). EVs are lipid bilayers nanoparticles, which size ranges from 20 to 200 nm, and that pinch off from the bacterial membrane (Hasegawa et al., 2015). These nanoparticles are widely described in Gram-negative bacteria and are considered as cargo to release proteins, lipids, DNA and RNA in the extracellular medium (Kim et al., 2015). Production of EVs is described as a conserved cross-kingdom secretion system, which plays an essential role in long-distance delivery of bacterial effectors such as virulence factors (Celluzzi and Masotti, 2016; Kim et al., 2015). EVs were shown to be involved in numerous biological processes, such as biofilm formation (He et al., 2017; Im et al., 2017; Wang et al., 2015), horizontal transfer of genes (Dorward and Garon, 1990; Fulsundar et al., 2014; Yaron et al., 2000), stress response (Kobayashi et al., 2000), elimination of useless components (Tashiro et al., 2009), threat avoidance (Manning and Kuehn, 2011; Reyes-Robles et al., 2018), quorum sensing, and interactions with bacterial communities (Manning and Kuehn, 2013; Mashburn and Whiteley, 2005; Toyofuku et al., 2017).

S. aureus EVs can trigger an inflammatory response similar to that of the producing parent bacteria *in vivo* (Askarian et al., 2018; Choi et al., 2015; Hong et al., 2011; Kim et al., 2012). EVs cargo includes various proteins, some of which are known as potent antigens. EVs have therefore been considered as potential candidates for the design of vaccines (Van der Pol et al., 2015). The protein cargo of EVs was shown to vary with environmental conditions as well as with the producing strain. However, biogenesis of EVs and the process that leads to their

selective cargo remain poorly characterized (Wolf and Casadevall, 2014). Despite strain-to-strain variations in EVs cargo, we hypothesized that all *S. aureus* strains share an EVs core proteome and that the EVs accessory proteome contains key elements reflecting their adaptation to different human or ruminant hosts.

This study thus aimed to investigate the EVs production capacities of *S. aureus* strains isolated from human, bovine, and ovine hosts and to identify core and accessory proteomes of *S. aureus* EVs. The protein content of EVs released into the culture supernatant of 6 well-characterized *S. aureus* strains was identified by mass spectrometry and discussed with regard to host specialization.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strains *S. aureus* (Table 1) were grown in Brain Heart Infusion (BHI) medium (Difco, pH 7.4) at 37°C under shaking (150 rpm). Concentrations and growing phases of the bacteria were estimated by spectrophotometric measurements of optical density at 600 nm (OD_{600}) (VWR V-1200 spectrophotometer). They were further routinely confirmed by counting of the colony forming units (CFU) on BHI agar using the micromethod (Baron et al., 2006).

Table 1: *S. aureus* strains used in this study.

Strain	Isolated	Host	Type of infection[1]	Origin	CC	Reference
S. aureus Newbould305 (N305) (ATCC 29740)	1958	Bovine	Mild mastitis	Canada	CC97	Bouchard et al., 2012; Prasad and Newbould, 1968
S. aureus RF122	1993	Bovine	Severe mastitis	Ireland	CC151	Herron et al. 2002; Herron-Olson et al. 2007
S. aureus O11	2002	Ewe	Gangrenous mastitis	France	CC130	Le Maréchal et al., 2011a; Vautor et al., 2009
S. aureus O46	2002	Ewe	Subclinical mastitis	France	CC130	Le Maréchal et al., 2011a; Vautor et al., 2009
S. aureus Mu50 (ATCC 700699)	1997	Human	Wound infection (HA-MRSA; HA-VRSA)	Japan	CC5	Kuroda et al. 2001
S. aureus MW2 (USA400)	1999	Human	Hospital pediatric infection (CA-MRSA)	United States	CC1	Baba et al., 2002

[1]HA, Hospital –acquired; CA, Community-acquired; VRSA, Vancomycin-resistant *S. aureus*; MRSA, methicillin-resistant *S. aureus.*

Purification of *S. aureus*-secreted EVs from culture supernatants

EVs were purified from *S. aureus* culture supernatants using a method adapted from previous reports (Gurung et al., 2011). Sub-cultured cells at the end of exponential phase (OD_{600}= ~3.0) were diluted in 1L of fresh BHI medium. Cultures were grown until the stationary phase

to optimize the number of recovered EVs. The cells were then pelleted at 6 000 *g* for 15 min and the culture supernatant was filtered through a 0.22 µm vacuum filter (PES). The filtrate was concentrated around 100-fold using Amicon ultrafiltration system (Millipore) with 100 kDa filter and subjected to ultracentrifugation at 150 000 *g* for 120 min at 4°C. The EVs fractions with density around 1.08 - 1.13 g/cm^3 were collected after centrifugation at 100 000 *g* for 150 min at 4°C using a discontinuous sucrose density gradient (8% - 68%). Then, the corresponding fractions were pooled and were centrifugated at 150 000 *g* for 120 min and suspended in Tris-Buffered Saline (TBS) (150 mM NaCl; 50 mM Tris-Cl, pH 7.5). *S. aureus* EVs were quantified using Bradford reagent (Bio-Rad) to determine the amount of proteins and stored at -20°C before use.

SDS-PAGE

EVs (5µg) were treated for 10 min at 100°C in Laemmli buffer (Laemmli, 1970) and electrophoresis was done with a 12% resolving gel, subsequently stained with Bio-Safe Coomassie (Biorad).

Negative staining electron microscopy (EM)

Transmission electron microscopy was performed at the Microscopy Rennes Imaging Center platform (MRic MET) (University of Rennes 1, Rennes, France). Purified EVs were placed to copped grids and negatively stained with 2% uranyl acetate prior the visualization. *S. aureus* EVs images were obtained on a transmission electron microscope Jeol 1400 TEM (Jeol, Tokyo, Japan) operating at 120 kv accelerating voltage.

Cryo-electron tomography (Cryo-ET)

Vitrification of purified *S. aureus* EVs was performed as previously described (Tartaglia et al) and only EVs recovered from bovine isolates were evaluated by Cryo-ET. Briefly, mix-capped gold nanoparticles of 10 nm in diameter (Duchesne et al., 2008) were added to the purified EVs at a final concentration of 80 nM. After mixed with the fiducial markers, EVs were deposited to glow-discharged electron microscope grids followed by blotting and vitrification by rapid freezing into liquid ethane (Dubochet and McDowall, 1981). Grids were transferred to a single-axis cryo-holder (model 626, Gatan) and were observed using a 200 kV electron microscope (Tecnai G^2 T20 Sphera, FEI) equipped with a 4kx4k CCD camera (model USC4000, Gatan). Single-axis tilt series, typically in the angular range ±60°, were acquired under low electron doses (~0.3 e$^-$/Å^2) using the camera in binning mode 2 and at a

nominal magnifications of 29,000x . Tomograms were reconstructed using the graphical user interface eTomo from the IMOD software package (Mastronarde, 1997). Slices through the tomograms were extracted using the graphical user interface 3dmod of the IMOD package. Measurements were performed using the measuring tools available in the slicer panel of 3dmod.

Nanoparticle Tracking Analysis (NTA)

NanoSight NS300 (Malvern Instruments, Unitted Kingdom) with a 488 nm laser module and sCMOS camera type were used for all measurements of *S. aureus* EVs. The samples were measured in flow mode using a syringe pump and measurements were obtained by capturing 5 videos of 60 s of each purified vesicles. EVs were thawed and diluted in TBS until an optimum visualization of a maximum number of vesicles. All quantification was performed with temperature controlled at 25°C, and the captured data of flow measurements were analyzed using NTA 3.3 software (Malvern Instruments).

Identification of proteins in *S. aureus* EVs

Three independent biological replicates of each *S. aureus* purified EVs were digested for NanoLC-ESI-MS/MS analysis, except *S. aureus* Mu50 that was performed only once. Approximately 50µg of were pelleted at 150 000 *g* for 2 h at 4°C and suspended with the solution of 6 M Guanidine-HCl (Sigma-Aldrich), 50 mM Tris-HCl (pH 8.0) (VWR C) and 2 mM DTT (Sigma-Aldrich). EVs were heated at 95ºC for 20 min and cooled in 50 mM NH_4HCO_3 (pH 7.8) (Sigma-Aldrich). Then, samples were digested in solution using sequencing grade-modified trypsin (Promega) with the ratio 1:50 of enzyme:protein for 15 h at 37°C, as previously described by Lee et al. (2009). After digestion, the peptides were stored at -20°C until further analysis. Nano-LC experiments were performed as previously reported (Le Maréchal et al., 2011b), with minor modifications. Briefly, the peptide mixture was loaded using a Dionex U3000-RSLC nanoLC system fitted to a Q-Exactive mass spectrometer (Thermo Scientific, USA) equipped with a nano-electrospray ion source (ESI) (Proxeon Biosystems A/S). Samples were first concentrated on a PepMap 100 reverse-phase column (C18, 5 µm, 300 µm inner diameter (i.d.) by 5 mm length) (Dionex). Peptides were then separated on a reverse phase PepMap column (C18, 3 µm, 75 µm i.d. by 250 mm length) (Dionex) using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) TFA in deionized water). A linear gradient from 5 to 85% of solvent B was

applied for the elution at a flow rate of 0.3 µL/min. MS data was acquired in positive mode and the spectra were collected in the selected mass range 250 to 2 000 m/z at a resolution of 70 000 for MS and at a resolution of 17 500 for MS/MS spectra. The peptides were identified from the MS/MS spectra using the X! Tandem pipeline software (Langella et al., 2017) and searching against the genome sequence of the bovine strains (*S. aureus* N305, *S. aureus* RF122), the ovine strains (*S. aureus* O11, *S. aureus* O46 and *S. aureus* ED133) and the human strains (*S. aureus* Mu50, *S. aureus* MW2 and *S. aureus* TW20). A minimum of two specific peptides per protein was imposed and a false discovery rate (FDR) was set to 0.02% for peptide and 0.3% for protein identifications.

Bioinformatics analysis

All proteins were searched against the NCBI (https://www.ncbi.nlm.nih.gov/) and Uniprot (http://www.uniprot.org/) databases. The proteins identified in purified *S. aureus* EVs were analyzed using the following prediction tools: PsortB version 3.0.2 (http://www.psort.org/psortb/) was used to predict subcellular localization, and SignalIP version 4.1 (http://www.cbs.dtu.dk/ services/SignalP/) was used to predict the cleavage of the signal peptide (Nielsen, 2017). The prediction of lipoproteins was inferred using LipoP version 1.0 (http://www.cbs.dtu.dk/services/LipoP/) (Rahman et al., 2008). The Clusters of Orthologous Groups of proteins (COGs) (Tatusov et al., 2000) was used to categorized *S. aureus* EVs proteins. The moonlight proteins were identified using MoonProt database (Mani et al., 2015).

RESULTS AND DISCUSSION

In this study, we identified the proteins released via EVs in six *S. aureus* strains. EVs enable a contact-independent molecular trade between bacteria and their host and thus play a key role in the pathogenesis process in many pathogenic bacteria. Their exact role in *S. aureus* pathogenesis is still poorly documented although they were shown to interact with host cells *in vitro* and to induce an immune response *in vivo* in animal models (Askarian et al., 2018; Choi et al., 2015; Gurung et al., 2011; Jeon et al., 2016; Jun et al., 2017; Kim et al., 2012; Tartaglia et al.; Thay et al., 2013). Beyond their involvement in pathogenesis, EVs might also play a role in bacterial adaptation to environmental variations, such as thermal, pH, or oxidative stresses (Schwechheimer and Kuehn, 2015). Whether they play a role in host adaptation has not been investigated so far. Comparative proteomic analysis was carried out here on the EVs produced in laboratory conditions by 2 human-, 2 bovine-, and 2 ovine- *S. aureus* strains.

EVs production from *S. aureus*

For the comparison of EVs proteomes, we selected six well-characterized strains of *S. aureus* that were widely studied in various models of infections. These included the bovine strain *S. aureus* RF122, two ovine strains *S. aureus* O11 and O46, and the highly virulent human MRSA *S. aureus* MW2 (USA400). These four strains were first evaluated for their capacity to produce EVs *in vitro*, in our experimental conditions. The bovine strain *S. aureus* N305 and the human strain *S. aureus* Mu50, in which EVs production was already shown, were added to the study (Gurung et al., 2011; Tartaglia et al.; Yuan et al., 2018). In all isolates, cell-free supernatants of stationary phase cultures were harvested to prepare EVs samples as described in materials and methods. Purity and quality of EVs samples were confirmed by transmission electron microscopy (TEM). All the *S. aureus* EVs presented classical features of extracellular vesicles, including nanoscale size, spherical structure, and cup-shaped morphology when visualized by TEM (Fig.1) (Raposo and Stoorvogel, 2013; Szatanek et al., 2017). It also revealed that EVs samples prepared from animal isolates exhibited cylindrical (nanotube-like) structures that were further observed by cryo-electron tomography (Cryo-ET) of *S. aureus* N305 EVs (Fig. 1). These nanotubes were observed in two forms, either closed with encapsed molecules, or as opened structures. Such nanoparticles were previously observed, although with some differences regarding the size of the objects (Dubey et al., 2016). They have been described as parts of a “mating” mechanism that enables the transfer of intracellular molecules and DNA between bacterial cells in proximity (Baidya et al., 2018;

García-Aljaro et al., 2017). It is plausible that EVs and nanotubes are produced due to similar machinery (Baidya et al., 2018). Their absence in human strains has not been elucidated. MRSA strains reportedly produce a thicker cell wall and that feature might account here for the absence of such nanotubes in both MW2 and Mu50.

The size distribution and concentration of all strains were evaluated using nanoparticle tracking analysis (NTA). The size of *S. aureus*-derived vesicles was 129.1 ± 3.1 nm (mean and standard deviation) in diameter for *S. aureus* RF122, 135 ± 2 nm for *S. aureus* N305, 172.1 ± 1.6 nm for *S. aureus* O46, 139.4 ± 2.7 nm for *S. aureus* O11 and 129.7 ± 1.2 nm for *S. aureus* MW2. The EVs isolated from the human strain *S. aureus* Mu50 had a lower mean diameter of 98 ± 0.9 nm. Although the grown conditions were similar, the concentration of EVs obtained differed between the isolates. Except for the ovine isolate *S. aureus* O11 ($1.2x10^7$ particles per mL of treated supernatant), the total nanoparticles amounts were higher in animal isolates (*S. aureus* N305, $2.3x10^8$ particles per mL; *S. aureus* RF122, $3.4x10^8$ particles per mL; *S. aureus* O46, $2.7x10^8$ particles per mL) when compared to human isolates (*S. aureus* MW2, $3x10^7$ particles per mL; *S. aureus* Mu50, $4.6x10^7$ particles per mL).

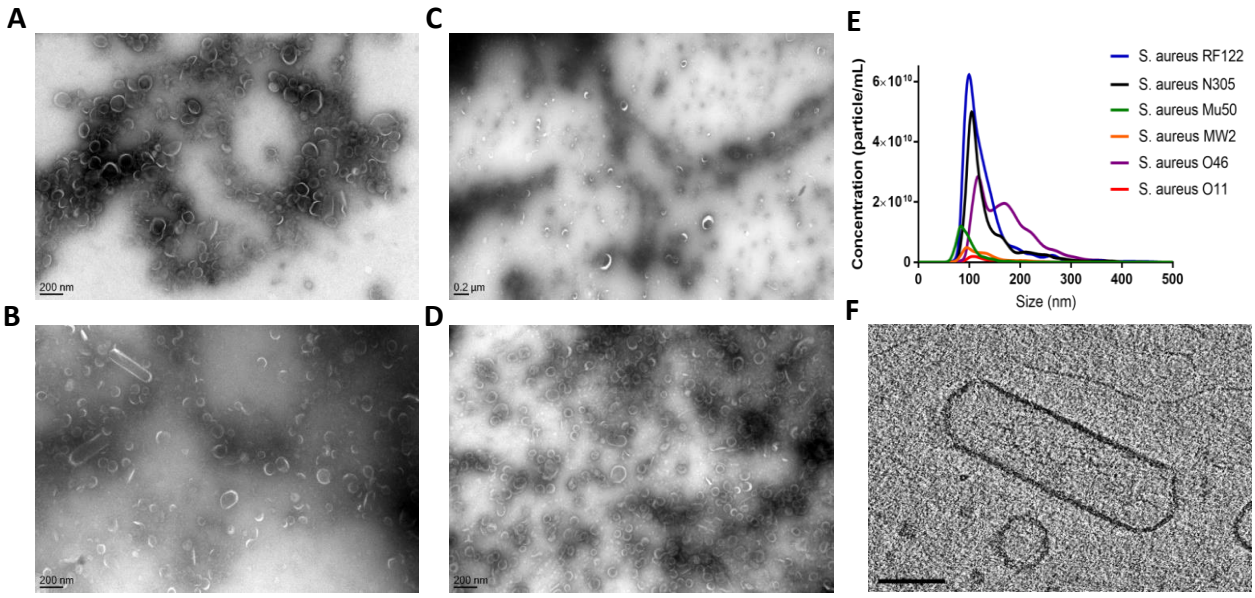

Figure 1. Purified *S. aureus* EVs in vitro. TEM of S. aureus purified EVs after negative staining (A) *S. aureus* O46; (B) *S. aureus* O11; (C) *S. aureus* MW2; (D) *S. aureus* RF122; (E) Representative graph of size distribuition of *S. aureus* EVs measured with nanoparticle tracking analysis (NTA) (F) Slice through a cryo-electron tomogram obtained from *S. aureus* N305 EVs with a nanotube-like structure (scale bar = 100 nm).

Identification of the *S. aureus* EVs proteins

S. aureus EVs were prepared from 3 independent cultures, except *S. aureus* Mu50 (one culture), and protein identification was carried out using Nano LC-ESI-MS/MS analysis. Proteomic analysis revealed a total 339 EVs proteins that were afterward screened according to their presence in at least two of the three biological replicates. Based on this screening, 261 proteins (77% of the total proteins identified) were further analyzed. This result shows that the overall protein cargo is well conserved and constant within the EVs production of individual strains. In summary, 97, 109, 104 and 96 proteins were analyzed for *S. aureus* MW2, *S. aureus* O46, *S. aureus* O11 and *S. aureus* RF122 EVs, respectively (Fig. 2). 173 and 149 proteins formed the vesicular cargo of *S. aureus* N305 and *S. aureus* Mu50, respectively. The 261 proteins identified in *S. aureus* EVs are listed in Supplementary Table 1.

Psort pipeline (Yu et al., 2010) was use to determine predicted subcellular localizations of the identified proteins. This enabled the classification of EVs proteins in cytoplasmic, cytoplasmic membrane, cell wall, extracellular and unknown. The majority of the proteins were predicted as cytoplasmic (n= 96) or cytoplasmic membrane-associated (n=82). 14 proteins were predicted as extracellular while 9 were determined as cell wall-associated (Fig. 3A). 21% (n = 54) of all the proteins identified presented positive predictions for a signal peptide using SignalP. To identify proteins that contain signal peptidase II cleavage site, we used LipoP tool (Rahman et al., 2008). According to this analysis, 16% (n=41) of the total EVs cargo were predicted as lipoproteins (*S. aureus* N305, n=30; *S. aureus* RF122, n=22; *S. aureus* MW2, n=26; *S. aureus* Mu50, n=23; *S. aureus* O46, n=21 and *S. aureus* O11, n=26) (Fig. 3A). They correspond to 52%, 39%, 43% and 34% of all the lipoproteins predicted in the whole proteomes of *S. aureus* N305, *S. aureus* RF122, *S. aureus* MW2 and *S. aureus* Mu50, respectively (Shahmirzadi et al., 2016; Tartaglia et al.) showing their relative enrichment in EVs. This class of surface proteins are recognized by Toll-like receptors 2 (TRL2) and can signal and modulate a host immune response (Nguyen and Götz, 2016). EVs enrichment with lipoproteins have also been previously reported in other pathogenic and non-pathogenic Gram-positive bacteria, such as *Streptococcus pneumoniae* (Olaya-Abril et al., 2014), *Streptococcus pyogenes* (Biagini et al., 2015; Resch et al., 2016), *Bacillus subtilis* (Brown et al., 2014) and *Mycobacterium tuberculosis H37Rv* (Lee et al., 2015).

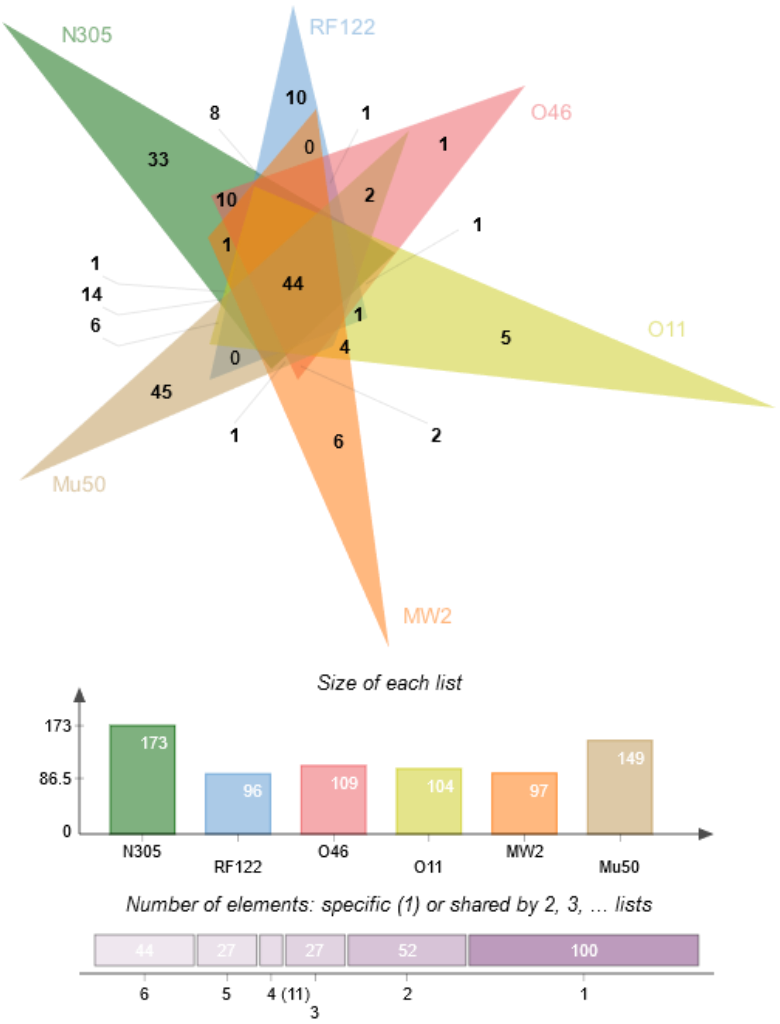

Figure 2. Venn diagram of the proteins identified in all S. aureus isolates. 44 proteins were common to the six isolates. Numbers in green regions and blue regions indicate the proteins identified in *S. aureus* N305 EVs (n=173) and *S. aureus* RF122 EVs (n=96), respectively; Numbers in pink and yellow areas correspond respectively to *S. aureus* O46 EVs (n=109) and *S. aureus* O11 EVs (n=104); Proteins identified in *S. aureus* MW2 and *S. aureus* Mu50 are shown respectively in orange (n= 97) and brown (n=149). The number of specific proteins identified in the set of proteins is indicated in the lower panel (Bardou et al., 2014).

Functional classifications of the *S. aureus* EVs proteins

The functional classification of the *S. aureus* EVs proteome was determined using a Clusters of Orthologous Groups analysis (COG). Overall, the majority of proteins was predicted as “translation, ribosomal structure and biogenesis” (16%; n=32) and “energy production and conversion” (10%; n=20). "Cell wall/membrane/envelope biogenesis", "inorganic ion transport and metabolism" and "carbohydrate transport and metabolism" each corresponded to 9% of the total of EVs proteins identified (Fig. 2C). Other studies have also shown that Gram-

positive-derived EVs contained many cytoplasmic proteins, including several ribosomal proteins and metabolic enzymes (Gurung et al., 2011; Jiang et al., 2014; Lee et al., 2009). Analysis of the functional categories of EVs proteins at the level of each single strain revealed subtle differences in the distribution of COGs. “Translation, ribosomal structure and biogenesis” was the majority group of the EVs proteins in most of the strains and corresponded to 14% in *S. aureus* Mu50, 14% in *S. aureus* MW2, 28% in *S. aureus* O46 and 21% in *S. aureus* N305 (Fig. 2D). COGs were overrepresented in EVs when compared with the whole proteome of *S. aureus* N305 (*e.g.* 21% versus 9% related to translation, ribosomal structure and biogenesis; 11% versus 5% related to energy production and conversion) (Peton et al., 2014; Tartaglia et al.). Conversely, translation, ribosomal structure and biogenesis form only 4% of the proteins found in *S. aureus* RF122-derived EVs, with an enrichment of proteins associated to energy production and conversion (20% versus 5%) and inorganic ion transport and metabolism (14% versus 7%) when compared to the whole proteome (Peton et al., 2014).

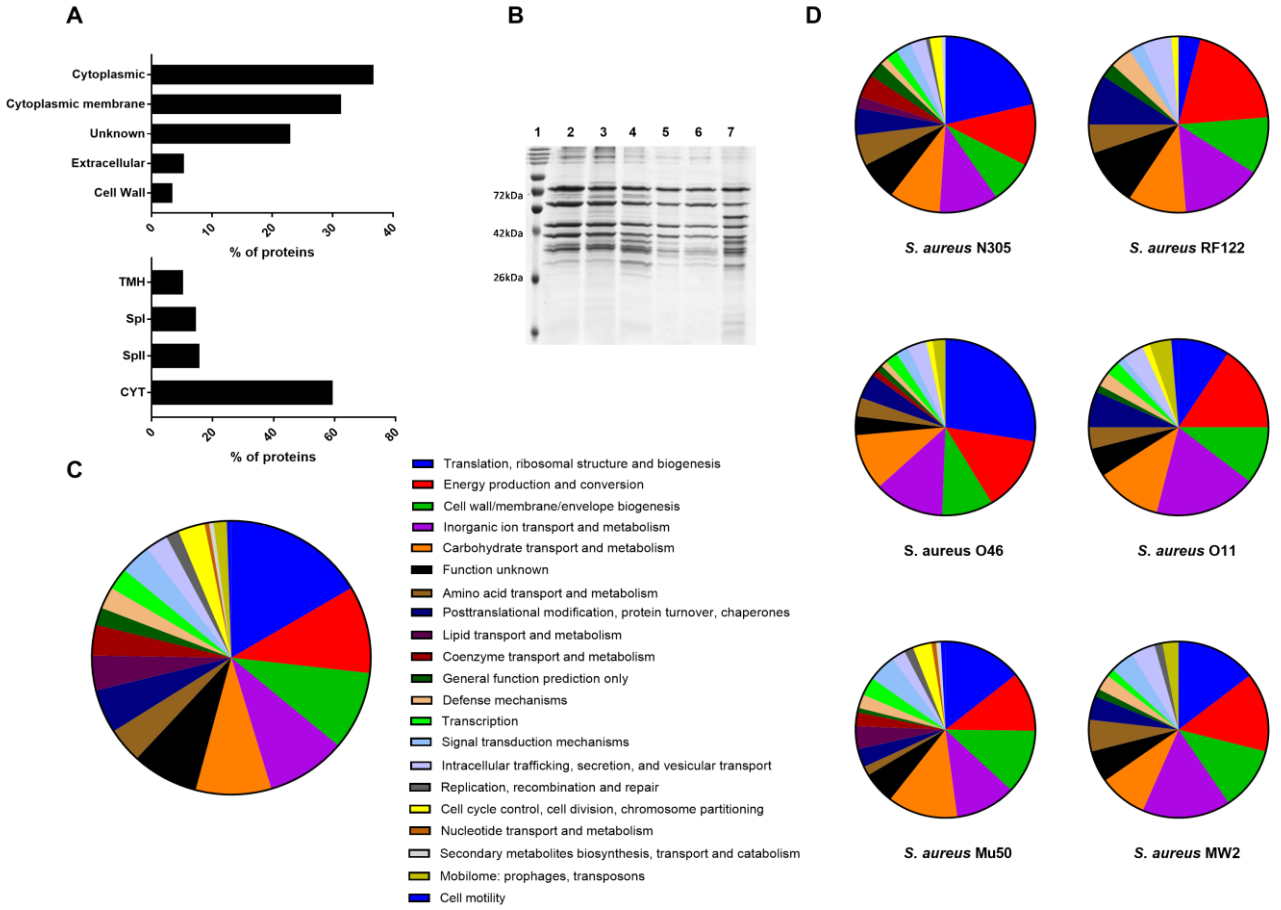

Figure 3. Protein distribution based on their COG annotation (IMG source). (A) Proteins distribution based on their localization (PsortB); Specific protein distribution based on their localization (LipoP). TMH: N-terminal transmembrane helices; SpI and II: signal peptidase I or II; CYT: cytoplasmic proteins. (B) SDS-PAGE (12%) protein separation. Lanes: MW, Molecular weight standards are indicated on the left (kDa); 1: *S. aureus* N305; 2*: S. aureus* RF122; 3: *S. aureus* O46; 4: *S. aureus* O11; 5: *S. aureus* MW2; 6: *S. aureus* Mu50. (C) Overall of the protein distribution based on their COG annotation (IMG source). (D) Proteins distribution based on their COG annotation according to the isolates (Bovine: *S. aureus* N305 and *S. aureus* RF122; Ovine: *S. aureus* O11 and *S. aureus* O46; Human: *S. aureus* Mu50 and *S. aureus* MW2).

Core proteome of *S. aureus* EVs proteins

A total of 44 EVs proteins (17% of the 261 proteins analyzed) were common to all strains and therefore compose what can be considered the core proteome of *S. aureus* EVs (Figure 2; Table 2). Most of these proteins belonged to the groups of "Energy production and conversion" (n=9), "Inorganic transport and metabolism" (n=7), "Cell wall/membrane/envelope biogenesis" (n=5) and "Carbohydrate transport and metabolism" (n=4). Of note, 25% (n=11) of these 44 EVs proteins were predicted as lipoproteins (Table 2). The high number of lipoproteins found here suggest that these proteins are particularly targeted during the biogenesis of EVs. Many lipoproteins were previously demonstrated as highly antigenic (Nguyen and Götz, 2016). The feature of *S. aureus* EVs make them of particular interest for the development of anti-*S. aureus* vaccine (Mariotti et al., 2013; Yang et al., 2018).

The proteins categorized in "Energy production and conversion" include, among others, the pyruvate dehydrogenase complex and ATP synthase proteins. *S. aureus* EVs also contained proteins involved in iron uptake and storage (*e.g.* ferritin, ferrichrome ABC transporter lipoprotein, ferric hydroxamate receptor, iron complex transport system), and metal ion binding proteins (*e.g.* zinc transport system substrate-binding protein, molybdate-binding protein). Iron is an essential element for bacterial growth and virulence during the infection process (Wooldridge, 1993). The release of EVs containing siderophores may support the growth of deficient strains in low iron medium, as observed in *Mycobacterium tuberculosis*-derived EVs containing mycobactin (Prados-Rosales et al., 2014). Other proteins, like penicillin-binding protein (PBP2), sortase A (SrtA), transcriptional attenuators (LytR family) and poly (glycerophosphate chain) D-alanine transfer protein (DltD), form a group of proteins associated to envelope biogenesis. Changes in the membrane-associated proteins and peptidoglycan (PG) cross-linking have been correlated to increase EVs production and yield in Gram-negative and Gram-positive bacteria. For example, mutation and deletion of OmpA, Lpp and TolA/P (Tol-Pal), membrane proteins involved in outer membrane stability in Gram-negative bacteria, result in an increase in outer membrane vesicles (OMVs) (Deatherage et al., 2009; Schwechheimer et al., 2013). Another example is the mutant of the murein hydrolase N-acetylmuramoyl-l-alanine amidase (Δ*sle1*) in *S. aureus* that showed a reduction in EVs production when compared to the wild-type strain (Wang et al., 2018).

Proteins belonging to the main secretion system (Sec translocase) and ABC transporters were also found in the core proteome of EVs (Lee et al., 2009). Peptidyl-prolyl cis/trans isomerase (PrsA) is a lipoprotein with post-translocational folding catalyst functions that also belongs to

this vesicular core proteome (Sarvas, 2004; Sibbald et al., 2006). Whether these proteins are exposed at the EVs surface as they are predicted to be in the bacterial cells is not known. Similarly, their role in EVs cargo might differ from the role they play in the bacterial cell physiology.

The majority of the shared EVs proteins belongs to functional categories described as evolutionary conserved (Luo et al., 2015). Some proteins comprised in this core proteome are so-called moonlighting proteins, a special class of multifunctional proteins, including the bifunctional autolysin (Atl), elongation factor Tu (Ef-Tu), and Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) (Heilmann et al., 2005; Modun and Williams, 1999; Pasztor et al., 2010; Widjaja et al., 2017). Surprisingly, Enolase protein, another well-documented moonlight protein, is present in all strains except *S. aureus* MW2, which genome nevertheless comprises the *eno* gene (Henderson and Martin, 2013).

Autolysin is a bifunctional murein hydrolase that is processed in the *N*-acetylmuramoyl-l-alanine amidase and endo-β-*N*-acetylglucosaminidase (Hussain et al., 2015; Oshida et al., 1995). Beside its activity as peptidoglycan hydrolase, this autolysin also has adhesive functions and contributes to *S. aureus* internalization into host cells (Hirschhausen et al., 2010). Similarly, GAPDH has been shown to bind to a variety of host ligands including fibronectin, plasminogen and transferrin (Henderson and Martin, 2013). In spite of a role in energy metabolism and adhesion to host cell surfaces, its most outstanding feature is the ability to catalyze the fusion of membranes (Glaser and Gross, 1995; Whitworth and Morgan, 2015). Interestingly, a synergy between the fusogenic proprieties of the enzyme GAPDH and OMVs activity was previously proposed in the non-pathogenic *Myxococcus xanthus* (Evans et al., 2012). Another moonlight protein is EF-Tu, that appears to be physically associated with bacterial surface and outer membrane vesicles (OMVs) of *Acinetobacter baumannii* mediating their ability to bind fibronectin (Dallo et al., 2012). In *Bacillus subtillis*, EF-Tu localizes in a helical pattern underneath the cell membrane and plays a second role in cell shape maintenance colocalizing and interacting with MreB, an actin-like cytoskeletal element (Defeu Soufo et al., 2010).

Furthermore, even well-studied proteins might harbor additional functions that need to be discovered (Huberts and van der Klei, 2010) and can be involved in the process of production and release of EVs. Six hypothetical proteins were conservatively associated with *S. aureus* -derived EVs. The enrichment of EVs with these peculiar hypothetical proteins might participate in the *S. aureus* pathogenic process or EVs biogenesis.

Altogether, the conservation of proteins, including adhesion and fusion factors, in a core proteome of EVs produced by phylogenetically distant *S. aureus* strains fortifies the hypothesis that EVs are not randomly formed but that a dedicated mechanism selects and packs proteins into EVs. Some of the proteins characterized in this study were also detected in EVs purified from other *S. aureus* strains (*e.g.* PBPs, N-acetylmuramoyl-L-alanine amidase, GAPDH) (Gurung et al., 2011; Lee et al., 2009) and other Gram-positive bacteria (*e.g.* N-acetylmuramoyl-L-alanine amidase, GAPDH) (Kim et al., 2016; Lee et al., 2015), confirming this orchestrated target selection in bacterial EVs.

Table 2. Proteins belonging to the core EVs proteome.

GI	Locus Tag	UniParc	COG[1]	Gene Product Name	Genome Name	LipoP[2]	Psort[3]	Gene name
Energy production and conversion								
14246831	SAV1061	UPI00000D7857	COG1622	Quinol oxidase polypeptide II QoxA	*S. aureus* Mu50	SpII	CM	qoxA
14246863	SAV1093	UPI000000978E	COG1071	Pyruvate dehydrogenase E1 component alpha subunit	*S. aureus* Mu50	CYT	C	pdhA
14246864	SAV1094	UPI0000054B50	COG0022	Pyruvate dehydrogenase E1 component beta subunit	*S. aureus* Mu50	CYT	C	phdB
14246866	SAV1096	UPI0000129471	COG1249	Dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3	*S. aureus* Mu50	CYT	C	pdhD
118573756	SAB1987c	UPI00000545EA	COG0055	ATP synthase F1 subcomplex beta subunit	*S. aureus* RF122	CYT	CM	atpD
123548233	SAB1991c	UPI00000D78AB	COG0711	ATP synthase F0 subcomplex B subunit	*S. aureus* RF122	CYT	CM	atpF
123548234	SAB1988c	UPI00005FE6D1	COG0224	ATP synthase F1 subcomplex gamma subunit	*S. aureus* RF122	CYT	U	atpG
124007215	SAB1989c	UPI00005FE6D2	COG0056	ATP synthase F1 subcomplex alpha subunit	*S. aureus* RF122	CYT	C	atpA
298694330	SAOV_1039	UPI00003B159A	COG0508	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	*S. aureus* ED133	CYT	C	NA
Inorganic ion transport and metabolism								
14248057	SAV2284	UPI00000547D7	COG0614	Similar to ferric hydroxamate receptor 1	*S. aureus* Mu50	SpII	CM	NA
21203739	MW0573	UPI00098EE875	COG0614	Iron complex transport system substrate-binding protein	*S. aureus* MW2	CYT	C	NA
21205368	MW2197	UPI00000D7772	COG0725	Probable molybdate-binding protein	*S. aureus* MW2	SpII	U	modA
21205500	MW2328	UPI00000D9FAC	COG3443	Ribulose-phosphate 3-epimerase/zinc transport system substrate-binding protein	*S. aureus* MW2	SpII	CM	NA
123768519	SAB1825	UPI0000054490	COG1528	Ferritin	*S. aureus* RF122	CYT	C	ftn
298695443	SAOV_2224c	UPI00005FE701	COG4594	Ferrichrome ABC transporter lipoprotein	*S. aureus* ED133	SpII	CM	NA
726968822	SAV0631	UPI000005228B	COG0803	Lipoprotein	*S. aureus* Mu50	CYT	CM	NA
Cell wall/membrane/envelope biogenesis								
14246704	SAV0935	UPI00000D4741	COG3966	Poly (glycerophosphate chain) D-alanine transfer protein	*S. aureus* Mu50	SpI	C	dltD
14246826	SAV1056	UPI00000D7738	COG1316	Transcriptional attenuator, LytR family	*S. aureus* Mu50	SpI	CM	NA
14247221	SAV1450	UPI00000CAACF	COG0744	PBP2	*S. aureus* Mu50	CYT	CM	pbp2
14248084	SAV2310	UPI00000D775D	COG1316	Transcriptional attenuator, LytR family	*S. aureus* Mu50	SpI	CM	NA

14248302	SAV2528	UPI00000CAB21	COG3764	Sortase A. Cysteine peptidase.	*S. aureus* Mu50	SpI	CM	srtA
Carbohydrate transport and metabolism								
14246541	SAV0772	UPI000005229B	COG0057	Glyceraldehyde-3-phosphate dehydrogenase (NAD+)	*S. aureus* Mu50	CYT	C	gap
14248381	SAV2607	UPI000012F4C1	COG0579	Malate:quinone oxidoreductase	*S. aureus* Mu50	CYT	CW	mqo2
269940277	SATW20_07750	UPI0000696BD9	COG1299	PTS transport system, fructose-specific IIABCcomponent	S. aureus TW20	CYT	CM	fruA
323438876	SAO11_2281	UPI0001FAD546	COG4193	Bifunctional autolysin	*S. aureus* O11	SpI	Extra	atl
Intracellular trafficking, secretion, and vesicular transport								
14246734	SAV0965	UPI000012E4AA	COG0681	Type-1 signal peptidase 1B	*S. aureus* Mu50	TMH	CW	spsB
14247409	SAV1637	UPI00000D7784	COG0341	Protein translocase subunit secD/protein translocase subunit secF	*S. aureus* Mu50	SpI	CM	secF
14247410	SAV1638	UPI000005426C	COG1862	Protein translocase subunit yajC/ conserved hypothetical protein	*S. aureus* Mu50	CYT	CM	NA
Amino acid transport and metabolism								
14248186	SAV2412	UPI0000054B9D	COG1126	Aamino acid ABC transporter ATP-binding protein	*S. aureus* Mu50	CYT	CM	NA
21204360	MW1192	UPI000012B701	COG0174	L-glutamine synthetase	*S. aureus* MW2	CYT	C	glnA
Translation, ribosomal structure and biogenesis								
115502797	SAB2115c	UPI00000D76A9	COG0197	50S ribosomal protein L16	*S. aureus* RF122	CYT	C	rplP
123754568	SAB0499	UPI0000054837	COG0050	Elongation factor Tu	*S. aureus* RF122	CYT	C	tuf
Posttranslational modification, protein turnover, chaperones								
14247500	SAV1728	UPI000005224B	COG0265	Similar to serine proteinase Do	*S. aureus* Mu50	TMH	CM	NA
14247613	SAV1841	UPI000005229F	COG0760	Peptidyl-prolyl cis/trans isomerase	*S. aureus* Mu50	SpII	CM	prsA
Defense mechanisms								

14247703	SAV1931	UPI00000CA978	COG1131	Similar to ABC transporter (ATP-binding protein)	*S. aureus* Mu50	CYT	CM	NA
Signal transduction mechanisms								
14248188	SAV2414	UPI00000CAB6A	COG0834	Amino acid ABC transporter substrate-binding protein	*S. aureus* Mu50	SpII	U	NA
General function prediction only								
323439847	SAO11_1262	UPI0001B70093	COG4851	Lipoprotein/Protein involved in sex pheromone biosynthesis	*S. aureus* O11	SpII	U	NA
Function unknown								
14247344	SAV1573	UPI00000CAC7C	COG4864	Hypothetical protein	*S. aureus* Mu50	TMH	C	SSP1183
14248232	SAV2458	UPI00000CAB9C	COG4808	Hypothetical protein	*S. aureus* Mu50	SpII	U	NA
Unclassified function								
123548096	SAB1806c	UPI00000D7827		Monofunctional glycosyltransferase	*S. aureus* RF122	CYT	CM	mgt
14246862	SAV1092	UPI00000CAA03		Hypothetical protein	*S. aureus* Mu50	SpII	U	NA
14247561	SAV1789	UPI00000CAABD		Hypothetical protein	*S. aureus* Mu50	CYT	U	NA
323438943	SAO11_2265	UPI00005FE322		Hypothetical protein	*S. aureus* O11	SpII	U	NA
323439299	SAO11_1892	UPI0001FAD4D7		Hypothetical protein	*S. aureus* O11	SpII	U	NA

[1]Proteins are classified in Gene Ontology functional classes. Names are given according to annotation of available genomes and verified against NCBI and uniprot database;
[2]LipoP prediction: SpII, SPaseII-cleaved proteins; SpI, SPaseI-cleaved proteins; CYT, cytoplasmic proteins; TMH, transmembrane proteins;
[3]PsortB prediction: Extra, extracellular; CM, cytoplasmic membrane; C, cytoplasmic; U, Unknow;
NA: No Available;

Accessory proteome of *S. aureus* EVs.

S. aureus virulence-associated genes (*e.g.* those encoding virulence factors and antibiotic resistance) are part of the set of strain specific proteins (Table 3). Proteins associated to antibiotic resistance are indeed found in some EVs only. Penicillin binding protein 2' (PBP2', encoded by *mecA*) is specifically found associated in *S. aureus* Mu50 EVs, although the replicates must be concluded to obtain data and reliable conclusions. TcaA protein associated to teicoplanin resistance is specifically found in *S. aureus* Mu50 EVs whereas TcaA is present in the predicted proteome of all the other strains. Whether and why some strains selectively pack some proteins and not others in their own EVs remains to be clarified. The presence of proteins involved in antibiotic resistance is in accordance with the hypothesis that EVs enable bacteria to share transient features (Chattopadhyay and Jagannadham, 2015; Schaar et al., 2011, 2013; Stentz et al., 2015). *S. aureus* MW2 EVs also carry beta-lactamase, which is consistent with previous works that showed *S. aureus* EVs carry biologically active beta-lactamase and mediate survival of ampicillin-susceptible Gram-negative and Gram-positive bacteria in presence of ampicillin (Lee et al., 2013).

Altogether, 100 proteins (38%) were strain-specific (Figure 2) of which 45 and 33 were exclusively found in the *S. aureus* Mu50 and N305 EVs cargos, respectively. Of note, these 2 strains are also those with the highest number of EVs proteins identified. Overall, the comparison of EVs proteomic content do not suggest a correlation between EVs content and the different types of infection induced by *S. aureus* strains in the host. However, this difference may be associated with other specialized mechanisms attributed to EVs, such as the delivery of bacterial RNAs (Tsatsaronis et al., 2018).

Among *S. aureus* Mu50-specific EVs proteins, several virulence-associated proteins were identified: ECM and plasma binding protein, VraE and VraD (a two-component system involved in nisin resistance), Staphylococcal accessory regulator protein (*sarR*), leukocidins and Staphylococcal secretory antigen A. Some of them may mediate EVs adhesion and cytotoxicity in the host cells. Furthermore, peptidoglycan-N-acetylmuramate O-acetyltransferase (OatA), a protein associated with persistence and colonization of skin and mucosal areas, was also specifically found in *S. aureus* Mu50 EVs (Bera et al., 2005). The highest amount of proteins was obtained in purified EVs from *S. aureus* N305. Among the 33 *S. aureus* N305-specific EVs proteins, none were obvious virulence related and 5 were predicted as lipoproteins. *S. aureus* N305-specific EVs proteins include alkaline shock protein 23 (asp23), ATP-dependent metallopeptidase (FtsH), chaperone protein (DnaK) mainly involved in tolerance of different types of stresses (Kuroda et al., 1995; Liu et al., 2017; Singh

et al., 2007). Strain-specific EVs proteins were also identified in the other strains studied (*S. aureus* RF122; n=10; *S. aureus* MW2, n=6; *S. aureus* O11, n=5; *S. aureus* O46, n=1). Overall, a significant proportion of these strain-specific EVs proteins are described as hypothetical or probable proteins (*S. aureus* Mu50, n=10; *S. aureus* N305, n=7; *S. aureus* RF122, n=4; *S. aureus* MW2, n=3; *S. aureus* O11, n=2). Virulence factors were also shared between different isolates. Interestingly, δ-hemolysin, a toxin encoded by the RNAIII of the *agr* system, was found in all the EVs except in those of the *agr* deficient *S. aureus* Mu50 (Tsompanidou et al., 2011). Likewise, phenol-soluble modulins (PSMα2 and α4), which production is *agr*-dependent, were also identified all the strains except *S. aureus* Mu50 (Table 3).

The biogenesis of *S. aureus* EVs was recently associated to the quorum-sensing *agr* system (Im et al., 2017). EVs production indeed reportedly occurs at the end of the exponential phase and the beginning of stationary phase (Hagemann et al., 2014; Im et al., 2017). EVs are produced from budding of the cytoplasmic membrane, a process promoted by *S. aureus* PSMα peptides, which have surfactant-like activity and induce the disruption of the membrane (Ebner et al., 2017; Wang et al., 2018). Interestingly, we observed the absence of PSMα2 and 4 in the *agr*-deficient Mu50 strain. Moreover, this strain produced EVs with a smaller size and at lower concentration than the other strains. The absence of PSMα peptides in EVs produced by an *agr*-mutant of *S. aureus* was recently reported by Wang et al. (2018) who also showed that a deletion of the *psmα* genes significantly reduced the size and production yield of *S. aureus* EVs (Wang et al., 2018). Our data on purified *S. aureus* Mu50 EVs corroborate these results and suggest the important role of the *agr* system in production and release of *S. aureus* EVs. Besides a functional role of EVs containing PSMα peptides in the pathogenesis process with regard to pro-inflammatory and cytolytic properties of PSMα peptides, they could also stimulate EVs production of nearby bacterial cells.

Table 3. Pathogenesis-related proteins identified in *S. aureus* EVs.

	GI	Locus Tag	UniParc	Gene Product Name	Gene name (1)	Function	Mu50[1] (2)	O46[2] (2)	O11[3] (2)	N305[4] (2)	RF122[5] (2)	MW2[6] (2)
INVASION AND ESTABLISHMENT OF AN INFECTION												
Toxins	172046784	SAV2035	UPI00001110E1	Delta-hemolysin (Hld)	hld	Lyses erythrocytes and many other mammalian cells	-	1E+11	3,2E+25	3E+07	1E+16	1E+09
	21204223	MW1056	UPI00000D9D8F	Phenol-soluble modulin beta class	PSMβ	Pathogenesis	-	3,64	2153,4	20,54	2,2E+08	2153,4
	206557787	MW0406.1 PSMA4_STAAW	UPI000161A44A	Phenol-soluble modulin alpha 4	PSMα4	Pathogenesis	-	9	999	99	30,62	9
	206557785	MW0406.3 PSMA2_STAAW	UPI00015FD703	Phenol-soluble modulin alpha 2	PSMα2	Pathogenesis	-	9	1E+09	999	99	99
	206557786	MW0406.2 PSMA3_STAAW	UPI000161A449	Phenol-soluble modulin alpha 3	PSMα3	Pathogenesis	-	-	2,50	-	-	-
	14247777	SAV2004	UPI00000CACAB	Hypothetical protein/ Leukocidin family protein	NA	Cytolysis in other organism; Pathogenesis	73,98	-	-	-	-	-
	14247778	SAV2005	UPI00000CAF1E	Hypothetical protein/ Leukocidin family protein	NA	Cytolysis in other organism; Pathogenesis	3,43	-	-	-	-	-
Evasion of host immune system	446715237	SAV2418	UPI0000358A34	Immunoglobulin-binding protein	sbi	Interacting selectively and non-covalently with an immunoglobulin	907,51	3,64	8,08	10,00	-	-
Adhesion/invasion and internalization	14247252	SAV1481	UPI00000CADE3	Elastin binding protein	ebpS	Promotes binding of soluble elastin peptides and tropoelastin to *S. aureus* cells	-	0,35	-	3,64	-	-
	14246582	SAV0813	UPI00000CAD7F	Extracellular ECM and plasma binding protein	ssp	Adhesin that binds to the host cell extracellular matrix proteins fibronectin, fibrinogen, collagen, and vitronectin	0,25	0	0	0	0	0
	14247095	SAV1324	UPI00000D77A5	Thermonuclease	nuc	Nuclease activity	0,93	-	-	25,82	-	-
	14248419	SAV2644	UPI00000CACD9	N-acetylmuramoyl-L-alanine amidase	NA	Amidase activity	3,06	-	1,72	-	-	-
	323438876	SAO11_2281	UPI0001FAD546	Bifunctional autolysin	atl	Hydrolase activity	2,54	5,30	23,17	21,38	1,92	5,81
	447090640	Newbould305_2629	UPI00005FE29C	ATP-dependent metallopeptidase	ftsH		-	-	-	1,51	-	-
Poorly characterized	14248072	SAV2299	UPI000005225C	Secretory antigen precursor SsaA homolog	ssaA	Immunogenic protein of unknown function	5,81	1,15	2,16	1,15	-	2,16

	14248318	SAV2544	UPI00000548B8	Similar to secretory antigen precursor SsaA	NA	Immunogenic protein of unknown function	0,58	-	-	-	-	-
	21205393		UPI000005434E	Staphylococcal secretory antigen A	NA	Immunogenic protein of unknown function	99	-	-	-	-	-
	14248343	SAV2569	UPI00000CAC78	Immunodominant antigen A	isaA	Hydrolase activity, acting on glycosyl bonds	2,16	-	2,16	-	-	-
Regulatory system	109894865	SAB2167c	UPI00000D7855	Staphylococcal accessory regulator protein	sarR	Negative regulator of sarA transcription at late exponential and stationary growth phases	99	-	-	-	-	-
	14248084	SAV2310	UPI00000D775D	Transcriptional attenuator, LytR family			145,77	9	37,31	7,25	2,83	45,41
	14248476	SAV2701	UPI0000054977	Similar to vraD protein	vraD	Antimicrobial resistance	18,30	-	-	-	-	-
	14248477	SAV2702	UPI00000CAE09	vraE protein	vraE	Antimicrobial resistance	0,33	-	-	-	-	-
	14247657	SAV1885	UPI00000CACC0	Two-component sensor histidine kinase	vraS	Member of the two-component regulatory system VraS/VraR involved in the control of the cell wall peptidoglycan biosynthesis	1,05	-	-	0,53	-	0,33
	323439373	SAO11_1797 SAV2147	UPI00000CAF2B	Membrane-embedded lytic regulatory protein	NA		-	1,15	9	0,66	40,5	0,66
CELL WALL, MEMBRANE AND ENVELOPE BIOGENESIS												
Resistance												
	14245808	SAV0041	UPI00000DA0B7	Penicillin binding protein 2 prime	mecA	Antibiotic resistance	133,59	-	-	-	-	-
	14247323	SAV1552	UPI00000CAAD1	Penicillin-binding protein 3 (PBP3)	pbp3	Antibiotic resistance	0,67	-	-	0,58	-	-
	14247221	SAV1450	UPI00000CAACF	Penicillin-binding protein 2 (PBP2)	pbp2	Antibiotic resistance	85,97	10,49	74,64	45,41	13,17	25,56
	323439395	SAO11_1696	UPI0001FADC52	Penicillin binding protein 4 (PBP4)	pbp4	Antibiotic resistance	-	-	1,15	0,58	-	0,58
	269940929	SATW20_14350	UPI000197AC14	Very large surface anchored protein	ebh	Pathogenesis	-	-	0,01	-	-	-
	123547713	SAB0668	UPI00005FE32B	Lipoteichoic acid synthase	ltaS	Catalyzes the polymerization of lipoteichoic acid (LTA) polyglycerol phosphate	-	-	-	0,77	0,25	-
	14248130	SAV2356	UPI00000CA97D	TcaA protein	tcaA	Plays a major role in decreasing resistance to glycopeptide antibiotics	1,23	-	-	-	-	-
	14246827	SAV1057	UPI00000D76EA	Autolysis and methicillin resistant-related protein	fmtA	Function unknown	1,56	-	-	0,23	0,36	-
	123549386	SAB2212	UPI00005FE767	Lysostaphin resistance protein A	lyrA	Lysostaphin resistance	-	-	-	-	0,29	-

Envelope biogenesis												
	269939772	SATW20_02560	UPI00003B137F	Putative teichoic acid biosynthesis protein	tarF	CDP-glycerol glycerophosphotransferase activity	-	-	-	2,48	-	-
	269942161	SATW20_27120	UPI0001BE5B83	Beta-lactamase	blaZ	Beta-lactamase activity		-	-	-	-	2,45

(1), Gene names are given according to annotation of *S. aureus* Mu50, *S. aureus* RF122, *S. aureus* Newbould 305 (N305), *S. aureus* O11, *S. aureus* O46, *S. aureus* MW2, *S. aureus* TW20 and *S. aureus* ED133. NA: No Available;
(2), Exponentially Modified Protein Abundance Index (emPAI);
[1]Proteins identified in *S. aureus* Mu50 strain, (-) protein absent;
[2]Proteins identified in *S. aureus* O46 strain, (-) protein absent;
[3]Proteins identified in *S. aureus* O11 strain, (-) protein absent;
[4]Proteins identified in *S. aureus* N305 strain, (-) protein absent;
[5]Proteins identified in *S. aureus* RF122 strain, (-) protein absent;
[6]Proteins identified in *S. aureus* MW2 strain, (-) protein absent;

Herein, we characterized the production and proteome of EVs produced by several isolates of *S. aureus*. We showed that phylogenetically distant *S. aureus* stains secrete common elements through EVs. This core EVs proteome comprises proteins involved in the first steps of adhesion and fusion with host cells. This set of well-characterized *S. aureus* strains originating from human, ovine and bovine hosts did not allow the identification of host-specific EVs protein content with regard to host-specificity of *S. aureus*. The package of bacterial antigens, including lipoproteins and proteins with membrane-binding capacity, confirm the potential of *S. aureus* EVs in vaccine formulations. Engineered EVs with increased fusogenic abilities, non-cytolytic toxins and enriched with membrane proteins constitute an interesting vaccine strategy against S. aureus infections. Altogether, our proteomic repertoire evidence some targets that might be investigated in future studies on S. aureus pathogenesis and for the development of new methods to control S. aureus infections.

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7. Chapter 4. Critical analysis and perspectives

7.1. General discussion and conclusions

Mastitis is a disease with a high incidence worldwide and corresponds to one of the main problems in dairy farming and milk production. Strain-dependent features of *S. aureus* determine a high variability of clinical manifestations in the host. One of the most important aspects of *S. aureus* mastitis in bovine herds is its propensity to recurrence and chronicity. To control this type of infection, drug therapy is widely used although it increases the risk of emergence and spread of resistant bacterial strains. Furthermore, prophylactic measures, including effective vaccine formulations and probiotic therapies, still require research efforts to fully control mastitis. For that purpose, EVs, as natural vesicles filled with a cocktail of antigens and self-adjuvants properties, appear to be a promising vaccine platform. However, though EVs have been applied in vaccine formulations of meningococcal infections, their use for producing staphylococcal vaccines remains unexplored.

In eukaryotes, several studies involving the induction of an immune response (Lee et al., 2016), intracellular communication (Zhang et al., 2015), metastasis (Isola and Chen, 2016), angiogenesis (Todorova et al., 2017) have helped to understand the role of EVs as a mechanism of cellular communication. Furthermore, although great advances have been made in the past decades regarding EVs released from Gram-negative bacteria, research on EVs from Gram-positive bacteria is still emerging and Gram-positive EVs are still poorly documented. The long interval of more than 40 years between the first works involving purified EVs of Gram-negative and Gram-positive bacteria illustrates the gap that exists in this research field. EVs are nano-sized particules, for which purification and concentration methods appear quite tricky and methodological limitations contribute to the difficulties in gaining more insight in this field.

Invasive bacteria have evolved different strategies for interacting with the host and one of these mechanisms obviously occurs via EVs. EVs represent a great advantage for the bacterium since they enable a delivery of concentrated factors to their target cells. Considering pathogenic bacteria, it may favor the early stages of colonization and tissue damage in the host. Despite their harmful potential, it is reasonable to assume that EVs can favor the survival of *S. aureus* during the infectious process.

To date, all studies involving purified *S. aureus* EVs have been carried out on human strains of nosocomial interest. In this project, we attempted to better understand the

mechanisms associated with *S. aureus* mastitis and to provide new insights into the mechanism of production and release of EVs by this Gram-positive bacterium.

EVs – host interaction

In the first steps of the project, we evaluated the ability of *S. aureus* N305 to release EVs. The purification procedure was optimized in order to recover a large amount of EVs produced by N305 grown in laboratory conditions. Vesicles with the well-documented and typical cup-shaped morphology (doughnut) were observed by MET and quantified using NTA and TRPS. It is worth noting that *S. aureus* N305, a bovine strain associated with moderate mastitis, can release a large amount of EVs when compared to clinical isolates under the same conditions of growth and purification. Cylindrical (nanotube-like) structures were also recovered from culture supernatants, although EVs presented a monodisperse size. Through a proteomic approach, we identified the protein cargo associated with *S. aureus* N305-derived EVs.

EVs were enriched in cytoplasmic and membrane-associated proteins. These results were consistent with the previous reports on EVs protein content derived from *S. aureus* human isolates (Gurung et al., 2011; Lee et al., 2009). *S. aureus* N305-derived EVs were filled with important determinants of virulence, such as immunoglobulin G-binding protein (Sbi), penicillin-binding protein (PBPs), phenol soluble modulins (PSMs), elastin binding protein (EbpS). Some of them can mediate the adhesion of the bacteria to components of the extracellular matrix (ECM) (*e.g.* EbpS and FnBP) (Foster et al., 2014; Fraunholz and Sinha, 2012; Gordon and Lowy, 2008). Furthermore, Sbi can contribute to the bacteria evasion of host immune responses (Atkins et al., 2008) and toxins can promote membrane damage and favor the bacterial spread in the surrounding tissues (Otto, 2014). EVs were also enriched with lipoproteins, which are agonists and modulate a TLR2 response. This set of virulence factors led us to hypothesize that *S. aureus* N305 are also able of induce a cytotoxic effect on the host cells. Although concentrated in toxins, *S. aureus* N305-derived EVs had no cytotoxic effect on two bovine mammary epithelial cell (bMEC) lines in vitro. This result can be explained by previous works that showed cytotoxicity induced by *S. aureus* EVs *in vitro* depended on cell types (Gurung et al., 2011; Jeon et al., 2016). Concentration of EVs, as well as their protein cargo, are also factors that can influence their cytotoxic effects *in vitro* (Jeon et al., 2016). Furthermore, this indicates that EVs may interact and induce host cells responses in a more subtle way. Some other studies have indeed described the role of the Gram-positive-

and Gram-negative-derived EVs in the host immune response in different murine models (Hong et al., 2011; Prados-Rosales et al., 2011; Söderblom et al., 2005; Surve et al., 2016). When evaluated *in vitro*, N305 EVs were able to induce an immunostimulatory response in bMEC with an induction of the expression of genes encoding pro-inflammatory cytokines, such as IL-1β, IL-8 and TNF-α. The antimicrobial peptide β defensin-1 was also induced following treatment with EVs. In accordance with our results, OMVs from *H. pylori* and *P. aeruginosa* also induced the production of the pro-inflammatory cytokine IL-8 in epithelial cells (Bauman and Kuehn, 2006; Ismail et al., 2003). These results showed that *S. aureus* N305 EVs contribute to the stimulation of the innate immune system of the mammary gland without cytotoxic effects, even after 24h of stimulation. More specifically, increasing doses of EVs induced a dose-dependent response in the expression of the pro-inflammatory cytokines. These *in vitro* results suggested that EVs might participate in the mammary inflammatory response during *S. aureus* infection.

Therefore, EVs were evaluated in a mice model of mastitis. This methodology was developed in the 1970s as an alternative to the difficult management and infrastructure in cattle experiments and the limited response provided by *in vitro* models (Chandler, 1970; Notebaert and Meyer, 2006). In a murine model of experimental mastitis, *S. aureus* N305-derived EVs induced a slight and localized inflammation when compared to live bacterium. EVs treatment induced an influx of immune cells into the alveoli in a dose-dependent manner, which correlated well with the cytokines levels measured in mammary tissues. In accordance, PSMs found in the intravesicular content can also interfere with the chemiotactic activity of the neutrophils and monocytes (Liles et al., 2001; Peschel and Otto, 2013). More importantly, *S. aureus* N305-derived EVs mainly induced chemokines (*e.g*. MCP-1 (CCL2), RANTES (CCL5), KC, and MIP-2). This could be seen as a bacterial mechanism favoring an intracellular lifestyle during the infectious process, although the molecular mechanisms associated with EVs interaction, fusion, and stimulation of host cell requires further investigations. These results clearly suggest that EVs play a role in the inflammatory response in the mammary context, mainly involved in the cell recruitment. Besides pro-inflammatory responses, Gram-negative OMVs were involved in B-cell activation, interaction with complement system, and were shown to trigger a cell-mediated immunity in the host (Chatterjee and Chaudhuri, 2012).

In summary, in this part of the project, we demonstrated that EVs are able to stimulate mammary cells *in vitro* and to induce an immune response *in vivo*.

During these experiments, additional samples were collected. Notably, we collected mammary gland tissue after the EVs injections. These samples will be useful to further investigate the host response to exposure to *S. aureus* EVs, heat killed, or live *S. aureus* in a context of mastitis. See Perspectives.

Comparative proteomics

The release of EVs by *S. aureus* has been evidenced in the last decade. This explains the gaps in knowledge and the extensive research efforts that aim at uncovering the functional role and the potential applications of EVs in *S. aureus* pathogenesis. A better understanding of the factors involved in *S. aureus* pathogenesis, including the delivery of bacterial effectors and the host immune modulation mediated by EVs, could help in the development of satisfactory therapies to prevent and combat mastitis. Besides, the mechanism involving the biogenesis of Gram-positive EVs is still undetermined.

The high adaptability of *S. aureus* strains and the ability to transfer resistance are the important features to consider in the development of therapeutic approaches against *S. aureus* infections. For instance, one of the mechanisms proposed for the transmission of resistance, even non-hereditary, is through EVs (Lee et al., 2013). To gain insight on secreted proteins that may participate in EVs production, we selected six *S. aureus* strains isolated from different hosts and involved in several types of infection. All isolates released spherical membrane-derived vesicles with nano-scale diameters. However, some differences were observed regarding the amount of EVs recovered after the purification steps. Beside the spherical nanoparticles, cylindrical structures were co-purified in the supernatants of the animal strains only, as mentioned above. Given the importance of secreted factors in bacterial infection, EVs were characterized in relation to their protein cargo. Overall, we identified 261 proteins (*S. aureus* N305; *S. aureus* RF122; n=96; =173; *S. aureus* Mu50; n= 149; *S. aureus* MW2; n=97; *S. aureus* O46; n=109; *S. aureus* O11; n=104). Most of them were predicted as cytoplasmic, membrane-associated and enriched with lipoproteins, as already described in *S. aureus* (Askarian et al., 2018) and other Gram-positive EVs (Biagini et al., 2015; Lee et al., 2015). The protein composition was dependent on the parent bacterial strain.

The proteins that were found common to all the EVs constitute the *S. aureus* EVs core proteome. This core proteome was determined on phylogenetically distant strains. However, its determination was based on the analysis of a limited number of strains (n=6). With the investigation of a higher number of strains, one might expect a different and more restricted core proteome, and a large *S. aureus* EVs panproteome. Interestingly, most of these core

proteins were classified in COGs that correspond to evolutionary conserved proteins. In this group, we found murein hydrolases that were recently associated with the steps of peptidoglycan cleavage which is required for the vesicular formation (Lee et al., 2009; Wang et al., 2018). Three other moonlight proteins with a previously shown ability to interact with host ligands were identified: enolase, EF-Tu, and GAPDH (Henderson and Martin, 2013). In addition, GAPDH can be involved in the initial stage of membrane fusion with host cells. This reinforced both hypotheses that EVs are not randomly formed and that do not simply result from a cell lysis process (McBroom et al., 2006). We identified proteins that may support the growth of strains under stress conditions. Indeed, EVs containing proteins involved in iron uptake and storage and metal ion binding proteins were shown to allow the growth non-EVs producing strains, in laboratory conditions. PrsA is also common in all *S. aureus* EVs and can be involved in post-translocational folding of vesicular proteins. PrsA may support the package of biologically active proteins.

Beside this core EVs proteome, we analyzed the EVs protein content with the prism of host origin. When looking at the accessory EVs proteome, it was not possible to associate EVs protein cargo with any kind of host-specificity of the *S. aureus* producing strains.

Regarding the accessory EVs proteome, differences in size, concentration, and proteins content can nevertheless be associated with strain-specific factors. Such information can be useful for selecting good candidates for vaccine platform. Considering vaccine formulation, EVs are naturally produced by bacteria, are capable to stimulate innate and adaptive immunity and have intrinsic adjuvant effects. Several studies have shown the adaptive memory immune response induced by Gram-negative OMVs and, as vaccine formulations, they have been mainly studied against serogroup B of *N. meningitidis* (Chatterjee and Chaudhuri, 2012). As adjutants, EVs can be used to overcome, for instance, the toxicity, sensibility, and adverse reactions associated with the major adjuvants in the market (Tan et al., 2018).

Although our results were inconclusive about host-specificity of *S. aureus* EVs, they highlighted a level of organization between EVs in terms of moonlight, membrane-associated and cytosolic proteins with membrane-binding capacity. Although still preliminary, this work suggests that the *S. aureus* EVs sorting is not random and is governed by dedicated mechanisms. It also opens new perspectives in diagnostic and prevention against *S. aureus* mastitis and other infections.

Overall, this work opens a new perspective on the investigation of *S. aureus* pathogenesis in the mastitis context. Considering the different mechanisms evolved by *S. aureus* and the intense research for alternative treatment and prophylactic measures, the study in EVs field becomes breathtaking and promising.

7.2. Perspectives

This thesis was the first project carried out on the "world" of EVs in STLO (INRA Agrocampus Rennes) and LGCM (UFMG, Belo Horizonte), the 2 research teams involved. One of the barriers in working with this field is the EVs preparation and purification. This is a heavy, energy- and time-consuming task. From a methodological point of view, a good challenge is to optimize the method of EVs purification in terms of amount recovery and elimination of contaminants, such as aggregated proteins. Furthermore, the methodology requires a large volume of culture, and the yield of the EVs sample preparation is insufficient for all experimental steps required for the standardization of the *in vivo* and *in vitro* assays. The main perspectives of this project are briefly summarized in figure 13.

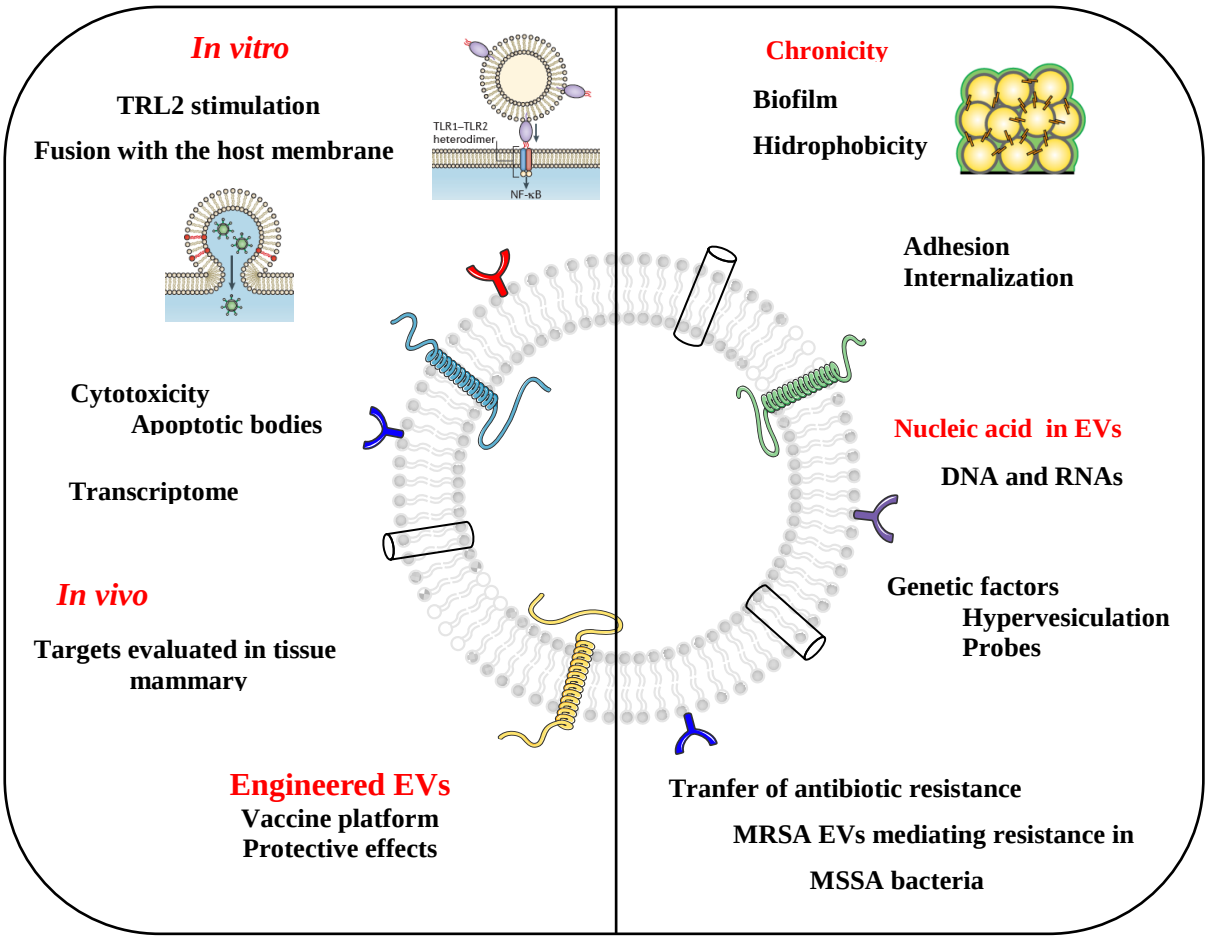

Figure 13. Schematic diagram with the main perspectives of this project.

Cargo characterization in terms of protein

Regarding these technical aspects, STLO has a huge expertise in membrane technology (notably, nano- and ultra-filtration) and it would be interesting to work with the in-house teams to set preparative protocols for EVs production and purification.

From a more scientific point of view, first, more isolates should be screened with regard to protein cargo to explore the possibility of a host adaptation via EVs. It would be interesting to insert in the experimental group EVs purified from goat strains. For a reliable identification of markers, the amount of the proteins should be reported in a quantitative manner. Furthermore, the enrichment of proteins should be reported in relation to the cytoplasmic membrane. In eukaryotes, the relative proportions of membrane-associated proteins between different subpopulation of EVs are different. It is reasonable that a similar relation can be observed in different bacterial strains. As mentioned above, proteomic analysis also should be reported correlated to the data of the whole cell lysate obtained from bacterial cells grown under the same laboratory conditions.

Cargo characterization in terms of nucleic acid

The lumen of EVs forms a favorable and protective environment for biologically active components, such as DNA and RNA. Whether or not *S. aureus* EVs contain nucleic acids remains to be elucidated. To date, only a few studies have been identified extracellular DNA (Jiang et al., 2014; Surve et al., 2016) and RNA (Resch et al., 2016; Surve et al., 2016) in Gram-positive-derived EVs. Nowadays, extensive studies have been carried out in extracellular RNAs as mediators of intercellular communication, although most of them have focused on eukaryotic exosomes. Interestingly, trans-kingdom gene regulation mediated by bacterial EVs has been recently postulated (Celluzzi and Masotti, 2016; Tsatsaronis et al., 2018). Analogously to eukaryotic exosomes, that deliver miRNA able to alter gene expression of the target cells, such EVs-based bacterial modulation may also occur through vesicular RNAs (Tsatsaronis et al., 2018).

We previously observed the presence of small RNA molecule in *S. aureus* EVs that were prepared at the very beginning of this Ph.D thesis, in collaboration with Zuzana Krupova (EXCILONE) during a training period at INRA Jouy en Josas. It would be very interesting to complete the proteomic analysis of *S. aureus* EVs with the analysis of their nucleic acid contents. DNA and RNA extraction and sequencing might indeed reveal features (*e.g.* RNA sequences that are complementary to DNA or RNA targets in eukaryotic genomes) related to

host specificity or that might be involved in the remodeling of gene expression profile of host cells.

Another feature that is poorly addressed so far in the studies on Gram-positive EVs is the lipid composition of the EV membrane. Whether this composition is similar to that of the cytoplasmic membrane of the producing bacterial cells is unknown. Likewise, it would be interesting to compare it to the membrane lipid composition of eukaryotic cytoplasmic membranes and or to that of eukaryotic exosomes. It is of particular interest in the case of mastitis since it is well-known that milk contains exosomes originating from the mammary gland tissues. Whether exosome and bacterial EVs can fuse together would be rather easy to check through in vitro experimental set up and the biological significance and impact of such fusion would be an exciting field of research.

Global transcriptome profiling of S. aureus infected udder

We have highlighted in our study that EVs purified from *S. aureus* N305 have an immunomodulatory effect in the host. This result indicates that EVs can in fact participate in the mastitis pathogenic process; however, more studies should be carried out to investigate in detail the molecular mechanisms involved in this phenomenon. First, the impact of *S. aureus* N305 EVs on the transcriptome of bovine mammary epithelial cells (and other cell types, such as those of the immune system) should be evaluated *in vitro*, on cell culture. This screening will enable the identification of gene targets for further research using mammary gland tissue we collected and conserved following the *in vivo* assays carried out on mice. These results will allow comparing the gene expression profile of the mammary gland after infection with EVs or with the live bacteria.

EVs and mammary infection

More *S. aureus* strains should be tested with regard to EVs released and to their immunomodulatory effects *in vitro* and *in vivo* in order to compare with the clinical profile of the infection. EVs of all strains were enriched with lipoproteins, which suggest (if we assume the reported antigenic properties of lipoproteins) that the immunostimulation in the host occurs via TRL2 (to be tested). However, it would be interesting to verify if the stimulation *in vitro* is similar when using purified EVs from strains originating from different hosts. Furthermore, the model proposed about EVs-mediated cytotoxicity involves the fusion of the vesicles with the membrane of the host cell and delivery of virulence factors in the eukaryotic cytoplasm. Considering the differences between the intravesicular content in terms of protein,

the cytotoxicity profile should also be different. Even if there is no induction of cytotoxicity *in vitro*, the formation of apoptotic bodies can be evaluated through immunofluorescent staining and microscopy.

In this work, EVs were able to stimulate mainly a chemotactic immune response *in vivo*. Considering the mammary infectious context, these results raise some questions regarded the biological signification of such observations:

How can an increase in the number of polymorphonuclear cells favor bacterial survival and or colonization?

Indeed, it would be interesting to evaluate whether EVs can facilitate the internalization or favor an intracellular lifestyle in professional and non-professional phagocytes.

Chronic infections are strongly correlated with the ability of strains to adhere and to internalize, to produce biofilm, and to induce cytotoxicity on host cells. Considering that *S. aureus* RF122 has lower internalization rate in bMEC when compared to *S. aureus* N305, it would be interesting to compare the level of bacterial adhesion and internalization after incubation with EVs purified from these two different strains.

The release of EVs favors the surface hydrophobicity of the Gram-negative bacteria, which enhances the biofilm formation (Baumgarten et al., 2012). Although *S. aureus* N305 and *S. aureus* RF122 not differ in terms of biofilm formation *in vitro* (Peton et al., 2014), they differ in terms of amount recovered of EVs. It could be interesting to compare the relation surface hydrophobicity and release of EVs in Gram-positive strains.

Comparative analysis between strains and under different environmental conditions

Considering that the content in proteins of *B. anthracis* EVs was correlated to their surface electrostatic charge, it would be interesting to compare the EVs from all isolates used in this project in relation to their charge. We observed differences in terms of the amount of proteins and repertoire of virulence factors. The zeta potential can be used to characterize the surface charge of EVs.

Different stress conditions can also be evaluated. It is interesting to characterize of EVs production of the different strains under iron limitation or conditions that mimic the stressful host environment. In this context, the protein profile packaged must be different which may help to understand the role of EVs in the pathogenesis.

Genetic factors involved in EVs release

Proteins associated with the cellular envelope were extensively associated with the biogenesis process of OMVs in Gram-negative bacteria. The identification of genes that can influence the levels of *S. aureus* vesiculation was initiated during my thesis project. In EVs, we identified proteins that were shown to enhance the adhesive proprieties of *S. aureus* cells, as GAPDH, enolase and, EF-Tu. The murein hydrolases (*e.g. Atl* and *sle*), PrsA, PBPs, PSMs are also relevant candidates, as some of them were recently shown to be involved in EVs release (Wang et al., 2018). The hypothetical proteins shared between the six strains can be evaluated as mutagenesis targets. Random mutagenesis approaches at the genome scale could also be used to unravel genetic determinants involved in EVs production if appropriate screening tools are developed.

Some targets can be marked (e.g Green Fluorescen Protein-GFP) and be used as probes in *in vitro* assays to investigate fusion of EVs with eukaryotic cells.

Mechanism of protein cargo selection

Identification of sequences motifs enriched in EVs through bioinformatics tools, which may act as a "zipcode" that targets molecules into bacterial EVs.

Community interactions

S. aureus has the ability to acquire multiple resistances to antibiotics, which explain why its epidemic success worldwide. We have highlighted that *S. aureus* EVs contained several proteins associated to antibiotic resistance and that some of them were strain specifics. Antibiotic resistance phenotype can be provided to non-resistant strains by EVs produced by antibiotic-resistant strains (Chattopadhyay and Jagannadham, 2015). Such transfer could easily be tested by incubating purified EVs from a MRSA strain (*e.g. S. aureus* MW2) with an MSSA strain.

EVs and mastitis prevention

To achieve the long-term perspective of applying EVs in mastitis vaccine formulations, more studies should be conducted to provide insights into the cargo selection, the efficacy of the protective response induced, and safety assays *in vivo*. A better understanding of the biogenesis process of EVs may help in the construction of genetically engineered mutants to package detoxified antigens into *S. aureus* EVs. This could facilitate the development of vaccines.

8. Thesis outputs

Published or submitted research articles

Tartaglia, N.R., Breyne, K., Meyer, E., Cauty, C., Jardin, J., Chrétien, D., Dupont, A., Demeyere, K., Berkova, N., Azevedo, V., Guédon, E., Le Loir, Y. *Staphylococcus aureus* extracellular vesicles elicit an immunostimulatory response in vivo on the murine mammary gland.

Santana-Jorge, K.T.O., Santos, T.M., **Tartaglia, N.R.,** Aguiar, E.L., Souza, R.F.S., Mariutti, R.B., Eberle, R.J., Arni, R.K., Portela, R.W., Meyer, R., et al. (2016). Putative virulence factors of *Corynebacterium pseudotuberculosis* FRC41: vaccine potential and protein expression. Microb. Cell Factories *15*.

Mariutti, R.B., Souza, T.A.C.B., Ullah, A., Caruso, I.P., de Moraes, F.R., Zanphorlin, L.M., **Tartaglia, N.R**., Seyffert, N., Azevedo, V., Le Loir, Y., et al. (2015). Crystal structure of *Staphylococcus aureus* exfoliative toxin D-like protein: Structural basis for the high specificity of exfoliative toxins. Biochem. Biophys. Res. Commun. *467*, 171–177.

Imanishi, I., Nicolas, A., Caetano, A.B., Castro, T.LP, **Tartaglia, N.R**., Mariutti, R., Guédon, E., Even, S., Berkova, N, Arni, R.K., Seyffert, N., Azevedo, V., Nishifuji, K., Le Loir, Y. Exfoliative toxin E, a new *Staphylococcus aureus* virulence factor with host specific activity.

Papers in Preparation

Tartaglia, N.R., Briard-Bion, V**,** Cauty, C., Jardin, J**.,** Krupova, Z, Chrétien, D**.,** Even, S., Berkova, N, Azevedo, V., Guédon, E., Le Loir, Y. Proteomic analysis of extracellular vesicles produced by *Staphylococcus aureus* strains isolated from human, ovine, and bovine hosts.

Book Chapter

Mariutti, R.B., **Tartaglia, N.R**., Seyffert, N., Castro, T.L. de P., Arni, R.K., Azevedo, V., Le Loir, Y., and Nishifuji, K. (2017). Exfoliative Toxins of *Staphylococcus aureus*. In The Rise of Virulence and Antibiotic Resistance in *Staphylococcus aureus*, S. Enany, and L.E. Crotty Alexander, eds. (InTech), p.

Conference Presentations

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Tartaglia. N.R. and Dupont, A. Congress Gen2Bio. Le Congrès Biotech du Grand Ouest. 22 march 2018. Oral presentation.

Event organization

V Genetics Meeting of Minas Gerais (VENGEMIG). 17-19 september 2014, Belo Horizonte - Brazil

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10. Extended abstract (Résumé étendu)

La mammite est une inflammation de la glande mammaire qui affecte les mammifères, y compris les bovins et les petits ruminants. Cette maladie a une incidence élevée dans le monde entier et est un problème majeur dans l'élevage laitier et la production de lait. *Staphylococcus aureus* est l'un des principaux agents étiologiques de mammites bovines et peut induire des manifestations cliniques d'intensités variées chez l'hôte. Cette forte variabilité des manifestations cliniques peut être associée à des caractéristiques dépendant de la souche de *S. aureus*. Dans le contexte mammaire, *S. aureus* remonte le canal du trayon, colonise, se multiplie et établit un processus infectieux dans la glande mammaire. Les profiles moléculaires associés aux microbes (MAMPs) sont reconnus dans les alvéoles mammaires par des récepteurs de reconnaissance de motifs (PRRs), tels que les récepteurs Toll (TLRs), qui induisent la production de cytokines et de médiateurs pour combattre les bactéries invasives (Oviedo Boyso et al., 2007, Sordillo et Streicher, 2002). Les cytokines pro-inflammatoires produites induisent l'expression de molécules d'adhésion dans les cellules épithéliales et favorisent par conséquent la chimiotaxie des neutrophiles. Progressivement, le tissu de la glande mammaire s'infecte. La progression de l'infection entraîne des lésions tissulaires avec perte de l'intégrité anatomique de l'alvéole et rupture de la barrière hémato-lactique. Ceci conduit ensuite à la contamination du lait par des fluides extracellulaires.

L'un des aspects les plus impactants des mammites à *S. aureus* dans les troupeaux laitiers bovins est leur propension à la récurrence et à la chronicité. Pour traiter ce type d'infection, l'antibiothérapie est largement utilisée, bien qu'elle augmente le risque d'émergence et de propagation de souches bactériennes résistantes. En outre, des mesures prophylactiques, incluant des formulations vaccinales efficaces et des thérapies probiotiques, nécessitent encore des efforts de recherche pour maîtriser complètement la mammite.

S. aureus produit un vaste arsenal de facteurs de virulence, structurels et sécrétés, jouant un rôle dans la pathogenèse. Les facteurs sécrétés sont des composants importants de la virulence de *S. aureus* et, de ce fait, la production de vésicules extracellulaires (VEs) par *S. aureus* a attiré l'attention au cours des dernières années.

Les VEs sont des particules de taille nanométrique formées par le bourgeonnement de la membrane plasmique. La production de VEs est un processus conservé dans toutes les branches de la vie : les eucaryotes, les archées et les bactéries. L'intérieur de ces vésicules constitue un environnement protecteur pour les composants biologiquement actifs et les VEs correspondent ainsi à une alternative au transport de molécules hautement dégradables, telles

que les acides nucléiques (ADN et ARN). D'autre part, la bicouche lipidique facilite encore la délivrance par la fusion avec la membrane cellulaire cible via divers composants de la surface cellulaire bactérienne, tels que les protéines membranaires bactériennes, les lipoprotéines et le peptidoglycane (Jan 2017, Szatanek et al. Valadi et al., 2007). Dans l'ensemble, la libération de ces VEs bactériennes est associée à la communication inter-espèces (Yaron et al., 2000) et inter-royaume (Yaron et al., 2000), à la détoxification et à l'élimination des protéines mal repliées (Kobayashi et al. (Manning et Kuehn 2011), au quorum sensing (Mashburn et Whiteley 2005), à l'élimination des organismes compétitifs (Z. Li, Clarke, et Beveridge 1998), à la formation de biofilm (Im et al., 2017), au transfert de matériel génétique (Dorward et Garon 1990) et à l'acquisition de nutriments (Ellis et Kuehn 2010, E.-Y. Lee et al 2009, K.-S. Park et al 2010, Toyofuku et al., 2015). Les VEs représentent un grand avantage pour la bactérie puisqu'ils permettent de délivrer des facteurs concentrés à leurs cellules cibles. Considérant les bactéries pathogènes, les VEs peuvent favoriser les stades précoces de la colonisation et les dommages tissulaires chez l'hôte.

Les premières preuves de la production de VEs chez les bactéries à Gram négatif ont été rapportées dans les années 1960. Par contre, ce mécanisme de sécrétion n'a été décrit que récemment chez les bactéries à Gram-positif. Bien que de grands progrès aient été réalisés au cours des dernières décennies en ce qui concerne les VEs libérées par les bactéries à Gram-négatif, la recherche sur les VEs des bactéries à Gram-positif est encore émergente et les VEs Gram-positif sont encore mal documentés. En outre, les mécanismes de biogenèse des VEs Gram-positifs sont encore inconnus. Ceci explique les importants efforts de recherche dédiés aux investigations sur leur rôle fonctionnel, dans la pathogenèse des bactéries à Gram positif et sur les applications potentielles des VEs dans les stratégies anti-infectieuses.

Les VEs, en tant que vésicules naturelles remplies d'un cocktail d'antigènes et de propriétés auto-adjuvantes, constituent une plate-forme vaccinale prometteuse. Cependant, bien que les VE aient été appliquées dans des formulations vaccinales contre des infections à méningocoques, leur utilisation pour la production de vaccins staphylococciques reste inexplorée. Une meilleure compréhension des facteurs impliqués dans la pathogenèse de *S. aureus*, y compris la délivrance d'effecteurs bactériens et la modulation de la réponse immunitaire de l'hôte médiée par les VEs, peut aider au développement de thérapies satisfaisantes pour prévenir et combattre la mammite. A ce jour, toutes les études impliquant des EVs purifiées de *S. aureus* ont été réalisées sur des souches humaines d'intérêt nosocomial.

Dans le cadre de ce projet, nous avons tenté de mieux comprendre les mécanismes associés à la mammite à *S. aureus* et de fournir de nouvelles informations sur le mécanisme de production et de libération des VEs par cette bactérie à Gram positif. Dans ce but, nous avons évalué *in vitro* et *in vivo* le potentiel immunomodulateur des VEs purifiées à partir de la souche bovine *S. aureus* Newbould 305 (N305) dans le contexte de la mammite. Dans la deuxième partie du projet, les teneurs en protéines des VEs produites par des souches de *S. aureus* isolées d'hôtes humains, bovins et ovins ont été comparées.

Effets immunomodulateurs induits par les vésicules extracellulaires de *S. aureus*

Les VEs purifiées de la souche bovine archétypale *S. aureus* N305 ont été caractérisées. Pour les étapes de purification des VEs, nous avons utilisé une méthode basée sur la filtration des surnageants de culture suivie d'étapes de lavage par ultracentrifugation. La centrifugation en gradient de densité a été utilisée pour éliminer les particules contaminantes. La procédure de purification a été optimisée afin de récupérer une grande quantité de VEs produites par *S. aureus* N305 cultivé dans des conditions de laboratoire. L'analyse par tomographie cryogénique (cryo-ET) a révélé des particules sphériques de forme homogène. Des vésicules avec la morphologie en forme de coupe bien documentée et typique (en donut) ont été observées par microscopie électronique à transmission (MET) et quantifiées en utilisant l'analyse NTA et la TRPS. La taille moyenne des VEs était de 67 ± 13 nm (moyenne et écart-type) pour le TRPS, de 91 ± 23 nm pour la cryo-ET et de 126 ± 2 nm pour le NTA. Le nombre de particules total évalué par TRPS et NTA était similaire et proche de 4×10^9 particules par mL de surnageant traité.

Les VEs purifiées (environ 50 µg) ont été digérées en solution puis analysées en NanoLC-ESI-MS / MS. Grâce à cette approche protéomique, nous avons identifié 222 protéines associées aux VE de *S. aureus* N305. Ces VEs se sont révélées enrichies en protéines cytoplasmiques (n = 89) et membranaires (n = 71). Ces dernières étaient surreprésentées dans les VE par rapport au protéome total prédit pour la membrane (32% contre 26%). Ces résultats sont en accord avec les rapports précédents sur la teneur en protéines des VEs dérivées d'isolats humains de *S. aureus* (Gurung et al., 2011, Lee et al., 2009).

Les VEs dérivées de *S. aureus* N305 portaient des déterminants importants pour la virulence, tels que la protéine de liaison à l'immunoglobuline G (Sbi), la protéine de liaison à la pénicilline (PBPs), les phenol soluble modulins (PSMs), la protéine de liaison à l'élastine (EbpS). Certains d'entre eux sont impliqués dans l'adhésion des bactéries aux composants de

la matrice extracellulaire (ECM) (par exemple EbpS et FnBP) (Foster et al., 2014, Fraunholz et Sinha, 2012, Gordon et Lowy, 2008). En outre, Sbi peut contribuer à l'échappement de *S. aureus* à la réponse immunitaire de l'hôte (Atkins et al., 2008) et les toxines peuvent infliger des dommages membranaires et favoriser la propagation bactérienne dans les tissus environnants (Otto, 2014). Les VEs sont également enrichies en lipoprotéines, qui sont des agonistes et modulent une réponse TLR2. Plus de la moitié (34/58) du nombre de lipoprotéines prédites sur le protéome entier (c'est-à-dire des protéines avec un site de clivage de signal peptidase II) ont été identifiées dans les VEs de N305, indiquant un enrichissement relatif de ces VEs en lipoprotéines.

Les VEs sécrétés par *S. aureus* N305 ne sont pas cytotoxiques *in vitro*

L'ensemble des facteurs de virulence identifiés au sein des VEs de *S. aureus* N305 nous a conduits à émettre l'hypothèse que ces VEs sont également capables d'induire un effet cytotoxique sur les cellules hôtes. La capacité des VEs dérivées de *S. aureus* N305 à induire une réponse immunitaire et un effet cytotoxique a été évaluée sur des cellules épithéliales mammaires bovines (CEMb). À cette fin, deux lignées de CEMb, MAC-T et PS, ont été traitées pendant 24 h avec des doses croissantes de VE : 0,01, 0,1, 1 et 10 μg par puits. Bien que concentrées en toxines, les VEs dérivées du *S. aureus* N305 n'ont eu aucun effet cytotoxique sur les lignées de CEMb dans les conditions testées. Ce résultat fait écho à des travaux antérieurs montrant que la cytotoxicité induite par les VEs de *S. aureus in vitro* dépendait du type cellulaire (Gurung et al., 2011, Jeon et al., 2016). La concentration de VEs, ainsi que leur contenu en protéines, sont également des facteurs qui peuvent influencer leurs effets cytotoxiques *in vitro* (Jeon et al., 2016). En outre, cela indique que les VEs peuvent interagir et induire des réponses de cellules hôtes d'une manière plus subtile.

Les VEs sécrétées par *S. aureus* N305 induisent une réponse immunitaire sur CEMb *in vitro*

Pour tester la capacité des VEs sécrétées par *S. aureus* N305 à induire une réponse immunitaire de l'hôte *in vitro*, en particulier les défenses immunes innées, des CEMb (lignée PS) ont été traitées pendant 3 h avec des VEs de *S. aureus* N305 (10 et 20 μg par puits). Les niveaux d'expression des gènes de CEMb codant pour les cytokines pro-inflammatoires clés (IL-1β, IL-8 et TNF-α) et pour les peptides antimicrobiens β défensine-1 (DEFβ1) ont été comparés à ceux des cellules PS non traitées. Des bactéries *S. aureus* N305 vivantes (MOI de 100:1), ou tuées à la chaleur ($N305_{HK}$) et 10μg d'acide lipotéichoïque staphylococcique (LTA)

ont également été utilisés comme témoins. Une induction significative de tous les gènes a été observée après traitement avec *S. aureus* vivant par rapport aux cellules PS non traitées. En revanche, aucune différence d'expression d'IL-1, TNF-α et DEFβ1 n'a été observée après un traitement avec des bactéries inactivées par la chaleur, alors que l'expression d'IL-8 était légèrement plus élevée que celle des cellules PS non traitées. Nos données *in vitro* ont montré que l'exposition de CEMb aux VEs sécrétées par *S. aureus* N305 entraîne une augmentation significative et dose-dépendante de l'expression de deux cytokines pro-inflammatoires (IL-1β, TNF-α), une chimiokine (IL-8) et un peptide bactéricide (DEFβ1). Lors d'études précédentes, il a été montré que, dans des conditions expérimentales semblables, les OMVs de bactéries à Gram-négatif (par exemple *H. pylori* et *P. aeruginosa*) induisent également la production de la cytokine pro-inflammatoire IL-8 par les cellules épithéliales (Bauman et Kuehn, 2006; Ismail et al. 2003).

Plus spécifiquement, les VEs de *S. aureus* N305 induisent une réponse dose-dépendante dans l'expression des cytokines pro-inflammatoires. Dans l'ensemble, nos résultats *in vitro* suggèrent que les VEs de *S. aureus* N305 contribuent à la stimulation du système immunitaire inné de la glande mammaire sans effets cytotoxiques et participent ainsi à l'induction de la réponse inflammatoire mammaire durant l'infection par *S. aureus*.

Les VEs de *S. aureus* N305 induisent une réponse inflammatoire locale et une réponse immunitaire innée *in vivo*

Dans cette partie du projet, les VEs de *S. aureus* N305 ont été évaluées pour leurs propriétés immunomodulatrices *in vivo*, en utilisant un modèle murin de mammite. D'autres études ont en effet décrit le rôle des VEs Gram-positives et Gram-négatives dans la réponse immunitaire de l'hôte dans différents modèles murins (Hong et al., 2011; Prados-Rosales et al., 2011; Söderblom et al., 2005, Surve et al., 2016). Quarante souris femelles lactantes de la lignée Hsd:ICR (CD-1) ont été inoculées par voie intraductale dans la quatrième paire de glandes mammaires. Six groupes de souris ont été inoculés : deux groupes ont reçu des VE de N305 (à des concentrations de 1 μg et 10 μg dans du PBS, tous deux n = 7) et comparés à un groupe témoin négatif PBS seulement (n = 7). Les groupes témoins positifs étaient : des cellules N305 vivantes (117 UFC) dans du PBS (n = 7), 100 UFC de *S. aureus* $N305_{HK}$ dans du PBS (n = 6) et 10 μg de LTA dans du PBS (n = 6). Vingt-quatre heures après l'infection (p.i.), les souris ont été anesthésiées et euthanasiées. Toutes les glandes mammaires ont été isolées et homogénéisées mécaniquement. Ensuite, les lysats ont été utilisés pour quantifier les niveaux d'IL-1α, IL-1β, IL-6, TNF-α, MCP-1, CXCL2 (MIP-2), RANTES, BAFF, CXCL1

(KC) et IL-17A. Les glandes mammaires ont également été colorées à l'hématoxyline et à l'éosine pour des analyses histologiques. Les VEs de *S. aureus* N305 ont induit une inflammation légère et localisée par rapport à cellules N305 vivantes. Cependant, elles ont induit une réponse *in vivo* plus élevée que le LTA et $N305_{HK}$, tant au niveau histologique que de la production de cytokines, ce qui suggère que les EVs jouent un rôle dans la pathogenèse de *S. aureus* N305 en tant que facteurs immunostimulants.

Par ailleurs, l'injection d'VEs a induit un afflux de cellules immunitaires dans les alvéoles d'une manière dose-dépendante, ce qui correspond bien aux niveaux de cytokines mesurés dans les tissus mammaires. L'influx de cellules immunitaires au niveau des sites inflammatoires est généralement associé à des taux élevés de chimiokines CXC (Zlotnik et Yoshie 2000). Nous avons, de fait, détecté une induction des chimiokines murines de type IL-8 KC (CXCL1) et MIP-2 (CXCL2) impliquées dans le recrutement des neutrophiles sur les sites d'inflammation (De Filippo et al., 2008; Leemans et al., 2003). De plus, les taux de MCP-1, un chimio-attractant monocytaire (Rollins 1997), RANTES, un chimio-attractant de monocytes, lymphocytes T, basophiles et éosinophiles (Arango Duque et Descoteaux 2014) et BAFF, un facteur d'activation des lymphocytes B, ont été augmenté par les injections de VEs. Ceci pourrait être vu comme un mécanisme bactérien déclenchant le recrutement de cellules capables d'internaliser *S. aureus* et, par conséquent, favorisant un mode de vie intracellulaire au cours du processus infectieux. Les mécanismes moléculaires associés à l'interaction, à la fusion et à la stimulation de la cellule hôte par les VEs nécessitent cependant des recherches plus poussées. Ces résultats suggèrent clairement que les VEs jouent un rôle dans la réponse inflammatoire dans le contexte mammite, principalement par le recrutement cellulaire.

Globalement, dans cette partie de l'étude, nous démontrons que les VEs sont produits par la souche bovine *S. aureus* N305 et qu'ils induisent une réponse immunitaire, à la fois *in vitro* sur les CEMb et *in vivo* dans un modèle murin de mammite. De plus, il fournit des preuves que les VEs sécrétées par *S. aureus* N305 modulent principalement le chimiotactisme des cellules immunitaires innées.

Protéomique comparative des VEs libérés par plusieurs isolats de *S. aureus*

Dans la seconde partie du projet, la capacité de six souches de *S. aureus* phylogénétiquement distantes à produire et à sécréter des VEs a été évaluée. Six souches bien caractérisées ont été sélectionnées en fonction de leur origine (bovine, ovine et humaine) et de leurs propriétés infectieuses. Parmi les isolats bovins, *S. aureus* RF122 cause des mammites

aiguës tandis que *S. aureus* N305 déclenche des mammites modérées et chroniques chez les vaches laitières (Bannerman et al., 2004; Peton et al., 2014). Les souches ovines *S. aureus* O11 et *S. aureus* O46 induisent de manière reproductible des symptômes sévères et subcliniques, respectivement, dans lors de mammites expérimentales. Les souches O11 et O46 induisent ces symptômes distincts malgré une parenté génotypique étroite (Le Maréchal et al., 2011). De plus, nous avons sélectionné deux isolats cliniques humains, *S. aureus* Mu50 résistant à la méthicilline et résistant à la vancomycine (MRSA, VRSA) et *S. aureus* MW2, résistant à la méthicilline (CA-MRSA).

La purification des VEs a été effectuée comme mentionné précédemment. Les échantillons ont également été visualisés par MET et quantifiés en utilisant la NTA. Tous les isolats ont libéré des VEs avec des diamètres d'échelle nanométrique. La taille des VEs était de 129,1 ± 3,1 nm (écart type et écart type) pour *S. aureus* RF122, 135 ± 2 nm pour *S. aureus* N305, 172,1 ± 1,6 nm pour *S. aureus* O46, 139,4 ± 2,7 nm pour *S. aureus* O11 et 129,7 ± 1,2 nm pour *S. aureus* MW2. Les VEs isolées de la souche humaine *S. aureus* Mu50 avaient un diamètre moyen inférieur, de 98 ± 0,9 nm. En plus des nanoparticules sphériques, des structures cylindriques ont été co-purifiées dans les surnageants des souches animales seulement.

Bien que les conditions de croissance soient similaires, la concentration de VEs obtenue différait entre souches. Sauf pour la souche ovine *S. aureus* O11 (1,2 x 10^7 particules par mL de surnageant traité), les quantités totales de nanoparticules étaient plus élevées dans les isolats animaux (N305, 2,3 x 10^8 particules par ml, RF122, 3,4 x 10^8 particules par mL, O46, 2,7 x 10^8 particules par mL) comparativement aux isolats humains (MW2, 3 x 10^7 particules par mL, Mu50, 4,6 x 10^7 particules par mL). Les souches bovines libéraient une grande quantité de VEs par rapport aux souches humaines dans les mêmes conditions de croissance et de purification. Étant donné l'importance des facteurs sécrétés dans l'infection bactérienne, les VEs ont été caractérisées par rapport à leur contenu en protéines. Trois réplicats biologiques indépendants de chaque préparation de VEs purifiées ont été digérés pour l'analyse NanoLC-ESI-MS / MS, à l'exception de *S. aureus* Mu50 qui n'a été effectuée qu'une seule fois. Dans l'ensemble, nous avons identifié 261 protéines (N305; n = 173; RF122; n = 96; Mu50; n = 149; MW2; n = 97; O46; n = 109; O11; n = 104). La plupart d'entre elles sont prédites cytoplasmiques et associées à la membrane. Ces contenus sont enrichis en lipoprotéines, comme déjà décrit chez *S. aureus* (Askarian et al., 2018) et d'autres VEs de bactéries à Gram positif (Biagini et al., 2015, Lee et al., 2015). La composition protéique dépend de la souche parentale. La classification fonctionnelle du protéome des VEs

a été déterminée à l'aide d'une analyse de groupes d'orthologues (COG). Dans l'ensemble, la majorité des protéines ont été prédites dans les catégories «traduction, structure ribosomale et biogenèse» (16%, n = 32) et «production et conversion d'énergie» (10%, n = 20). «Paroi cellulaire, membrane, biogenèse de l'enveloppe», «transport et métabolisme des ions inorganiques» et «transport et métabolisme des hydrates de carbone» correspondaient chacun à 9% du total des protéines de VEs identifiées. Un total de 44 protéines de ces VEs (17% des 261 protéines analysées) étaient communes à toutes les souches et composent donc ce qui peut être considéré comme le protéome de base (ou protéome cœur) des VEs de *S. aureus*. La plupart de ces protéines appartiennent aux groupes «Production et conversion d'énergie» (n = 9), «Transport et métabolisme inorganique» (n = 7), «paroi cellulaire, membrane, biogenèse de l'enveloppe» (n = 5) et «transport et métabolisme des carbohydrates» (n = 4). Fait à noter, 25% (n = 11) de ces 44 protéines de VEs sont prédites lipoprotéines. Fait intéressant, la plupart de ces protéines de base ont été classés en COG qui correspondent à des protéines conservées au niveau évolutif. Ce groupe contient des protéines impliquées dans l'assimilation et le stockage du fer (ferritine, transporteur ferrochrome ABC, récepteur de l'hydroxamate ferrique, système de transport du complexe ferrique) et des protéines de liaison aux ions métalliques (protéine de liaison au substrat du système de transport du zinc, protéine liant le molybdate). Le fer est un élément essentiel de la croissance bactérienne et de la virulence au cours du processus d'infection (Wooldridge, 1993). La libération de VEs contenant des sidérophores peut favoriser la croissance de souches déficientes dans un milieu à faible teneur en fer, comme cela a été observé avec des VEs dérivés de *Mycobacterium tuberculosis* contenant de la mycobactine (Prados-Rosales et al., 2014). PrsA est également commun à tous les VEs de *S. aureus* et peut être impliqué dans le repliement post-translocationnel des protéines vésiculaires et peut ainsi intervenir dans conformation de protéines biologiquement actives. D'autres protéines, comme la protéine liant la pénicilline (PBP2), la SortaseA (SrtA), les atténuateurs transcriptionnels (famille LytR) et la protéine de transfert de la D-alanine aux chaines de poly-glycérophosphate (DltD) forment un groupe de protéines associées à la biogenèse de l'enveloppe. Dans des études antérieures, les modifications des protéines associées à la membrane et la réticulation du peptidoglycane ont été corrélées à la production et au rendement des VEs chez les bactéries à Gram-négatif et à Gram-positif. Certaines protéines comprises dans ce protéome de base sont des protéines dites « moonlight », une classe spéciale de protéines multifonctionnelles, comprenant l'autolysine bifonctionnelle (Atl), le facteur d'élongation Tu (Ef-Tu) et la Glycéraldéhyde-3-phosphate déshydrogénase (GAPDH) (Heilmann et al., 2005, Modun et Williams, 1999, Pasztor et al., 2010, Widjaja et

al., 2017). Des hydrolases de muréine peuvent être impliquées dans le clivage du peptidoglycane requis pour la formation et la libération vésiculaire chez les bactéries à Gram-positif (Lee et al., 2009; Wang et al., 2018). Les autres protéines « moonlight », énolase, EF-Tu et GAPDH, sont capables d'interagir avec plusieurs ligands de l'hôte (Henderson et Martin, 2013). En outre, GAPDH peut être impliquée dans le stade initial de la fusion membranaire avec des cellules hôtes. Cela renforce l'hypothèse selon laquelle les VEs ne sont pas formées au hasard et ne résultent pas simplement d'un processus de lyse cellulaire (McBroom et al., 2006). De plus, même des protéines bien étudiées pourraient avoir des fonctions supplémentaires à découvrir (Huberts et van der Klei, 2010) et pourraient être impliquées dans le processus de production et de libération des VEs. Six protéines hypothétiques étaient associées de façon conservative aux VEs dérivées de *S. aureus*. Ces protéines hypothétiques pourraient participer au processus pathogène de *S. aureus* ou à la biogenèse des VEs.

Lors de l'analyse du protéome accessoire des VEs, il n'a pas été possible d'associer le contenu en protéines accessoires des VEs à une quelconque spécificité d'hôte des souches productrices. Concernant cette catégorie de protéines accessoires, les protéines associées à la résistance aux antibiotiques sont retrouvées dans certains VEs seulement. La protéine de liaison à la pénicilline 2 '(PBP2', codée par *mecA*) est spécifiquement associée aux VEs de *S. aureus* Mu50, bien que des réplicats doivent être produits et analyser pour obtenir des données et des conclusions fiables. La présence de protéines impliquées dans la résistance aux antibiotiques est en accord avec l'hypothèse selon laquelle les VEs permettent aux bactéries de partager des caractéristiques transitoires (Chattopadhyay et Jagannadham, 2015, Schaar et al., 2011, 2013, Stentz et al., 2015). Les VEs de *S. aureus* MW2 portent également la bêta-lactamase, ce qui est cohérent avec les travaux précédents qui ont montré que les VEs de *S. aureus* transportent des bêta-lactamases biologiquement actives et assurent la survie de bactéries à Gram négatif et à Gram positif sensibles à l'ampicilline en présence d'ampicilline (Lee et al., 2013). Au total, 100 protéines (38%) étaient souche-spécifiques dont 45 et 33 étaient exclusivement présentes dans les contenus de Mu50 et N305, respectivement. Il convient de noter que ces 2 souches sont également celles qui ont le plus grand nombre de protéines identifiées dans leurs VEs. Des protéines de VEs souche-spécifiques ont également été identifiées dans les autres souches étudiées (RF122, n = 10, MW2, n = 6, O11, n = 5, O46, n = 1). Dans l'ensemble, une proportion significative de ces protéines souche-spécifiques sont décrites comme des protéines hypothétiques ou probables (Mu50, n = 10, N305, n = 7, RF122, n = 4, MW2, n = 3, O11, n = 2). Les facteurs de virulence étaient également partagés entre différentes VEs. Fait intéressant, la δ-hémolysine, une toxine codée par l'ARN III du

système *agr*, a été retrouvée dans toutes les VE sauf dans celles de *S. aureus* Mu50, souche déficiente pour *agr* (Tsompanidou et al., 2011). De même, les PSMα2 et α4, dont la production est dépendante du système *agr*, ont également été identifiées dans toutes les souches sauf *S. aureus* Mu50. Les peptides *S. aureus* PSMα, qui ont une activité analogue à un surfactant et induisent la rupture de la membrane, peuvent favoriser le bourgeonnement de la membrane cytoplasmique et la production de VEs (Ebner et al., 2017; Wang et al., 2018). De plus, la souche Mu50 produit des VEs de taille plus petite et à de concentration plus faible que les autres souches. L'absence de peptides PSMα dans les VEs produits par un mutant *agr* de *S. aureus* a été récemment décrite par Wang et al. (2018) qui ont également montré qu'une délétion des gènes *psmα* réduisait significativement la taille et le rendement de production des VEs de *S. aureus* (Wang et al., 2018). Ces résultats suggèrent fortement un rôle important du système *agr*, et donc du quorum sensing, dans la production et la libération des VEs de *S. aureus*.

Concernant la formulation de vaccins, les VEs sont naturellement produits par des bactéries, capables de stimuler l'immunité innée et adaptative et ont des effets adjuvants intrinsèques. Plusieurs études ont montré que les OMVs de bactéries à Gram négatif peuvent induire une réponse immunitaire adaptative. En tant que formulations vaccinales, elles ont principalement été étudiées contre le sérogroupe B de *N. meningitidis* (Chatterjee et Chaudhuri, 2012). En tant qu'adjuvants, les VEs peuvent être utilisées pour éviter, par exemple, la toxicité, la sensibilité et les effets indésirables associés aux principaux adjuvants sur le marché (Tan et al., 2018). Dans le contexte de la mammite, les VEs modifiées dotés de capacités fusogènes accrues, de toxines non cytolytiques et enrichies de protéines membranaires immunogènes constituent une stratégie vaccinale intéressante contre les infections à *S. aureus*.

Globalement, la conservation des protéines, y compris les facteurs d'adhésion et de fusion, dans le protéome de base des VEs produites par des souches de *S. aureus* phylogénétiquement distantes renforce l'hypothèse que les VEs ne se forment pas au hasard mais qu'un mécanisme dédié sélectionne et empaquète les protéines dans les VEs. Bien que nos résultats ne soient pas concluants quant à la spécificité d'hôte des VEs de *S. aureus*, ils ont mis en évidence un niveau d'organisation entre VEs en termes de protéines « moonlight », membranaires et cytoplasmiques avec une capacité de liaison membranaire. Bien que préliminaire, ce travail ouvre de nouvelles perspectives en matière de diagnostic et de prévention contre les mammites à *S. aureus* et d'autres infections.

Dans l'ensemble, ce travail a démontré que les VEs sont des facteurs importants dans la réponse immunitaire lors de mammite à *S. aureus* et il ouvre de nouvelles perspectives sur l'étude de la pathogenèse de *S. aureus* en contexte mammite. Les champs de recherche associés aux mécanismes de production des VEs par *S. aureus* et les besoins cruciaux pour des traitements alternatifs aux antibiotiques et des mesures prophylactiques, font de l'étude des VEs un domaine passionnant et porteur d'innovations.

11. Annexes

Annex 1. Supplementary data (Chapter 2)

Annex 2. Supplementary data (Chapter 3)

The project "Structural and Functional Characterization of Homologous Exfoliative Toxin D of *Staphylococcus aureus* involved in subclinical mastitis of small ruminants" was started during my master's degree in Brazil.

In a previous work, *S. aureus* strains O46 (subclinical mastitis) and O11 (gangrenous mastitis) were compared using a serological proteome analysis (SERPA) (Le Maréchal et al., 2011). Through this method, a protein homologous to *S. aureus* exfoliative toxin D (ETD-like) was identified as immunoreactive only in *S. aureus* O46. The ETD-like was characterized and the crystal structure was determined (Annex 5). Furthermore, this new exfoliative toxin displayed host specific activity and was then named ETE (Annex 3).

Annex 3. Exfoliative toxin E, a new *Staphylococcus aureus* virulence factor with host specific activity (Imanishi et al)

Annex 4. Book chapter
Exfoliative Toxins of *Staphylococcus aureus* (Mariutti et al., 2017)

Annex 5. Crystal structure of *Staphylococcus aureus* exfoliative toxin D-like protein: Structural basis for the high specificity of exfoliative toxins (Mariutti et al., 2016)

Annex 6. Putative virulence factors of *Corynebacterium pseudotuberculosis* FRC41: vaccine potential and protein expression (Santana-Jorge et al., 2016)

Annex 1

Supplementary data (Chapter 2)

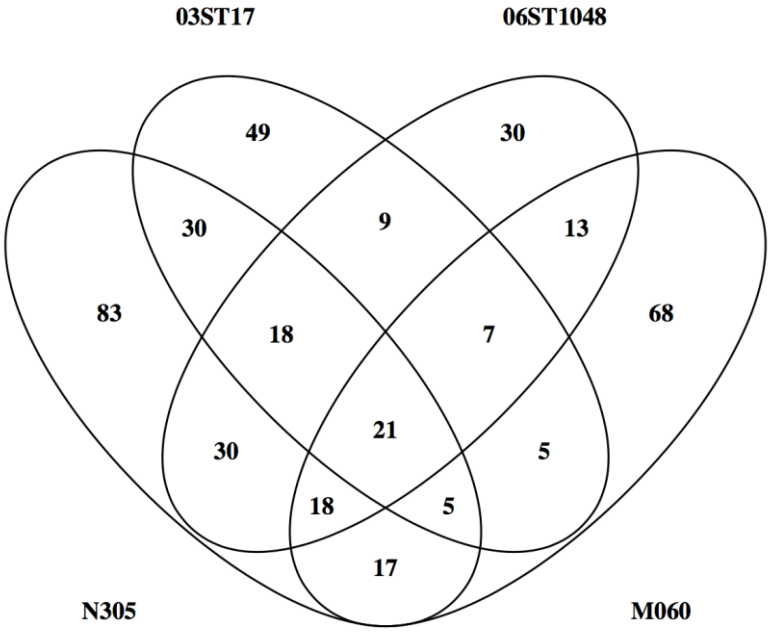

Figure S1. Venn diagram of proteins identified in EVs from *S. aureus* N305, 03ST17, 06ST1048 and M060 isolates. The number of proteins identified in the four different *S. aureus* EVs is presented. Proteins identified in EVs derived from *S. aureus* 03ST17, 06ST1048 and M060 were obtained from Jeon et al., 2016 (Jeon H, Oh MH, Jun SH, Kim SI, Choi CW, Kwon HI, Na SH, Kim YJ, Nicholas A, Selasi GN, Lee JC. Variation among Staphylococcus aureus membrane vesicle proteomes affects cytotoxicity of host cells. Microb Pathog. 2016 Apr;93:185-93. doi: 10.1016/j.micpath.2016.02.014.). Venn diagram was generated with Venny 2.1 (Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams; http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Table S1. Primers used for cDNA quantification by real-time PCR in the PS bovine mammary epithelial cells.

Target cDNA	Sequence	Product size (pb)	Reference
PPIA	Forward: 5'- ATGGCAAGACCAGCAAGAAG - 3' Reverse: 5' – CTTGGAGGGGGATAAGGAAA - 3'	201	Deplanche et al., 2016
RPL19	Forward: 5'- TACTGCCAATGCTCGAATGC- 3' Reverse: 5' – TGATACATGTGGCGGTCAATC- 3'	114	Deplanche et al., 2016
YWHA	Forward: 5'- GTAGGAGCCCGTAGGTCATC- 3' Reverse: 5' – GCTTGTGAAGCGTTGGGGAT- 3'	182	This work
IL-8	Forward: 5'- TGGGCCACACTGTGAAAAT- 3' Reverse: 5' – TCATGGATCTTGCTTCTCAGC- 3'	92	Deplanche et al., 2016
TNF-α	Forward: 5'- TCTTCTCAAGCCTCAAGTAACAAGC- 3' Reverse: 5' – CCATGAGGGCATTGGCATAC - 3'	104	Bougarn et al., 2011
IL-1β	Forward: 5'- CTCTCACAGGAAATGAACCGAG - 3' Reverse: 5' – GCTGCAGGGTGGGCGTATCACC - 3'	152	Bougarn et al., 2011
DEFβ1	Forward: 5'- CTTCTCTTCCTGGTACTGTCT - 3' Reverse: 5' – GGCGTGAAACAGGTGCCAATC - 3'	140	Bougarn et al., 2011

Table S2. List of proteins identified in EVs of *Staphylococcus aureus* Newbould 305.

Uniparc	Locus Tag	Protein/description (1)	COG	SL (2)	LipoP (3)	NTD (4)	Coverage	kDa (5)	Gene name (6)	emPAI (7)
Translation, ribosomal structure and biogenesis										
UPI0000135022	Newbould305_0167	30S ribosomal protein S4	COG0522	C	CYT		68	22,9	rpsD	22,1
UPI00005FE57B	Newbould305_0206	Threonine--tRNA ligase	COG0441	C	CYT		4	74,3	thrS	0,2
UPI000012D34E	Newbould305_0208	Translation initiation factor IF-3	COG0290	C	CYT		44	20,1	infC	12,9
UPI0000054C25	Newbould305_0210	50S ribosomal protein L20	COG0292	C	CYT		16	13,6	rplT	4,6
UPI00024E3F9A	Newbould305_0224	Valine-tRNA ligase	COG0525	C	CYT		5	101,5	valS	0,2
UPI00000D766C	Newbould305_0232	50S ribosomal protein L21	COG0261	U	CYT		51	11,3	rplU	30,6
UPI0000136CFD	Newbould305_0240	Queuine tRNA-ribosyltransferase	COG0343	C	CYT		5	43,2	tgt	0,7
UPI000002E8EC	Newbould305_0314	Glycine--tRNA ligase	COG0423	C	CYT		7	53,5	glyS	0,3
UPI000004802A	Newbould305_0924	30S ribosomal protein S18	COG0238	C	CYT		27	9,2	rpsR	315,2
UPI0000054841	Newbould305_1063	50S ribosomal protein L1	COG0081	C	CYT		40	24,6	rplA	34,9
UPI0000054840	Newbould305_1064	50S ribosomal protein L10	COG0244	C	CYT		51	17,6	rplJ	9,0
UPI0000048026	Newbould305_1070	30S ribosomal protein S12	COG0048	C	CYT		23	15,2	rpsL	16,8
UPI000004801A	Newbould305_1071	30S ribosomal protein S7	COG0049	C	CYT		33	17,7	rpsG	3,6
UPI0000052287	Newbould305_1072	Elongation factor G (EF-G)	COG0480	C	CYT		34	76,5	fusA	5,6
UPI0000054837	Newbould305_1073	Elongation factor Tu (EF-Tu)	COG0050	C	CYT		81	43,0	tufA	38,8
UPI0000054B7F	Newbould305_1578	50S ribosomal protein L13	COG0102	U	CYT		66	16,2	rplM	45,4
UPI0000048025	Newbould305_1584	30S ribosomal protein S11	COG0100	C	CYT		72	13,8	rpsK	99,0
UPI0000054C04	Newbould305_1590	50S ribosomal protein L15	COG0200	U	CYT		21	15,5	rplO	2,2
UPI0000048021	Newbould305_1592	30S ribosomal protein S5	COG0098	C	CYT		41	17,7	rpsE	6,7
UPI0000054C2C	Newbould305_1594	50S ribosomal protein L6	COG0097	C	CYT		50	19,7	rplF	6,5
UPI000004801B	Newbould305_1595	30S ribosomal protein S8	COG0096	C	CYT		19	14,7	rpsH	1,2
UPI00005FE723	Newbould305_1596	30S ribosomal protein S14 type Z	COG0199	C	CYT		22	6,7	rpsZ	0
UPI00000D76D0	Newbould305_1597	50S ribosomal protein L5	COG0094	C	CYT		38	20,2	rplE	2,2
UPI0000054C69	Newbould305_1599	50S ribosomal protein L14	COG0093	C	CYT		17	13,1	rplN	1,5
UPI000004801D	Newbould305_1600	30S ribosomal protein S17	COG0186	C	CYT		28	10,1	rpsQ	9,0

UPI0000133E90	Newbould305_1601	50S ribosomal protein L29	COG0255	C	CYT		32	8,0	rpmC	9,0
UPI00000D76A9	Newbould305_1602	50S ribosomal protein L16	COG0197	C	CYT		66	16,2	rplP	630,0
UPI0000134FAF	Newbould305_1603	30S ribosomal protein S3	COG0092	C	CYT		64	24,0	rpsC	132,4
UPI0000054ADA	Newbould305_1604	50S ribosomal protein L22	COG0091	C	CYT		55	12,8	rplV	14,8
UPI00000522A9	Newbould305_1606	50S ribosomal protein L2	COG0090	C	CYT		65	30,1	rplB	49,1
UPI00000549A8	Newbould305_1607	50S ribosomal protein L23	COG0089	C	CYT		35	10,5	rplW	3,6
UPI00000545D9	Newbould305_1608	50S ribosomal protein L4	COG0088	U	CYT		32	22,4	rplD	9,0
UPI0000048024	Newbould305_1610	30S ribosomal protein S10	COG0051	C	CYT		38	11,5	rpsJ	3,6
UPI0000054AE5	Newbould305_1950	50S ribosomal protein L19	COG0335	C	CYT		64	13,3	rplS	630,0
UPI0000134F21	Newbould305_1964	30S ribosomal protein S2	COG0052	C	CYT		53	29,0	rpsB	24,1
UPI000004801C	Newbould305_1984	30S ribosomal protein S15	COG0184	C	CYT		48	10,5	rpsO	99,0
UPI000012B12E	Newbould305_2423	Aspartyl/glutamyl-tRNA amidotransferase subunit A (Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit A)	COG0154	C	CYT		9	52,7	gatA	0,4
Energy production and conversion										
UPI000012529A	Newbould305_0176	Acetate kinase	COG0282	C	CYT		10	43,9	ackA	0,5
UPI00000548FB	Newbould305_0465	L-lactate dehydrogenase (L-LDH)	COG0039	C	SpI		10	34,3	ldh	0,4
UPI00005FE17F	Newbould305_0688	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	COG1454	C	CYT		7	94,8	adhE	0,4
UPI00000546A4	Newbould305_0764	Formate C-acetyltransferase	COG1882	C	CYT		31	85,3	NA	2,8
UPI000012E2D4	Newbould305_0783	L-lactate dehydrogenase (L-LDH)	COG0039	C	SpI		20	34,5	lctE	1,6
UPI0000054716	Newbould305_1381	NADH dehydrogenase	COG1252	CM	CYT		26	44,0	yumB	3,9
UPI0000054B74	Newbould305_1502	Cytochrome aa3 quinol oxidase, subunit I (Cytochrome aa3-600 quinol oxidase (Subunit I))	COG0843	CM	TMH	15	3	75,1	qoxB	0,5
UPI0001D169A5	Newbould305_1503	Quinol oxidase subunit II	COG1622	CM	CYT	2	53	36,6	NA	99,0
UPI0000054329	Newbould305_1668	FAD binding domain protein (Salicylate hydroxylase)/Hypothetical protein	COG0654	C	CYT		15	41,8	nagX	2,2
UPI00000543F4	Newbould305_1786	Succinate dehydrogenase (Flavoprotein subunit)	COG1053	C	SpI		19	65,4	sdhA	0,6
UPI00004B5BD1	Newbould305_2012	Glycerol-3-phosphate dehydrogenase	COG0578	C	CYT		43	62,2	glpD	7,5
UPI0000129471	Newbould305_2507	Dihydrolipoamide dehydrogenase	COG1249	C	CYT		98	49,3	pdhD	189573564,2
UPI0000054B50	Newbould305_2509	Pyruvate dehydrogenase E1 component subunit beta	COG0022	C	CYT		97	35,1	pdhB	7196855,7

UPI000000978E	Newbould305_2510	Pyruvate dehydrogenase E1 component alpha subunit	COG1071	C	CYT		93	41,3	pdhA	28942661246,2
UPI00000D78AB	Newbould305_2680	ATP synthase subunit B	COG0711	CM	CYT	1	69	19,4	atpF	1466,8
UPI00000545EC	Newbould305_2682	ATP synthase subunit alpha	COG0056	C	CYT		39	54,5	atpA	7,7
UPI0001DA20F1	Newbould305_2683	ATP synthase gamma chain	COG0224	U	CYT		55	30,2	NA	11,1
UPI00000545EA	Newbould305_2684	ATP synthase subunit beta	COG0055	CM	CYT		71	51,3	atpD	8,1
UPI00000545E9	Newbould305_2685	ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit) (F-ATPase epsilon subunit)	COG0355	C	CYT		22	14,8	atpC	9
Cell wall, membrane and envelope biogenesis										
UPI00000BC8F2	Newbould305_0327	Penicillin-binding protein 3 (PBP3)	COG0768	CM	CYT	1	17	77,1	pbpF	0,7
UPI00000D9FF9	Newbould305_0388	LPXTG specific sortase A (Sortase A transpeptidase)	COG3764	CM	SpI	1	50	23,5	srtA	6,7
UPI00000548B8	Newbould305_0406	CHAP domain protein (Secretory antigen SsaA)	COG3942	Extra	SpI		20	16,8	ssaA2	5,3
UPI00005FE858	Newbould305_0516	N-acetylmuramoyl-L-alanine amidase	COG1705	Extra	SpI		2	69,2	NA	0,2
UPI0000052300	Newbould305_1163	N-acetylglucosaminyldiphosphoundecaprenol N-acetyl-beta-D-mannosaminyltransferase	COG1922	C	CYT		19	29,0	tarA	2,7
UPI00004E153B	Newbould305_1169	Penicillin binding protein 4 (PBP4)	COG1686	CM	SpI	1	12	48,1	pbp4	0,8
UPI00005FE32B	Newbould305_1248	Lipoteichoic acid synthase (LTA synthase)	COG1368	CM	TMH	5	15	74,2	ltaS	1,0
UPI000005471C	Newbould305_1375	D-alanyl-lipoteichoic acid biosynthesis protein DltD	COG3966	C	SpI	1	53	44,8	dltD	24,1
UPI00005FE3D5	Newbould305_1498	Putative transcriptional regulator LytR	COG1316	CM	CYT		9	46,0	NA	0,8
UPI000005225C	Newbould305_1662	Staphylococcal secretory antigen ssaA2	COG3942	Extra	SpI		12	29,2	ssaA2	1,2
UPI0000696CCA	Newbould305_1676	Putative transcriptional regulator LytR	COG1316	CM	SpI	1	30	33,7	NA	7,3
UPI000250690A	Newbould305_1824	Penicillin-binding protein	COG0768	CM	CYT		6	78,5	pbpA	0,3
UPI00000543A2	Newbould305_1866	Serine/threonine-protein kinase PrkC	COG2815	CM	CYT	1	9	74,2	prkC	0,7
UPI00000549E5	Newbould305_2136	Peptide methionine sulfoxide reductase regulator MsrR	COG1316	CM	CYT	1	26	36,9	msrR	2,5
UPI0000054A63	Newbould305_2192	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	COG0707	CM	CYT		7	39,6	murG	0,3
UPI00004E1539	Newbould305_2227	Penicillin-binding protein 2 (PBP2)	COG0744	CM	CYT	1	75	80,3	pbp2	162,0
UPI00000CA966	Newbould305_2559	Glycine betaine/carnitine/choline ABC transporter	COG1732	CM	SpII	1	32	34,7	opuCC	2,4
UPI00000522A6	Newbould305_2697	Membrane protein insertase YidC	COG0706	CM	SpII	6	4	33,5	yidC	0,9

Inorganic ion transport and metabolism											
UPI00000542DF	Newbould305_0346	Putative sulfur transferase (Rhodanese-like domain protein)	COG0607	U	CYT	1	34	14,7	yibN	14,8
UPI00000522E5	Newbould305_0654	Lipoprotein SirA	COG0614	CM	SpII		57	36,6	sirA	116,9
UPI00000546F6	Newbould305_0683	Phosphate/phosphite/phosphonate ABC transporter, periplasmic binding family protein	COG3221	U	SpII	1	15	34,9	phnD	0,7
UPI00000CAA63	Newbould305_0763	Iron ABC transporter substrate-binding protein	COG1840	CM	SpII		10	36,8	NA	0,4
UPI00026C2324	Newbould305_1024	Membrane lipoprotein	COG1464	CM	SpII		29	31,1	NA	3,4
UPI00005FE2E1	Newbould305_1137	ABC transporter substrate-binding protein	COG0614	C	CYT		67	33,2	NA	41,2
UPI000005228B	Newbould305_1158	Manganese-binding protein	COG0803	CM	SpII		87	34,6	mntC	3162276,7
UPI00000DCA56	Newbould305_1160	Manganese ABC transporter/Phosphonate ABC transporter ATP-binding protein	COG1121	CM	CYT		17	27,9	mntA	1,2
UPI0000054792	Newbould305_1268	Iron compound ABC uptake transporter substrate-binding protein (Periplasmic binding protein)/hypothetical protein	COG4607	CM	SpII		84	37,7	yclQ	54116,0
UPI00000D9D29	Newbould305_1350	Lipoprotein/methionine ABC transporter substrate-binding protein	COG1464	CM	SpII		27	30,2	metQ1	2,9
UPI00000CAB53	Newbould305_1545	ABC transporter substrate-binding protein	COG4594	CM	SpII		69	36,5	yhfQ1	609,5
UPI00024E41C7	Newbould305_1643	Molybdenum ABC transporter	COG0725	U	SpII		67	28,7	modA	27824,6
UPI00000547D7	Newbould305_1648	Fe ABC transporter	COG0614	CM	SpII		80	33,9	fhuD2	6308,6
UPI00024E438D	Newbould305_1709	Hypothetical protein/sodium ABC transporter permease	COG1668	CM	SpI	7	6	45,8	NA	0,5
UPI00000D9FAC	Newbould305_1772	Zn-binding lipoprotein adcA-like protein/zinc ABC transporter substrate-binding protein	COG3443	CM	SpII		53	59,0	zinT	88,1
UPI0000054230	Newbould305_2377	Ferrichrome-binding protein FhuD	COG0614	CM	SpII		26	34,7	fhuD	1,5
UPI0000054490	Newbould305_2432	non-heme ferritin	COG1528	C	CYT		55	19,5	ftnA	30,6
Carbohydrate transport and metabolism											
UPI0000054360	Newbould305_0110	Peptidoglycan endo-beta-N-acetylglucosaminidase	COG4193	Extra	CYT	1	55	33,7	NA	10,7
UPI0000054C39	Newbould305_0189	ATP-dependent 6-phosphofructokinase (ATP-PFK)	COG0205	C	CYT		11	34,7	pfkA	0,7
UPI00000522A3	Newbould305_0190	Pyruvate kinase	COG0469	C	CYT		42	63,0	pykA	3,3
UPI00005FE807	Newbould305_0400	PTS system glucoside-specific EIICBA component	COG1263	CM	TMH		12	74,2	glcB	1,5
UPI000012F4C1	Newbould305_0471	Probable malate:quinone oxidoreductase	COG0579	CW	CYT		48	55,9	mqo2	19,8

UPI00005FE855	Newbould305_0513	PTS system fructose-specific II component	COG1299	CM	CYT	8	13	69,8	NA	1,4
UPI00026C21A7	Newbould305_0729	PTS glucose EIICBA component	COG1263	CM	TMH	10	26	74,3	ptsG	18,7
UPI00000522B0	Newbould305_0753	Maltose ABC transporter substrate-binding protein	COG2182	U	SpII		4	47,7	NA	0,3
UPI00026C2327	Newbould305_1034	PTS system trehalose-specific IIBC component	COG1263	CM	CYT	9	4	50,9	NA	0,8
UPI00000547EE	Newbould305_1132	Alcohol dehydrogenase/zinc-dependent alcohol dehydrogenase	COG1064	C	CYT		61	35,9	adhA	13,7
UPI00026C23D8	Newbould305_1230	PTS system fructose-specific transporter subunit IIABC	COG1299	CM	CYT	9	33	68,6	fruA2	47,7
UPI000005229B	Newbould305_1307	Glyceraldehyde-3-phosphate dehydrogenase	COG0057	C	CYT		64	36,2	gapC	48,2
UPI00024E3FAB	Newbould305_1308	Phosphoglycerate kinase	COG0126	C	CYT		38	42,5	NA	9,0
UPI0000054764	Newbould305_1311	Enolase	COG0148	C	CYT		57	47,0	eno	9,0
UPI00026C2301	Newbould305_1495	Autolysin	COG4193	Extra	SpI		49	137,2	atl	37,3
UPI00005FE48D	Newbould305_2114	Transketolase	COG0021	U	CYT		5	72,2	NA	0,3
Function unknown										
UPI0000054388	Newbould305_0145	Hypothetical protein/DUF948 domain containing protein	COG4768	C	CYT	1	59	17,9	ytxG	1,0
UPI00000542B3	Newbould305_0306	UPF0365 protein BN1321_260211/hypothetical protein	COG4864	C	TMH	2	58	35,1	yqfA	14,4
UPI00005FE857	Newbould305_0515	Conserved membrane protein/hgE/Pip domain-containing protein	COG1511	CM	SpI	6	7	108,3	NA	0,2
UPI00000D76CF	Newbould305_0930	Lipoprotein/Peptidase	COG3212	U	SpII		75	21,2	NA	157,5
UPI0000054BEA	Newbould305_1551	Alkaline shock protein 23	COG1302	U	CYT		20	19,1	asp23	2,7
UPI00005FE70B	Newbould305_1558	Hypothetical protein/alpha/beta hydrolase	COG4814	U	TMH	1	16	33,0	NA	1,5
UPI00026C23C9	Newbould305_1711	Hypothetical protein/DUF805 domain-containing protein	COG3152	CM	CYT	4	17	25,8	NA	4,6
UPI00026C23CB	Newbould305_1724	Membrane-associated protein TcaA	COG4640	U	CYT	1	8	52,0	tcaA	0,4
UPI00000D774D	Newbould305_2115	UPF0154 protein SAB1201/hypothetical protein	COG3763	CM	TMH	1	29	9,2	NA	24,1
UPI00000D7723	Newbould305_2410	Hypothetical protein/UPF0316 protein ERS072738_02115	COG4843	CM	TMH	3	8	22,9	NA	1,5
UPI0001AE9AC5	Newbould305_2442	Ribonuclease BN /YihY/virulence factor BrkB family protein	COG1295	CM	CYT	6	15	45,5	yihY	3,0
UPI00000D9FCA	Newbould305_2546	Lipoprotein/hypothetical protein	COG4808	U	SpII		46	17,2	yehR	9999,0
UPI0000054628	Newbould305_2732	Putative uncharacterized protein orf2 (YbbR-like family protein) (YbbR-like protein)/hypothetical protein	COG4856	U	SpI	1	12	34,5	orf2	0,6

Amino acid transport and metabolism										
UPI00005FE424	Newbould305_1809	Ornithine carbamoyltransferase	COG0078	C	CYT		31	37,4	argF	2,5
UPI000005492C	Newbould305_0507	Arginine deiminase (ADI)	COG2235	C	CYT		16	46,8	arcA	0,6
UPI00005FE1C8	Newbould305_0779	Nickel ABC transporter substrate-binding protein	COG0747	CW	SpII		6	55,2	NA	0,3
UPI00000D78A5	Newbould305_1400	Glutamate dehydrogenase	COG0334	C	CYT		11	45,6	gudB/gluD	0,5
UPI00003B1A87	Newbould305_1433	Periplasmic oligopeptide-binding protein oppA/Peptide ABC transporter substrate-binding protein (Lipoproteins)	COG4166	U	SpII		7	61,4	oppA	0,6
UPI000012B701	Newbould305_2020	Glutamine synthetase	COG0174	C	CYT		23	50,7	glnA	2,4
UPI00026C222B	Newbould305_2537	Cobalt and nickel transporter Cnt	COG0747	CW	SpII		52	59,1	opp1A	13,9
UPI0002506E5E	Newbould305_2595	Extracellular amino acid binding ABC transporter/amino acid ABC transporter substrate-binding protein	COG0834	U	CYT		84	27,9	NA	1211526,7
UPI0000054B9D	Newbould305_2597	Amino acid ABC transporter, ATP-binding protein	COG1126	CM	CYT		58	27,1	tcyC	9,0
UPI00000545F5	Newbould305_2674	Serine hydroxymethyltransferase (SHMT) (Serine methylase)	COG0112	C	CYT		20	45,1	glyA	2,0
Defense mechanisms										
UPI00000D9CDC	Newbould305_1170	Lipid A export ATP-binding/permease protein MsbA	COG1132	CM	TMH	6	2	63,9	msbA1	0,3
UPI0000054B77	Newbould305_1499	Protein FmtA	COG1680	CM	CYT	1	14	45,9	fmtA	0,7
UPI00005FE729	Newbould305_1623	Acriflavin resistance transport protein/AcrB/AcrD/AcrF family protein [Staphylococcus aureus]	COG0841	CM	CYT	11	5	114,5	NA	0,4
UPI0000054464	Newbould305_2390	ABC transporter ATP-binding protein	COG1131	CM	CYT		32	32,8	ybhF	2,5
Posttranslational modification, protein turnover and chaperones										
UPI000005229F	Newbould305_0020	Foldase protein PrsA	COG0760	CM	SpII		82	35,5	prsA	193069771,9
UPI000005224B	Newbould305_0158	Serine protease/PDZ domain-containing protein	COG0265	CM	TMH		28	45,7	degP	7,1
UPI00005FE543	Newbould305_0299	Chaperone protein DnaK (HSP70)	COG0443	C	CYT		30	66,2	dnaK	1,8
UPI00000D7685	Newbould305_0300	Chaperone protein DnaJ	COG0484	C	CYT		7	41,6	dnaJ	0,4
UPI00000CACA2	Newbould305_1417	Chaperone protein ClpB	COG0542	C	CYT		4	98,2	clpB	0,1

UPI0000054B9A	Newbould305_1775	Disulfide bond protein A (Protein-disulfide isomerase, DsbA-like protein)	COG1651	CW	SpII		41	23,0	dsbA	9,0
UPI0000054A61	Newbould305_2194	Serina protease/Carboxy-terminal processing proteinase ctpA	COG0793	CM	CYT	1	23	55,1	ctpA	1,8
UPI00000CAC92	Newbould305_2237	Zinc metallopeptidase (Putative membrane protease YugP)	COG2738	CM	CYT	3	31	25,2	yugP	9,0
UPI00004B5C68	Newbould305_2348	60 kDa chaperonin (GroEL protein) (Protein Cpn60)	COG0459	C	CYT		10	57,5	groEL	0,3
UPI000002EA3A	Newbould305_2629	ATP-dependent zinc metalloprotease FtsH	COG0465	CM	TMH	2	19	77,7	ftsH	2,0
Lipid transport and metabolism										
UPI0000054395	Newbould305_0159	1-acyl-sn-glycerol-3-phosphate acyltransferase (EC 2.3.1.-) (EC 2.3.1.51) (Acyltransferase)	COG0204	C	CYT		11	23,0	plsC	0,5
UPI00024E4C72	Newbould305_0797	Teichoic acid biosynthesis protein F	COG1887	CM	CYT		36	45,9	NA	3,6
UPI0000054B16	Newbould305_1427	beta-ketoacyl-[acyl-carrier-protein] synthase II	COG0304	CM	CYT		9	43,6	fabF	0,6
UPI00005FE44D	Newbould305_1875	Phosphate acyltransferase	COG0416	C	CYT		6	35,3	plsX	0,4
UPI00021AE7EF	Newbould305_2089	Cardiolipin synthase/Phospholipase D/transphosphatidylase	COG1502	CM	TMH	2	4	56,3	NA	0,3
Coenzyme transport and metabolism										
UPI00000D9FFF	Newbould305_0401	Pyruvate oxidase	COG0028	CM	CYT		8	63,5	poxB	0,4
UPI0001FAD51F	Newbould305_1042	Pyridoxal biosynthesis lyase PdxS	COG0214	C	CYT		27	30,5	NA	2,5
UPI00000CAD7B	Newbould305_1487	1,4-dihydroxy-2-naphthoyl-CoA synthase (DHNA-CoA synthase)	COG0447	C	CYT		12	30,3	menB	1,5
UPI00024E4C6C	Newbould305_1576	Acetolactate synthase	COG0028	CM	CYT		16	61,0	NA	1,0
UPI00026C23A9	Newbould305_2246	Ubiquinone/menaquinone biosynthesis methyltransferase	COG2226	U	CYT		42	27,3	NA	5,8
General function prediction only										
UPI00020F307E	Newbould305_0857	5'-nucleotidase, lipoprotein e(P4) family	COG2503	U	SpI		90	33,3	hel	71968567299,1
UPI00005FE76D	Newbould305_1710	Probable sodium ABC transporter ATP-binding protein	COG4152	CM	CYT		24	33,6	NA	2,0
UPI00026C23F4	Newbould305_1811	TRAP family protein/Hypothetical protein	COG1288	CM	SpI	13	18	56,5	NA	2,5
UPI00005FE660	Newbould305_2419	Probable lipoprotein/CamS family sex pheromone protein	COG4851	U	SpII		44	45,3	NA	16,2
UPI0000054610	Newbould305_2645	Putative thiol-disulfide oxidoreductase, DCC family/hypothetical protein	COG3011	CM	CYT		75	16,2	NA	99,0

Signal transduction mechanisms										
UPI0000054C46	Newbould305_0177	Putative universal stress protein	COG0589	C	CYT			18,4	NA	2,6
UPI00005FE502	Newbould305_2270	Sensor proteins SrrB	COG5002	CM	CYT	2	4	66,0	srrB	0,2
UPI000005449B	Newbould305_2440	Sensor protein VraS	COG4585	CM	TMH	2	17	39,9	vraS	1,1
Transcription										
UPI00000D771C	Newbould305_1067	DNA-directed RNA polymerase subunit beta	COG0085	C	CYT		25	133,0	rpoB	2,1
UPI00005FE2AF	Newbould305_1068	DNA-directed RNA polymerase subunit beta' (RNAP subunit beta')	COG0086	C	CYT		38	135,2	rpoC	3,4
UPI0000054BBD	Newbould305_1583	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha)	COG0202	C	CYT		9	34,9	rpoA	0,8
UPI00000D9DAF	Newbould305_1971	Membrane-associated zinc metalloprotease/Putative zinc metalloprotease	COG0750	CM	TMH	5	17	48,0	NA	1,9
Cell cycle control, cell division and chromosome partitioning										
UPI0000054C4F	Newbould305_0169	Septation ring formation regulator EzrA	COG4477	CM	CYT	1	35	66,1	ezrA	2,3
UPI00000543C7	Newbould305_1829	Cell division protein FtsZ	COG0206	C	CYT		34	40,9	ftsZ	3,1
Intracellular trafficking, secretion and vesicular transport										
UPI00005FE565	Newbould305_0242	Protein-export membrane protein/protein translocase subunit SecDF	COG0342	CM	CYT	10	18	84,1	NA	11,3
UPI000005226D	Newbould305_1286	Protein translocase subunit SecA	COG0653	C	CYT		12	95,8	secA	0,6
UPI0001AB18F3	Newbould305_1407	Signal peptidase IB	COG0681	CW	TMH	1	61	21,9	spsB	99,0
Replication, recombination and repair										
UPI00000CAD47	Newbould305_0594	DNA gyrase subunit B	COG0187	C	CYT		4	72,4	gyrB	0,18
UPI00026C2307	Newbould305_2095	Thermonuclease/ hypothetical protein	COG1525	Extra	SpI	1	48	21,8	NA	22,71
UPI00000D7761	Newbould305_2707	DEAD-box ATP-dependent RNA helicase CshA	COG0513	C	CYT		11	56,8	cshA	1,07
Nucleotide transport and metabolism										
UPI0000054548	Newbould305_0946	Inosine-5'-monophosphate dehydrogenase (IMP dehydrogenase) (IMPD) (IMPDH)	COG0516	C	CYT		6	52,7	guaB	0,20
UPI000012BD6F	Newbould305_0947	GMP synthase [glutamine-hydrolyzing]	COG0519	C	CYT		4	58,1	guaA	0,39

Secondary metabolites biosynthesis, transport and catabolism										
UPI0000054A71	Newbould305_2179	Hypothetical protein/5-bromo-4-chloroindolyl phosphate hydrolysis protein	COG4915	C	TMH	2	12	24,6	NA	1,4
Unclassified function										
UPI00000CAA99	Newbould305_2508	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex		C	CYT		87	46,2	pdhC	1,84785E+11
UPI0000505A18	Newbould305_1925	Phage capsid protein (Phage major capsid protein)		U	CYT		97	33,5	NA	23712,7
UPI0001FAD63B	Newbould305_2342	delta-hemolysin		Extra	CYT		96	3,2	NA	99999999,0
UPI00000D9D73	Newbould305_2511	Lipoprotein (Cell-wall binding lipoprotein)		U	SpII		79	23,8	NA	1668099,5
UPI00026C2258	Newbould305_2589	IgG-binding protein SBI/ hypothetical protein		U	SpI		68	50,1	sbi	221,8
UPI00000D9D8F	Newbould305_1816	Beta-class phenol-soluble modulin (PSMβ1)		U	CYT		88	4,4	psmβ1	99,0
UPI0000054555	Newbould305_0935	Lipoprotein (Putative lipoprotein)		U	SpII		78	23,6	NA	99999,0
UPI00005FE419	Newbould305_1791	Fibrinogen-binding protein		Extra	SpI		40	12,5	fnbP	561,3
UPI000005426C	Newbould305_0241	Component of the preprotein translocase (Preprotein translocase subunit YajC) (Preprotein translocase, YajC subunit)		U	CYT		59	9,6	yajC	31621,8
UPI00005FE322	Newbould305_1238	Uncharacterized protein/hypothetical protein		U	SpII		52	16,0	NA	192,1
UPI00026C2293	Newbould305_0083	Exported protein/hypothetical protein		U	CYT	1	43	34,9	NA	5,7
UPI00026C21D9	Newbould305_1885	Hypothetical protein		U	SpII		38	18,6	NA	99,0
UPI0000054A3F	Newbould305_2223	Cell cycle protein GpsB (Guiding PBP1-shuttling protein)		C	CYT		78	13,1	gpsB	1777,3
UPI0001AE9B38	Newbould305_2258	Elastin binding protein ebpS		CM	CYT	1	26	53,3	ebpS	4,6
UPI00026C23CA	Newbould305_1721	Hypothetical protein/ HlyD family secretion protein		CM	SpI	1	53	22,9	NA	41,2
UPI00000D76AE	Newbould305_1141	Exported protein (Uncharacterised protein)/ hypothetical protein		U	SpI		50	18,5	NA	30,6
UPI00024E41B3	Newbould305_1737	Hypothetical protein		CM	SpII		32	23,3	NA	5,8
UPI00000D7827	Newbould305_2451	Monofunctional glycosyltransferase (MGT) (Peptidoglycan TGase)		CM	CYT	1	31	31,4	mgt	7,4
UPI00024E4AEC	Newbould305_0824	Hypothetical protein		U	SpI	1	30	57,7	NA	3,3
UPI00021AE82F	Newbould305_0088	Hypothetical protein		U	SpII		60	13,3	NA	9,0
UPI00000CAF2B	Newbould305_2638	Lytic regulatory protein/hypothetical protein		CM	TMH	6	13	40,5	NA	9,0

UPI0000505A13	Newbould305_1921	Phage portal protein (Portal protein, phage associated)	C	CYT		18	54,9	NA	0,7
UPI00000548D6	Newbould305_1212	Hypothetical protein	U	CYT		20	26,6	NA	16,8
UPI00000522D5	Newbould305_2500	Membrane protein/Hypothetical protein	CM	TMH	3	22	39,9	NA	2,2
UPI00026C23D2	Newbould305_2311	Hypothetical protein	U	SpII		42	21,4	NA	6,2
UPI0000054770	Newbould305_1753	Exported protein/Hypothetical protein	U	SpI	1	37	13,9	NA	9,0
UPI0000505A1F	Newbould305_1931	Phage protein/Hypothetical protein	C	CYT		30	20,4	NA	1,8
UPI0000054640	Newbould305_1751	Hipothetical protein/DUF4889 domain-containing protein	U	SpII	1	32	13,3	NA	14,8
UPI00000543D5	Newbould305_1817	Beta-class phenol-soluble modulin (PSMβ2)	U	CYT		88	4,4	psmβ2	30,6
UPI00026C225E	Newbould305_0033	Hypothetical protein	U	CYT		15	17,6	NA	3,0
UPI00000D9DCA	Newbould305_2096	Uncharacterised protein/hypothetical protein	U	CYT	1	11	32,7	NA	0,6
UPI000005449E	Newbould305_2443	Exported protein (Membrane associated protein) (Putative staphylococcal protein)/hypothetical protein	C	CYT	1	47	10,2	NA	9,0
UPI00005FE67D	Newbould305_2380	Uncharacterized leukocidin-like protein 2	Extra	SpI	1	9	40,4	NA	0,7
UPI00026C22FD	Newbould305_1006	Hypothetical protein	U	SpII	1	8	32,9	NA	1,2
UPI00026C23B2	Newbould305_2269	Lipoprotein/ Hypothetical protein	U	SpII		15	35,1	NA	1,0
UPI00000DA0F2	Newbould305_1776	ABC transporter ATP-binding protein/Lipoprotein/cystatin-like fold lipoprotein	CM	SpII		9	14,2	NA	3,0
UPI000004802C	Newbould305_0304	30S ribosomal protein S21	C	CYT		33	6,9	rpsU	9,0
UPI00005FE707	Newbould305_1553	Probable membrane-bound oxidoreductase/hypothetical protein	U	CYT	1	20	22,9	NA	3,0
UPI00000CAD76	Newbould305_2573	Membrane protein (Putative membrane protein)	U	CYT	1	24	25,7	NA	0,9
UPI00005FE56A	Newbould305_0229	Hypothetical protein	U	CYT	1	33	18,2	NA	1,7
UPI00005FE1D7	Newbould305_0802	Probable glycosyl transferase/hypothetical protein	C	CYT		4	66,1	NA	0,2
UPI000005446B	Newbould305_2397	Hypothetical protein	CM	SpI	1	24	6,5	NA	9,0
UPI00015FD704	PSMA1_STAAB	Alpha-class phenol-soluble modulin	U	CYT		95	2,2	psmα1	30,6
UPI00015FD703	PSMA2_STAAB	Alpha-class phenol-soluble modulin alpha 2	U	CYT		95	2,2	psmα2	999,0
UPI000161A44A	PSMA4_STAAB	Alpha-class phenol-soluble modulin alpha 4	U	CYT		71	2,1	psmα4	34,0

(1), Proteins are classified in Gene Ontology functional classes. Names are given according to annotation of available *S.aureus* Newbould N305 and verified againts NCBI and uniprot database.

(2), PsortB prediction: Extra, extracellular; CM, cytoplasmic membrane; C, cytoplasmic; U, Unknow

(3), LipoP prediction: SpII, SPaseII-cleaved proteins; SpI, SPaseI-cleaved proteins; CYT, cytoplasmic; TMH, transmembrane

(4), Number of Transmembrane domain (THMM prediction)

(5), Molecular weight calculated from the protein sequence

(6), Gene names are given according to annotation of *S.aureus* Newbould 305 (N305). NA: No Available

(7), Exponentially Modified Protein Abundance Index (emPAI)

Table S3. Proteins identified in the extracellular vesicles derived from *S. aureus* N305, 03ST17, 06ST1048 and M060.

Locus tag TW20	Locus tag N305	Gene name	N305 (1)	03ST17 (1) (2)	06ST1048 (1) (2)	M060 (1) (2)
SATW20_07180	Newbould305_1170	abcA	1	0	0	1
SATW20_17020	Newbould305_0176	ackA	1	0	0	1
SATW20_04480	Newbould305_0938	ahpC	0	0	0	1
SATW20_15760	Newbould305_0299	alaS	1	0	1	0
SATW20_17000	Newbould305_0178	ald1	0	0	0	1
SATW20_01780	Newbould305_0707	aldA	0	0	0	1
SATW20_27730	Newbould305_0507	arcA	1	0	0	1
SATW20_27720	Newbould305_0506	arcB	0	0	0	1
SATW20_11610	Newbould305_1809	argF	1	0	0	0
SATW20_15170	Newbould305_2301	argR	0	0	1	0
SATW20_08810	Newbould305_1341	arsC	0	0	0	1
SATW20_14530	Newbould305_2231	asnS	0	0	0	1
SATW20_23200	Newbould305_1551	asp23	1	0	0	1
SATW20_10490	Newbould305_1495	atl	1	1	1	1
SATW20_22430	Newbould305_2682	atpA	1	1	1	0
SATW20_22410	Newbould305_2684	atpD	1	1	1	0
SATW20_22450	Newbould305_2680	atpF	1	1	1	0
SATW20_22440	Newbould305_2681	atpH	0	1	1	0
SATW20_15120	Newbould305_2296	bfmB	0	0	0	1
SATW20_27120	NA	blaZ	0	0	1	0
SATW20_16840	Newbould305_0195	citC	0	0	0	1
SATW20_16850	Newbould305_0194	citZ	0	0	0	1
SATW20_09740	Newbould305_1417	clpB	1	0	1	0
SATW20_08430	Newbould305_1303	clpP	0	0	1	1
SATW20_26790	Newbould305_0420	copA	0	1	0	0
SATW20_05810	Newbould305_2631	cysK	0	0	0	1
SATW20_22760	Newbould305_2647	deoD	0	0	0	1
SATW20_09350	Newbould305_1375	dltD	1	1	1	0
SATW20_15750	Newbould305_0300	dnaJ	1	0	1	0
SATW20_00020	Newbould305_0591	dnaN	0	0	0	1
SATW20_22770	Newbould305_2646	dps	0	0	0	1
SATW20_11470	Newbould305_1791	ecb	1	0	1	0
SATW20_08510	Newbould305_1311	eno	1	1	1	1
SATW20_02850	Newbould305_0830	esaA	0	1	0	0
SATW20_02880	Newbould305_0833	essB	0	1	0	0
SATW20_17080	Newbould305_0169	ezrA	1	1	0	0
SATW20_12250	Newbould305_1877	fabG	0	0	1	0
SATW20_10080	Newbould305_1453	fabI	0	0	1	1
SATW20_22370	Newbould305_2688	fabZ	0	0	1	1
SATW20_22630	Newbould305_2661	fbaA	0	0	0	1
SATW20_26370	Newbould305_0376	fbp	0	0	0	1

SATW20_13740	Newbould305_2148	femA	0	0	0	1
SATW20_13750	Newbould305_2149	femB	0	1	0	1
SATW20_12310	Newbould305_1883	ffh	0	0	1	0
SATW20_17220	Newbould305_0153	fhs	0	0	0	1
SATW20_24160	Newbould305_1648	fhuD	1	1	1	0
SATW20_23950	Newbould305_1624	fmhB	0	0	1	0
SATW20_07750	Newbould305_1230	fruA	1	0	1	0
SATW20_06170	Newbould305_1072	fus	1	0	1	1
SATW20_08470	Newbould305_1307	gap1	1	0	1	1
SATW20_16770	Newbould305_0202	gap2	0	0	0	1
SATW20_18950	Newbould305_2423	gatA	1	0	1	0
SATW20_15320	Newbould305_0344	gcvPA	0	0	0	1
SATW20_15310	Newbould305_0345	gcvPB	0	0	0	1
SATW20_01990	Newbould305_0729	glcA	1	1	0	0
SATW20_26590	Newbould305_0400	glcB	1	0	0	0
SATW20_22900	Newbould305_2739	glmS	0	0	0	1
SATW20_13020	Newbould305_2020	glnA	1	1	1	1
SATW20_12940	Newbould305_2012	glpD	1	1	1	1
SATW20_12920	Newbould305_2010	glpF	0	1	0	0
SATW20_12930	Newbould305_2011	glpK	0	0	0	1
SATW20_05400	Newbould305_1032	gltA	0	1	0	0
SATW20_22510	Newbould305_2674	glyA	1	0	0	1
SATW20_15080	Newbould305_2290	gnd	0	0	0	1
SATW20_25460	Newbould305_2592	gpmA	0	0	0	1
SATW20_13560	Newbould305_2129	grlA	0	0	1	0
SATW20_13550	Newbould305_2128	grlB	0	0	1	0
SATW20_04560	Newbould305_0946	guaB	1	0	0	1
SATW20_00060	Newbould305_0595	gyrA	0	0	1	1
SATW20_00050	Newbould305_0594	gyrB	1	0	1	0
SATW20_16590	Newbould305_0220	hemB	0	0	0	1
SATW20_18580	Newbould305_2461	hemL	0	0	0	1
SATW20_25490	Newbould305_2588	hlgA	0	1	1	1
SATW20_25510	Newbould305_2586	hlgB	0	1	1	1
SATW20_25500	Newbould305_2587	hlgC	0	1	1	1
SATW20_08350	Newbould305_1294	hprK	0	1	0	1
SATW20_14700	Newbould305_2249	hup	0	0	1	1
SATW20_11870	Newbould305_1836	ileS	0	0	0	1
SATW20_16710	Newbould305_0208	infC	1	0	1	0
SATW20_13360	Newbould305_2105	katA	0	0	0	1
SATW20_02430	Newbould305_0783	ldh1	1	0	0	1
SATW20_27410	Newbould305_0465	ldh2	1	0	0	1
SATW20_01220	Newbould305_0649	lldP1	0	0	1	0
SATW20_24980	Newbould305_1735	lldP2	0	1	0	0
SATW20_05850	Newbould305_2637	lysS	0	0	0	1

SATW20_00740	NA	mecA	0	1	0	0
SATW20_10420	Newbould305_1487	menB	1	0	0	1
SATW20_14680	Newbould305_2246	menH	1	0	1	0
SATW20_07610	Newbould305_1216	mgrA	0	0	0	1
SATW20_09490	Newbould305_1390	mnhD	0	1	0	0
SATW20_07060	Newbould305_1158	mntC	1	1	1	1
SATW20_11000	Newbould305_2498	mntH	0	1	0	0
SATW20_24110	Newbould305_1643	modA	1	1	0	0
SATW20_13600	Newbould305_2134	mprF	0	1	0	0
SATW20_27460	Newbould305_0471	mqo2	1	0	1	1
SATW20_11720	Newbould305_1821	mraZ	0	0	0	1
SATW20_13490	Newbould305_2119	mscL	0	0	1	0
SATW20_22910	Newbould305_2738	mtlA	0	1	0	0
SATW20_14190	Newbould305_2192	murG	1	0	0	1
SATW20_11440	Newbould305_1788	murI	0	0	0	1
SATW20_26680	Newbould305_0408	mvaS	0	0	0	1
SATW20_14660	Newbould305_2244	ndk	0	0	0	1
SATW20_15530	Newbould305_0322	nfo	0	0	0	1
SATW20_13260	Newbould305_2095	nucI	1	1	0	0
SATW20_05900	Newbould305_1044	nupC	0	1	1	0
SATW20_14140	Newbould305_2187	odhA	0	1	0	1
SATW20_14130	Newbould305_2186	odhB	0	1	0	1
SATW20_22290	Newbould305_2697	oxaA	1	1	1	0
SATW20_27380	Newbould305_0462	panB	0	0	0	1
SATW20_14490	Newbould305_2227	pbp2	1	0	1	0
SATW20_15480	Newbould305_0327	pbpF	1	1	0	0
SATW20_17800	Newbould305_0080	pckA	0	0	0	1
SATW20_10880	Newbould305_2510	pdhA	1	1	1	1
SATW20_10890	Newbould305_2509	pdhB	1	1	1	1
SATW20_10900	Newbould305_2508	pdhC	1	1	1	1
SATW20_10910	Newbould305_2507	pdhD	1	1	1	1
SATW20_05870	Newbould305_1042	pdxS	1	0	1	0
SATW20_09420	Newbould305_1382	pepA	0	0	0	1
SATW20_16900	Newbould305_0189	pfkA	1	0	1	0
SATW20_09620	Newbould305_1404	pgi	0	0	0	1
SATW20_12770	Newbould305_1995	pgsA	0	1	0	0
SATW20_12140	Newbould305_1866	pknB	1	1	0	0
SATW20_12680	Newbould305_1985	pnpA	0	0	0	1
SATW20_16800	Newbould305_0199	polA	0	0	1	0
SATW20_12570	Newbould305_1972	proS	0	0	1	0
SATW20_05680	Newbould305_2618	prs	0	0	1	1
SATW20_18350	Newbould305_0020	prsA	1	1	1	1
SATW20_05201	PSMA4_STAAB	psmA4	1	0	0	1
SATW20_06570	Newbould305_1114	pta	0	0	0	1

SATW20_05640	Newbould305_2613	purR	0	0	1	0
SATW20_18970	Newbould305_2420	putP	0	1	0	0
SATW20_16890	Newbould305_0190	pyk	1	0	1	1
SATW20_22740	Newbould305_2649	pyn	0	0	0	1
SATW20_12520	Newbould305_1967	pyrH	0	0	0	1
SATW20_11920	Newbould305_1842	pyrR	0	0	0	1
SATW20_10570	Newbould305_1503	qoxA	1	1	1	0
SATW20_10560	Newbould305_1502	qoxB	1	1	1	0
SATW20_17580	Newbould305_0117	ribH	0	1	1	0
SATW20_12120	Newbould305_1864	rlmN	0	0	1	0
SATW20_14910	Newbould305_2272	rluB	0	0	1	0
SATW20_26760	Newbould305_0417	rocA	0	0	0	1
SATW20_17520	Newbould305_0121	rot	0	1	1	0
SATW20_06080	Newbould305_1063	rplA	1	0	1	1
SATW20_23810	Newbould305_1606	rplB	1	1	1	1
SATW20_23840	Newbould305_1609	rplC	0	0	1	0
SATW20_23720	Newbould305_1597	rplE	1	1	1	1
SATW20_23690	Newbould305_1594	rplF	1	0	1	0
SATW20_06090	Newbould305_1064	rplJ	1	0	1	0
SATW20_23520	Newbould305_1578	rplM	1	0	1	0
SATW20_23740	Newbould305_1599	rplN	1	0	1	1
SATW20_23650	Newbould305_1590	rplO	1	0	1	0
SATW20_23770	Newbould305_1602	rplP	1	0	1	0
SATW20_23570	Newbould305_1582	rplQ	0	0	1	0
SATW20_23680	Newbould305_1593	rplR	0	0	1	0
SATW20_12350	Newbould305_1950	rplS	1	0	1	1
SATW20_16690	Newbould305_0210	rplT	1	0	1	1
SATW20_16410	Newbould305_0232	rplU	1	0	1	1
SATW20_23790	Newbould305_1604	rplV	1	0	1	1
SATW20_23820	Newbould305_1607	rplW	1	0	1	0
SATW20_05690	Newbould305_2619	rplY	0	0	1	1
SATW20_11220	Newbould305_2476	rpmF	0	0	0	1
SATW20_23580	Newbould305_1583	rpoA	1	0	1	1
SATW20_06120	Newbould305_1067	rpoB	1	0	1	1
SATW20_06130	Newbould305_1068	rpoC	1	1	1	1
SATW20_12500	Newbould305_1964	rpsB	1	0	1	1
SATW20_23780	Newbould305_1603	rpsC	1	0	1	1
SATW20_17100	Newbould305_0167	rpsD	1	0	1	1
SATW20_23670	Newbould305_1592	rpsE	1	1	1	1
SATW20_06160	Newbould305_1071	rpsG	1	1	1	1
SATW20_23510	Newbould305_1577	rpsI	0	0	1	1
SATW20_23850	Newbould305_1610	rpsJ	1	0	1	0
SATW20_23590	Newbould305_1584	rpsK	1	0	1	0
SATW20_06150	Newbould305_1070	rpsL	1	0	1	1

SATW20_23600	Newbould305_1585	rpsM	0	0	1	0
SATW20_12670	Newbould305_1984	rpsO	1	0	1	0
SATW20_23750	Newbould305_1600	rpsQ	1	0	1	1
SATW20_04450	Newbould305_0935	rpsR	1	1	1	1
SATW20_22050	Newbould305_2724	rsbW	0	0	0	1
SATW20_11730	Newbould305_1822	rsmH	0	0	1	0
SATW20_07830	Newbould305_1238	saeP	1	1	0	1
SATW20_07820	Newbould305_1237	saeQ	0	1	0	0
SATW20_06930	Newbould305_1144	sarA	0	0	1	1
SATW20_24260	Newbould305_1658	sarR	0	1	1	1
SATW20_01240	Newbould305_0651	sarS	0	1	1	1
SATW20_02020	Newbould305_0732		0	1	0	0
SATW20_02380	Newbould305_0778		0	1	0	0
SATW20_02410	Newbould305_0781		0	1	0	0
SATW20_02790	Newbould305_0821		0	1	0	0
SATW20_03970	Newbould305_0883		0	1	0	0
SATW20_04980	Newbould305_0978		0	1	0	0
SATW20_05310	Newbould305_1023		0	0	1	0
SATW20_05330	Newbould305_1025		0	0	1	0
SATW20_05960	Newbould305_1050		0	1	0	0
SATW20_06560	Newbould305_1113		0	0	0	1
SATW20_08520	Newbould305_1312		0	1	1	0
SATW20_08640	Newbould305_1324		0	0	1	0
SATW20_09140	Newbould305_1353		0	1	1	0
SATW20_10840	Newbould305_1531		0	1	1	1
SATW20_11010	Newbould305_2497		0	0	1	1
SATW20_11090	Newbould305_2489		0	1	1	0
SATW20_12190	Newbould305_1871		0	0	0	1
SATW20_12200	Newbould305_1872		0	0	1	0
SATW20_12400	Newbould305_1956		0	1	0	1
SATW20_12690	Newbould305_1987		0	0	1	1
SATW20_13530	Newbould305_2124		0	0	0	1
SATW20_13570	Newbould305_2130		0	1	0	0
SATW20_13660	Newbould305_2140		0	0	0	1
SATW20_14050	Newbould305_2180		0	0	0	1
SATW20_14260	Newbould305_2199		0	1	0	0
SATW20_15220	Newbould305_2306		0	0	0	1
SATW20_15690	Newbould305_0306		1	1	0	0
SATW20_16970	Newbould305_0182		0	1	1	1
SATW20_16980	Newbould305_0181		0	0	0	1
SATW20_17350	Newbould305_0139		0	0	0	1
SATW20_17690	Newbould305_0106		0	1	0	0
SATW20_17890	Newbould305_0072		0	1	0	0
SATW20_18160	NA		0	1	0	0

SATW20_18250	Newbould305_0031		0	1	0	0
SATW20_18360	Newbould305_0019		0	0	0	1
SATW20_18370	Newbould305_0018		0	1	1	0
SATW20_18410	Newbould305_0014		0	0	1	1
SATW20_19220	Newbould305_2393		0	1	0	0
SATW20_19240	Newbould305_2391		0	1	0	0
SATW20_19560	NA		0	1	0	0
SATW20_21590	NA		0	1	0	0
SATW20_21600	NA		0	1	0	0
SATW20_21670	NA		0	1	0	0
SATW20_21710	NA		0	1	0	0
SATW20_21760	NA		0	1	0	0
SATW20_22260	Newbould305_2701		0	0	0	1
SATW20_22730	Newbould305_2650		0	1	0	0
SATW20_23120	Newbould305_1545		1	1	1	1
SATW20_23220	Newbould305_1553		1	0	0	1
SATW20_24320	Newbould305_1664		0	0	1	0
SATW20_24330	Newbould305_1665		0	1	0	0
SATW20_24350	Newbould305_1667		0	0	0	1
SATW20_24450	Newbould305_1680		0	1	0	0
SATW20_24540	Newbould305_1689		0	0	1	0
SATW20_24670	Newbould305_1702		0	1	0	0
SATW20_24820	Newbould305_1719		0	1	0	0
SATW20_25430	Newbould305_2596		0	0	1	0
SATW20_25620	Newbould305_2573		1	1	0	0
SATW20_25780	Newbould305_2556		0	0	0	1
SATW20_25840	Newbould305_2549		0	0	0	1
SATW20_25870	Newbould305_2546		1	1	0	0
SATW20_26350	Newbould305_0374		0	1	1	0
SATW20_26650	Newbould305_0406		1	0	1	0
SATW20_26920	Newbould305_0435		0	1	0	0
SATW20_27660	Newbould305_0500		0	0	0	1
SATW20_27790	Newbould305_0513		1	1	0	0
SATW20_27960	Newbould305_0531		0	1	0	0
SATW20_25480	Newbould305_2589	sbi	1	1	1	1
SATW20_19040	Newbould305_2413	scpA	0	0	0	1
SATW20_11420	Newbould305_1786	sdhA	1	1	0	1
SATW20_11430	Newbould305_1787	sdhB	0	0	0	1
SATW20_08280	Newbould305_1286	secA	1	0	1	0
SATW20_16310	Newbould305_0242	secF	1	1	1	0
SATW20_08530	Newbould305_1313	secG	0	1	0	0
SATW20_23640	Newbould305_1589	secY	0	0	1	0
SATW20_01270	Newbould305_0654	sirA	1	1	1	0
SATW20_05660	Newbould305_2615	spoVG	0	0	0	1

SATW20_09650	Newbould305_1407	spsB	1	1	1	1
SATW20_14890	Newbould305_2270	srrB	1	1	0	0
SATW20_26490	Newbould305_0388	srtA	1	0	1	0
SATW20_04340	Newbould305_0923	ssb	0	0	0	1
SATW20_08110	Newbould305_1268	sstD	1	1	1	0
SATW20_12390	Newbould305_1955	sucC	0	0	0	1
SATW20_07110	Newbould305_1163	tagA	1	0	1	0
SATW20_02600	Newbould305_0802	tagE	1	0	0	1
SATW20_07130	Newbould305_1165	tagG	0	1	0	0
SATW20_07120	Newbould305_1164	tagH	0	0	1	0
SATW20_04220	Newbould305_0910	thl	0	0	0	1
SATW20_13440	Newbould305_2114	tkt	1	0	0	1
SATW20_23880	Newbould305_1613	topB	0	0	1	0
SATW20_06180	Newbould305_1073	tuf	1	0	1	1
SATW20_22500	Newbould305_2675	upp	0	0	1	1
SATW20_08340	Newbould305_1292	uvrA	0	0	1	0
NA	NA	ybeZ	0	0	0	1
NA	Newbould305_1824		1	0	0	0
SATW20_11680	Newbould305_1817		1	0	0	0
SATW20_27810	Newbould305_0515		1	0	0	0
SATW20_27820	Newbould305_0516		1	0	0	0
SATW20_01370	Newbould305_0666		0	1	0	0
SATW20_01540	Newbould305_0683		1	1	0	0
SATW20_02260	Newbould305_0764		1	1	0	0
SATW20_02820	Newbould305_0824		1	1	0	0
SATW20_05420	Newbould305_1034		1	1	0	0
SATW20_08020	Newbould305_1257		0	1	0	0
SATW20_10810	Newbould305_1528		0	1	0	0
SATW20_10980	Newbould305_2500		1	1	0	0
SATW20_11630	Newbould305_1811		1	1	0	0
SATW20_12070	Newbould305_1859		0	1	0	0
SATW20_12860	Newbould305_2004		0	1	0	0
SATW20_14590	Newbould305_2237		1	1	0	0
SATW20_17650	Newbould305_0110		1	1	0	0
SATW20_18760	Newbould305_2443		1	1	0	0
SATW20_18980	Newbould305_2419		1	1	0	0
SATW20_19060	Newbould305_2410		1	1	0	0
SATW20_20020	Newbould305_2380		1	1	0	0
SATW20_22970	Newbould305_2732		1	1	0	0
SATW20_24720	Newbould305_1709		1	1	0	0
SATW20_24730	Newbould305_1710		1	1	0	0
SATW20_25000	Newbould305_1737		1	1	0	0
SATW20_25150	Newbould305_1753		1	1	0	0
SATW20_25360	Newbould305_1772		1	1	0	0

SATW20_25390	Newbould305_1775	1	1	0	0
SATW20_06860	Newbould305_1137	1	0	1	0
SATW20_10870	Newbould305_2511	1	0	1	0
SATW20_22200	Newbould305_2707	1	0	1	0
SATW20_24300	Newbould305_1662	1	0	1	0
SATW20_25420	Newbould305_2597	1	0	1	0
SATW20_03120	Newbould305_0857	1	1	1	0
SATW20_12560	Newbould305_1971	1	1	1	0
SATW20_15270	Newbould305_2311	1	1	1	0
SATW20_15300	Newbould305_0346	1	1	1	0
SATW20_16320	Newbould305_0241	1	1	1	0
SATW20_17290	Newbould305_0145	1	1	1	0
SATW20_17780	Newbould305_0083	1	1	1	0
SATW20_06900	Newbould305_1141	1	0	0	1
SATW20_09410	Newbould305_1381	1	0	0	1
SATW20_24410	Newbould305_1676	1	0	0	1
SATW20_17010	Newbould305_0177	1	1	0	1
SATW20_19180	Newbould305_2397	1	1	0	1
SATW20_19250	Newbould305_2390	1	1	0	1
SATW20_18870	Newbould305_2432	1	1	1	1
SATW20_25440	Newbould305_2595	1	1	1	1
NA	Newbould305_1885	1	0	0	0
NA	Newbould305_1925	1	0	0	0
NA	Newbould305_0033	1	0	0	0
SATW20_01590	Newbould305_0688	1	0	0	0
SATW20_02150	Newbould305_0753	1	0	0	0
SATW20_02250	Newbould305_0763	1	0	0	0
SATW20_02390	Newbould305_0779	1	0	0	0
SATW20_02560	Newbould305_0797	1	0	0	0
SATW20_03630	Newbould305_1921	1	0	0	0
SATW20_03730	Newbould305_1931	1	0	0	0
SATW20_04350	Newbould305_0924	1	0	0	0
SATW20_04400	Newbould305_0930	1	0	0	0
SATW20_04570	Newbould305_0947	1	0	0	0
SATW20_05130	Newbould305_1006	1	0	0	0
SATW20_05203	PSMA2_STAAB	1	0	0	0
SATW20_05204	PSMA1_STAAB	1	0	0	0
SATW20_05320	Newbould305_1024	1	0	0	0
SATW20_05790	Newbould305_2629	1	0	0	0
SATW20_06820	Newbould305_1132	1	0	0	0
SATW20_07080	Newbould305_1160	1	0	0	0
SATW20_07170	Newbould305_1169	1	0	0	0
SATW20_07570	Newbould305_1212	1	0	0	0
SATW20_07940	Newbould305_1248	1	0	0	0

SATW20_08480	Newbould305_1308	1	0	0	0
SATW20_08880	Newbould305_1350	1	0	0	0
SATW20_09580	Newbould305_1400	1	0	0	0
SATW20_09830	Newbould305_1427	1	0	0	0
SATW20_09880	Newbould305_1433	1	0	0	0
SATW20_10520	Newbould305_1498	1	0	0	0
SATW20_10530	Newbould305_1499	1	0	0	0
SATW20_11670	Newbould305_1816	1	0	0	0
SATW20_11800	Newbould305_1829	1	0	0	0
SATW20_12230	Newbould305_1875	1	0	0	0
SATW20_13200	Newbould305_2089	1	0	0	0
SATW20_13270	Newbould305_2096	1	0	0	0
SATW20_13450	Newbould305_2115	1	0	0	0
SATW20_13620	Newbould305_2136	1	0	0	0
SATW20_14040	Newbould305_2179	1	0	0	0
SATW20_14210	Newbould305_2194	1	0	0	0
SATW20_14450	Newbould305_2223	1	0	0	0
SATW20_14780	Newbould305_2258	1	0	0	0
SATW20_14830	Newbould305_2269	1	0	0	0
SATW20_15610	Newbould305_0314	1	0	0	0
SATW20_15710	Newbould305_0304	1	0	0	0
SATW20_16130	Newbould305_0262	0	0	0	0
SATW20_16330	Newbould305_0240	1	0	0	0
SATW20_16440	Newbould305_0229	1	0	0	0
SATW20_16550	Newbould305_0224	1	0	0	0
SATW20_16730	Newbould305_0206	1	0	0	0
SATW20_17170	Newbould305_0159	1	0	0	0
SATW20_17180	Newbould305_0158	1	0	0	0
SATW20_18680	Newbould305_2451	1	0	0	0
SATW20_18770	Newbould305_2442	1	0	0	0
SATW20_18790	Newbould305_2440	1	0	0	0
SATW20_20060	Newbould305_2377	1	0	0	0
SATW20_20120	Newbould305_2348	1	0	0	0
SATW20_20180	Newbould305_2342	1	0	0	0
SATW20_22400	Newbould305_2685	1	0	0	0
SATW20_22420	Newbould305_2683	1	0	0	0
SATW20_22780	Newbould305_2645	1	0	0	0
SATW20_22850	Newbould305_2638	1	0	0	0
SATW20_23260	Newbould305_1558	1	0	0	0
SATW20_23450	Newbould305_1576	1	0	0	0
SATW20_23700	Newbould305_1595	1	0	0	0
SATW20_23710	Newbould305_1596	1	0	0	0
SATW20_23760	Newbould305_1601	1	0	0	0
SATW20_23830	Newbould305_1608	1	0	0	0

SATW20_23940	Newbould305_1623	1	0	0	0
SATW20_24360	Newbould305_1668	1	0	0	0
SATW20_24740	Newbould305_1711	1	0	0	0
SATW20_24840	Newbould305_1721	1	0	0	0
SATW20_24870	Newbould305_1724	1	0	0	0
SATW20_25130	Newbould305_1751	1	0	0	0
SATW20_25400	Newbould305_1776	1	0	0	0
SATW20_25750	Newbould305_2559	1	0	0	0
SATW20_25950	Newbould305_2537	1	0	0	0
SATW20_26600	Newbould305_0401	1	0	0	0
SATW20_27130	Newbould305_0088	1	0	0	0

(1), occurrence of proteins: 0, absent; 1, present.

(2), data obtained from Jeon *et al.*, 2016 (Jeon H, Oh MH, Jun SH, Kim SI, Choi CW, Kwon HI, Na SH, Kim YJ, Nicholas A, Selasi GN, Lee JC. Variation among Staphylococcus aureus membrane vesicle proteomes affects cytotoxicity of host cells. Microb Pathog. 2016 Apr;93:185-93. doi: 10.1016/j.micpath.2016.02.014.).

Annex 2

Supplementary data (Chapter 3)

Table S1. List of proteins identified in *S. aureus* EVs.

GI	Locus Tag	UniParc	COG (1)	Gene Product Name	Genome Name	Gene name (2)	LipoP (3)	Psort (4)	SigIP (5)	Mu50 (6)*	O46 (7)*	O11 (8)*	N305 (9)*	RF122 (10)*	MW2 (11)*
Translation, ribosomal structure and biogenesis															
123754568	SAB0499	UPI0000054837	COG0050	Elongation factor Tu	S. aureus RF122	tuf	CYT	C	No	1	1	1	1	1	1
123768552	SAB1118	UPI0000134F21	COG0052	30S ribosomal protein S2	S. aureus RF122	rpsB	CYT	C	No	1	1	1	1	0	1
119368770	SAB0498	UPI0000052287	COG0480	Elongation factor G	S. aureus RF122	fus	CYT	C	No	1	1	1	1	0	1
115502797	SAB2115c	UPI00000D76A9	COG0197	50S ribosomal protein L16	S. aureus RF122	rplP	CYT	C	No	1	1	1	1	1	1
118572741	SAB2119c	UPI00000522A9	COG0090	50S ribosomal protein L2	S. aureus RF122	rplB	CYT	C	No	0	1	0	1	1	1
14248017	SAV2244	UPI0000134FAF	COG0092	30S ribosomal protein S3	S. aureus Mu50	rpsC	CYT	C	No	1	1	0	1	0	0
14246306	SAV0538	UPI0000054841	COG0081	50S ribosomal protein L1	S. aureus Mu50	rplA	CYT	C	No	0	1	1	1	0	1
14248006	SAV2233	UPI0000048021	COG0098	30S ribosomal protein S5	S. aureus Mu50	rpsE	CYT	C	No	1	1	1	1	0	1
14246307	SAV0539	UPI0000054840	COG0244	50S ribosomal protein L10	S. aureus Mu50	rplJ	CYT	C	No	1	1	1	1	0	0
123754661	SAB2120c	UPI00000549A8	COG0089	50S ribosomal protein L23	S. aureus RF122	rplW	CYT	C	No	0	1	0	1	0	1
119367024	SAB2109c	UPI0000048028	COG0199	30S ribosomal protein S14 type Z	S. aureus RF122	rpsN	CYT	C	No	0	0	0	1	0	1
123740967	SAB1540c	UPI000012D34E	COG0290	Translation initiation factor IF-3	S. aureus RF122	infC	CYT	C	No	0	1	0	1	0	0
14247491	SAV1719	UPI0000135022	COG0522	30S ribosomal protein S4	S. aureus Mu50	rpsD	CYT	C	No	0	1	0	1	0	0
21204292	MW1124	UPI0000054AE5	COG0335	50S ribosomal protein L19	S. aureus MW20	rplS	CYT	C	No	0	1	0	1	0	0
119365987	SAB1516c	UPI00000D766C	COG0261	50S ribosomal protein L21	S. aureus RF122	rplU	CYT	U	No	0	1	0	1	0	0
14247998	SAV2225	UPI0000048025	COG0100	30S ribosomal protein S11	S. aureus Mu50	rpsK	CYT	C	No	0	1	0	1	0	0
118597469	SAB1135	UPI000004801C	COG0184	30S ribosomal protein S15	S. aureus RF122	rpsO	CYT	C	No	1	1	0	1	0	0
118573651	SAB2107c	UPI0000054C2C	COG0097	50S ribosomal protein L6	S. aureus RF122	rplF	CYT	C	No	0	1	0	1	0	0
118597325	SAB2123c	UPI0000048024	COG0051	30S ribosomal protein S10	S. aureus RF122	rpsJ	CYT	C	No	0	1	0	1	0	1
109893156	SAB1538c	UPI0000054C25	COG0292	50S ribosomal protein L20	S. aureus RF122	rplT	CYT	C	No	1	1	0	1	0	0
109893730	SAB2110c	UPI00000D76D0	COG0094	50S ribosomal protein L5	S. aureus RF122	rplE	CYT	C	No	1	0	0	1	0	0
118597396	SAB0496	UPI0000048026	COG0048	30S ribosomal protein S12	S. aureus RF122	rpsL	CYT	C	No	0	1	0	1	0	0
123741000	SAB0318	UPI000004802A	COG0238	30S ribosomal protein S18	S. aureus RF122	rpsR	CYT	C	No	1	1	0	1	0	0
123549318	SAB2091c	UPI0000054B7F	COG0102	50S ribosomal protein L13	S. aureus RF122	rplM	CYT	U	No	1	0	0	1	0	0
14246314	SAV0546	UPI000004801A	COG0049	30S ribosomal protein S7	S. aureus Mu50	rpsG	CYT	C	No	1	1	0	0	0	0
109893641	SAB2121c	UPI00000545D9	COG0088	50S ribosomal protein L4	S. aureus RF122	rplD	CYT	U	No	0	1	0	1	0	0

109893241	SAB2117c	UPI0000054ADA	COG0091	50S ribosomal protein L22	S. aureus RF122	rplV	CYT	C	No	0	0	0	1	0	0
14248009	SAV2236	UPI000004801B	COG0096	30S ribosomal protein S8	S. aureus Mu50	rpsH	CYT	C	No	1	0	0	0	0	0
14247672	SAV1900	UPI000012B12C	COG0154	Glutamyl-tRNAGln amidotransferase subunit A	S. aureus Mu50	gatA	CYT	C	No	0	1	0	1	0	0
115502711	SAB2103c	UPI0000054C04	COG0200	50S ribosomal protein L15	S. aureus RF122	rplO	CYT	U	No	0	0	0	1	0	0
123768505	SAB1437	UPI00005FE53A	COG0423	Glycyl-tRNA synthetase	S. aureus RF122	glyS	CYT	C	No	0	0	0	1	0	0
123549320	SAB2114c	UPI0000133E90	COG0255	50S ribosomal protein L29	S. aureus RF122	rpmC	CYT	C	No	0	0	0	1	0	0
Energy production and conversion																
298694330	SAOV_1039	UPI00003B159A	COG0508	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	S. aureus ED133	NA	CYT	C	No	1	1	1	1	1	1
14246866	SAV1096	UPI0000129471	COG1249	Dihydrolipoamide dehydrogenase component of pyruvate dehydrogenase E3	S. aureus Mu50	pdhD	CYT	C	No	1	1	1	1	1	1
14246864	SAV1094	UPI0000054B50	COG0022	Pyruvate dehydrogenase E1 component beta subunit	S. aureus Mu50	phdB	CYT	C	No	1	1	1	1	1	1
14246863	SAV1093	UPI000000978E	COG1071	Pyruvate dehydrogenase E1 component alpha subunit	S. aureus Mu50	pdhA	CYT	C	No	1	1	1	1	1	1
122063487	SAB1162	UPI0000EADFB8	COG0578	Aerobic glycerol-3-phosphate dehydrogenase	S. aureus RF122	glpD	CYT	C	No	1	1	1	1	0	1
14246831	SAV1061	UPI00000D7857	COG1622	Quinol oxidase polypeptide II QoxA	S. aureus Mu50	qoxA	SpII	CM	No	1	1	1	1	1	1
118573756	SAB1987c	UPI00000545EA	COG0055	ATP synthase F1 subcomplex beta subunit	S. aureus RF122	atpD	CYT	CM	No	1	1	1	1	1	1
123548233	SAB1991c	UPI00000D78AB	COG0711	ATP synthase subunit b	S. aureus RF122	atpF	CYT	CM	No	1	1	1	1	1	1
14246710	SAV0941	UPI0000054716	COG1252	Putative NADH dehydrogenase	S. aureus Mu50	NA	CYT	CM	No	1	1	0	1	0	0
124007215	SAB1989c	UPI00005FE6D2	COG0056	ATP synthase subunit alpha	S. aureus RF122	atpA	CYT	C	No	1	1	1	1	1	1
123548234	SAB1988c	UPI00005FE6D1	COG0224	ATP synthase gamma chain	S. aureus RF122	atpG	CYT	U	No	1	1	1	1	1	1
122064915	SAB0164	UPI00000D789B	COG1882	Formate acetyltransferase	S. aureus RF122	pflB	CYT	C	No	0	0	1	1	1	0
123727470	SAB0926c	UPI0000054B74	COG0843	Probable quinol oxidase subunit 1	S. aureus RF122	qoxB	TMH	CM	No	0	1	0	0	1	0
116256295	SAB0180	UPI00005FE1CB	COG0039	L-lactate dehydrogenase	S. aureus RF122	ldh1	SpI	C	No	1	0	0	1	0	0
119367791	SAB1986c	UPI00000545E9	COG0355	ATP synthase epsilon chain	S. aureus RF122	atpC	CYT	C	No	0	0	1	1	1	0
14245915	SAV0148	UPI00000D7748	COG1454	Alcohol-acetaldehyde dehydrogenase	S. aureus Mu50	adhE	CYT	C	No	0	0	0	1	1	0
14246918	SAV1148	UPI00000543F4	COG1053	Succinate dehydrogenase flavoprotein subunit	S. aureus Mu50	sdhA	SpI	C	No	0	0	0	0	1	0
123547902	SAB1570c	UPI000012529B	COG0282	Acetate kinase	S. aureus RF122	ackA	CYT	C	No	0	0	0	1	0	0
323440700	SAO11_0552	UPI0001FAD539	COG0479	Succinate dehydrogenase iron-sulfur subunit	S. aureus O11	sdhB	CYT	C	No	0	0	0	0	1	0
14246356	SAV0588	UPI000005480C	COG0280	Phosphotransacetylase	S. aureus Mu50	pta	CYT	C	No	1	0	0	0	0	0

Cell wall/membrane/envelope biogenesis

14247221	SAV1450	UPI00000CAACF	COG0744	PBP2	S. aureus Mu50	pbp2	CYT	CM	No	1	1	1	1	1	1
14248084	SAV2310	UPI00000D775D	COG1316	Similar to transcription antiterminator LytR /transcriptional attenuator, LytR family	S. aureus Mu50	NA	SpI	CM	No	1	1	1	1	1	1
14246704	SAV0935	UPI00000D4741	COG3966	Poly (glycerophosphate chain) D-alanine transfer protein	S. aureus Mu50	dltD	SpI	C	No	1	1	1	1	1	1
14246826	SAV1056	UPI00000D7738	COG1316	Conserved hypothetical protein/transcriptional attenuator, LytR family	S. aureus Mu50	NA	SpI	CM	Yes	1	1	1	1	1	1
14247927	SAV2154	UPI0000165A31	COG0449	Glutamine--fructose-6-phosphate transaminase	S. aureus Mu50	glmS	CYT	C	No	1	0	0	0	0	0
14248476	SAV2701	UPI0000054977	COG1136	Similar to vraD protein	S. aureus Mu50	vraD	CYT	CM	No	1	0	0	0	0	0
14248220	SAV2446	UPI00000CA966	COG1732	Glycine betaine/carnitine/choline ABC transporter	S. aureus Mu50	opuCC	SpII	CM	Yes	0	1	1	1	1	1
14248302	SAV2528	UPI00000CAB21	COG3764	Sortase A	S. aureus Mu50	srtA	SpI	CM	No	1	1	1	1	1	1
14248072	SAV2299	UPI000005225C	COG3942	Secretory antigen precursor SsaA homolog	S. aureus Mu50	ssaA	SpI	Extra	Yes	1	1	1	1	0	1
123547678	SAB0714	UPI00005FE346	COG0682	Prolipoprotein diacylglyceryl transferase	S. aureus RF122	lgt	TMH	CM	No	1	0	0	0	0	0
14247133	SAV1362	UPI00000CAEDB	COG1316	Peptide methionine sulfoxide reductase regulator/transcriptional attenuator, LytR family	S. aureus Mu50	msrR	CYT	CM	No	1	1	0	1	0	0
123548228	SAB1974c	UPI00000522A6	COG0706	Membrane-embedded lipoprotein precursor	S. aureus RF122	yidC	SpII	CM	No	0	0	0	1	1	0
323439395	SAO11_1696	UPI0001FADC52	COG1686	Penicillin binding protein 4	S. aureus O11	Pbp4	SpI	CM	Yes	0	0	1	1	0	1
123547713	SAB0668	UPI00005FE32B	COG1368	Lipoteichoic acid synthase	S. aureus RF122	ltaS	TMH	CM	No	0	0	0	1	1	0
14248318	SAV2544	UPI00000548B8	COG3942	Similar to secretory antigen precursor SsaA	S. aureus Mu50		SpI	Extra	Yes	1	0	0	0	0	0
14248341	SAV2567	UPI00000548D5	COG1835	peptidoglycan-N-acetylmuramate O-acetyltransferase	S. aureus Mu50	oatA	TMH	CM	No	1	0	0	0	0	0
14246430	SAV0661	UPI00000D77ED	COG1136	ABC transporter ATP-binding protein	S. aureus Mu50	vraF	CYT	CM	No	1	0	0	0	0	0
21205393	ssaA	UPI000005434E	COG3942	Hypothetical protein, similar to secretory antigen precursor SsaA	S. aureus MW20	NA	SpI	Extra	Yes	1	0	0	0	0	0

Inorganic ion transport and metabolism

726968822	SAV0631	UPI000005228B	COG0803	Lipoprotein/Manganese Abc Transporter Substrate-binding Protein Mntc	S. aureus Mu50	NA	CYT	CM	No	1	1	1	1	1	1
14248057	SAV2284	UPI00000547D7	COG0614	Similar to ferric hydroxamate receptor 1	S. aureus Mu50	NA	SpII	CM	Yes	1	1	1	1	1	1
14246505	SAV0736	UPI0000054792	COG4607	Lipoprotein	S. aureus Mu50	NA	SpII	CM	Yes	1	1	1	1	0	1
298695443	SAOV_2224c	UPI00005FE701	COG4594	Ferrichrome ABC transporter lipoprotein	S. aureus ED133	NA	SpII	CM	No	1	1	1	1	1	1

21205368	MW2197	UPI00000D7772	COG0725	Probable molybdate-binding protein	S. aureus MW20	modA	SpII	U	Yes	1	1	1	1	1	1
21205500	MW2328	UPI00000D9FAC	COG3443	Ribulose-phosphate 3-epimerase/zinc transport system substrate-binding protein/GO:metal ion transporte	S. aureus MW20		SpII	CM	Yes	1	1	1	1	1	1
14245882	SAV0115	UPI00000522E5	COG0614	Lipoprotein	S. aureus Mu50	sirA	SpII	CM	Yes	1	0	1	1	1	1
123768519	SAB1825	UPI0000054490	COG1528	Bacterial non-heme ferritin/ferritin	S. aureus RF122	ftn	CYT	C	No	1	1	1	1	1	1
21203739	MW0573	UPI00098EE875	COG0614	MW0573/ABC transporter susbtrate-binding protein	S. aureus MW20	NA	CYT	C	Yes	1	1	1	1	1	1
323438485	SAO11_2671	UPI0001FAD723	COG1464	substrate-binding ABC transporter protein	S. aureus O11	NA	SpII	CM	Yes	1	1	1	1	0	1
14246232	SAV0464	UPI00000CAAB8	COG1464	Lactococcal lipoprotein	S. aureus Mu50	NA	SpII	CM	Yes	1	1	1	1	1	0
14247305	SAV1534	UPI00000542DF	COG0607	Similar to rhodanese family protein	S. aureus Mu50	NA	CYT	U	No	0	1	1	1	1	1
1151012790	SAB0044c	UPI00098F9278	COG1283	Na/Pi cotransporter	S. aureus RF122	NA	TMH	CM	No	1	0	0	0	0	0
123549144	SAB1245c	UPI00005FE4B2	COG0226	Phosphate ABC transporter substrate-binding protein	S. aureus RF122	pstS	SpII	CM	No	1	0	1	0	0	0
14245910	SAV0143	UPI00000CAA28	COG3221	Alkylphosphonate ABC transporter	S. aureus Mu50	NA	SpII	U	Yes	0	0	1	1	0	0
14246572	SAV0803	UPI00000CAE7B	COG0614	Ferric hydroxamate receptor 1	S. aureus Mu50	NA	SpII	CM	Yes	0	0	0	1	0	0
14248114	SAV2340	UPI00000CAB9E	COG1668	Conserved hypothetical protein	S. aureus Mu50	NA	SpI	CM	No	0	0	0	0	1	0
14246402	SAV0633	UPI00000DCA56	COG1121	Similar to ABC transporter ATP-binding protein	S. aureus Mu50	NA	CYT	CM	No	0	0	0	1	0	0
Carbohydrate transport and metabolism																
323438876	SAO11_2281	UPI0001FAD546	COG4193	Autolysin/bifunctional autolysin	S. aureus O11	atl	SpI	Extra	Yes	1	1	1	1	1	1
14246541	SAV0772	UPI000005229B	COG0057	Glyceraldehyde-3-phosphate dehydrogenase (NAD+)	S. aureus Mu50	gap	CYT	C	No	1	1	1	1	1	1
119369402	SAB0732	UPI0000054764	COG0148	Enolase	S. aureus RF122	eno	CYT	C	No	1	1	1	1	1	0
14248381	SAV2607	UPI000012F4C1	COG0579	Malate:quinone oxidoreductase	S. aureus Mu50	mqo2	CYT	CW	No	1	1	1	1	1	1
14245956	SAV0189	UPI00000CA960	COG1264	PTS enzyme II	S. aureus Mu50	glcA	TMH	CM	No	1	1	0	1	1	1
269940277	SATW20_07750	UPI0000696BD9	COG1299	PTS transport system, fructose-specific IIABCcomponent	S. aureus TW20	fruA	CYT	CM	No	1	1	1	1	1	1
14246373	SAV0605	UPI00000CAD6C	COG1064	Alcohol dehydrogenase	S. aureus Mu50	adh1	CYT	C	No	1	0	1	1	1	0
394330464	Newbould305_1308	UPI00024E3FAB	COG0126	phosphoglycerate kinase	S. aureus N305	NA	CYT	C	No	1	1	1	1	0	0
123547911	SAB1556c	UPI00005FE587	COG0469	Pyruvate kinase	S. aureus RF122	pyk	CYT	C	No	1	1	0	1	0	0
21204885	MW1715	UPI00000BF2A9	COG4193	Conserved hypothetical protein	S. aureus MW20	NA	SpI	Extra	No	0	1	1	1	1	1
14247113	SAV1342	UPI00000543E4	COG0021	Transketolase	S. aureus Mu50	tkt	CYT	U	No	1	0	1	0	0	0
123547910	SAB1557c	UPI0000054C39	COG0205	6-phosphofructokinase (EC 2.7.1.11)	S. aureus RF122	pfkA	CYT	C	No	1	0	0	1	0	0

14247193	SAV1422	UPI00000CAC76	COG2190	Glucose-specific enzyme II, PTS system A component	S. aureus Mu50	NA	CYT	C	No	1	0	0	0	0	0
14246242	SAV0474	UPI00000CAB77	COG1263	Phosphoenolpyruvate-dependent and trehalose-specific PTS enzyme II	S. aureus Mu50	treP	CYT	CM	No	0	0	0	1	0	0
323438631	SAO11_2522	UPI0001FAD4E2	COG0149	Triosephosphate isomerase	S. aureus O11	tpiA	CYT	C	No	1	0	0	0	0	0
14247898	SAV2125	UPI00000D7773	COG0191	Fructose-bisphosphate aldolase	S. aureus Mu50	fbaA	CYT	C	No	1	0	0	0	0	0
14245981	SAV0214	UPI00000522B0	COG2182	Similar to maltose/maltodextrin transport system	S. aureus Mu50	NA	SpII	U	Yes	1	0	0	1	0	0
Function unknown															
14248232	SAV2458	UPI00000CAB9C	COG4808	Hypothetical protein	S. aureus Mu50	NA	SpII	U	Yes	1	1	1	1	1	1
14246140	SAV0372	UPI00000D76CF	COG3212	Conserved hypothetical protein	S. aureus Mu50	NA	SpII	U	Yes	0	0	1	1	1	1
14247344	SAV1573	UPI00000CAC7C	COG4864	Conserved hypothetical protein	S. aureus Mu50	NA	TMH	C	No	1	1	1	1	1	1
123547950	SAB1599c	UPI0000054388	COG4768	Probable general stress response protein	S. aureus RF122	NA	CYT	C	No	0	1	1	1	1	1
14247655	SAV1883	UPI00000CAEFB	COG1295	Similar to transporter	S. aureus Mu50	NA	CYT	CM	No	1	0	0	1	1	0
14248130	SAV2356	UPI00000CA97D	COG4640	TcaA protein	S. aureus Mu50	tcaA	CYT	U	No	1	0	0	0	0	0
1151012754	SaO11_01228	UPI00086EB7BA	COG3763	Hypothetical protein	S. aureus O11	NA	THM	CM	No	0	0	0	1	0	0
115311988	SAB1848c	UPI00005FE667	COG4843	Probable membrane protein	S. aureus RF122	NA	TMH	CM	No	1	0	0	1	1	0
123549281	SAB2063c	UPI0000054BEA	COG1302	Alkaline shock protein 23	S. aureus RF122	asp23	CYT	U	No	0	0	0	1	0	0
446749011		UPI00005FE62D	COG3763	Hypothetical protein	S. aureus	NA	SpII	U	Yes	0	0	0	0	1	0
14247961	SAV2188	UPI00000D7704	COG4814	Conserved hypothetical protein	S. aureus Mu50	NA	SpI	U	Yes	1	0	0	1	0	0
14247935	SAV2162	UPI0000054628	COG4856	Conserved hypothetical protein	S. aureus Mu50	NA	SpI	U	No	1	0	0	0	0	0
14248418	SAV2643	UPI00000CAB7A	COG1511	Similar to phage infection protein	S. aureus Mu50	NA	SpI	CM	No	1	0	0	0	0	0
298695602	SAOV_2384c	UPI0001DA2285	COG3152	Probable membrane protein	S. aureus ED133	NA	TMH	CM	No	0	0	0	1	0	0
14246415	SAV0646	UPI00000CACB3	COG1284	Conserved hypothetical protein	S. aureus Mu50	NA	TMH	CM	No	0	0	0	0	1	0
Posttranslational modification, protein turnover, chaperones															
14247613	SAV1841	UPI000005229F	COG0760	Peptidyl-prolyl cis/trans isomerase	S. aureus Mu50	prsA	SpII	CM	Yes	1	1	1	1	1	1
14247500	SAV1728	UPI000005224B	COG0265	Similar to serine proteinase Do	S. aureus Mu50	NA	TMH	CM	No	1	1	1	1	1	1
21204313	MW1145	UPI00000D9DAF	COG0750	Conserved hypotehtical protein	S. aureus MW20	NA	TMH	CM	No	1	0	1	1	1	0
14248183	SAV2409	UPI00000D7774	COG1651	Putative protein-disulfide isomerase	S. aureus Mu50	NA	SpII	CW	Yes	0	1	1	1	1	0
446264281	SAB1275c	UPI00005FE4C7	COG0793	Serine protease	S. aureus RF122	NA	CYT	CM	No	1	0	0	1	1	0

14247231	SAV1460	UPI00000CAC92	COG2738	Conserved hypothetical protein	S. aureus Mu50	NA	SpI	CM	No	0	0	0	1	1	1
123547849	SAB1452c	UPI00005FE543	COG0443	Chaperone protein	S. aureus RF122	dnaK	CYT	C	No	0	0	0	1	0	0
119366269	SAB1913c	UPI00005FE69B	COG0459	60 kDa chaperonin protein	S. aureus RF122	groEL	CYT	C	No	0	0	1	0	0	0
123549386	SAB2212	UPI00005FE767	COG1266	Probable membrane protein	S. aureus RF122	lyrA	TMH	CM	No	0	0	0	0	1	0
122063322	SAB0475	UPI00005FE2A5	COG0542	ATP-dependent Clp protease ATP-binding subunit ClpC	S. aureus RF122	clpC	CYT	C	No	0	1	0	0	0	0
Amino acid transport and metabolism															
21204360	MW1192	UPI000012B701	COG0174	Glutamine-ammonia ligase	S. aureus MW20	glnA	CYT	C	No	1	1	1	1	1	1
14248186	SAV2412	UPI0000054B9D	COG1126	ABC transporter (ATP binding subunit)	S. aureus Mu50	NA	CYT	CM	No	1	1	1	1	1	1
394329129	Newbould305_2537	UPI00026C222B	COG0747	Oligopeptide transporter substrate binding protein	S. aureus N305	opp1A	SpII	CW	No	0	0	1	1	0	1
14247886	SAV2113	UPI00000545F5	COG0112	Serine hydroxymethyltransferase	S. aureus Mu50	glyA	CYT	C	No	0	1	0	1	0	1
14246939	SAV1169	UPI00000CA9E3	COG0078	Ornithine carbamoyltransferase	S. aureus Mu50	argF	CYT	C	No	0	0	0	1	1	0
123549453	SAB2510c	UPI000005492C	COG2235	Arginine deiminase	S. aureus RF122	arcA	CYT	C	No	0	0	0	1	1	0
14246759	SAV0990	UPI00000CAA43	COG4166	Similar to peptide binding protein OppA	S. aureus Mu50	NA	SpII	CW	No	0	0	0	1	0	0
14246727	SAV0958	UPI00000D78A5	COG0334	NAD-specific glutamate dehydrogenase	S. aureus Mu50	gudB	CYT	C	No	0	0	0	1	0	0
Lipid transport and metabolism															
14246088	SAV0320	UPI00000D2F88	COG1075	Glycerol ester hydrolase	S. aureus Mu50	geh	SpI	Extra	Yes	1	0	0	0	0	0
269939772	SATW20_02560	UPI00003B137F	COG1887	Putative teichoic acid biosynthesis protein	S. aureus TW20	tarF	CYT	CM	No	0	0	0	1	0	0
14246753	SAV0984	UPI00000CAAB7	COG0304	3-oxoacyl synthase	S. aureus Mu50	NA	CYT	CM	No	1	0	0	0	0	0
298694619	SAOV_1331	UPI0001DA24FF	COG1502	Cardiolipin synthase	S. aureus ED133	NA	TMH	CM	No	0	0	0	1	0	0
14246781	SAV1011	UPI00000D774F	COG0623	Trans-2-enoyl-ACP reductase	S. aureus Mu50	fabI	CYT	CM	No	1	0	0	0	0	0
14247499	SAV1727	UPI0000054395	COG0204	Acylglycerol-3-phosphate O-acyltransferase-like	S. aureus Mu50	NA	CYT	C	No	0	0	0	1	0	0
14246999	SAV1229	UPI0000131C70	COG0416	Fatty acid/phospholipid synthesis protein	S. aureus Mu50	plsX	CYT	C	No	1	0	0	0	0	0
14247076	SAV1306	UPI0000054618	COG0386	Glutathione peroxidase	S. aureus Mu50	bsaA	CYT	U	No	1	0	0	0	0	0
Coenzyme transport and metabolism															
14247242	SAV1471	UPI0000054C6C	COG2226	Menaquinone biosynthesis methyltransferase	S. aureus Mu50	gerCB	CYT	U	No	1	1	0	1	0	0
14248080	SAV2306	UPI00000CAD12	COG0654	Similar to monooxygenase	S. aureus Mu50	NA	CYT	C	No	0	0	0	1	0	0

123768514	SAB1625c	UPI0000054369	COG0054	6,7-dimethyl-8-ribityllumazine synthase (EC 2.5.1.78)	S. aureus RF122	ribH	CYT	C	No	1	0	0	0	0	0
14246287	SAV0519	UPI0000054856	COG0214	Putative pyridoxine biosynthesis protein	S. aureus Mu50	pdxS	CYT	C	No	1	0	0	1	0	0
323441712	SAO46_2330	UPI0001FAD53F	COG0447	Naphthoate synthase	S. aureus O46	menB	CYT	C	No	0	0	0	1	0	0
394329991	Newbould305_1576	UPI00024E4C6C	COG0028	Acetolactate synthase	S. aureus N305	NA	CYT	CM	No	0	0	0	1	0	0
14248313	SAV2539	UPI00000CAB2E	COG0028	Pyruvate oxidase	S. aureus Mu50	NA	CYT	CM	No	0	0	0	1	0	0
Signal transduction mechanisms															
14248188	SAV2414	UPI00000CAB6A	COG0834	ABC transporter/amino acid ABC transporter substrate-binding protein	S. aureus Mu50	NA	SpII	U	Yes	1	1	1	1	1	1
123768509	SAB1569	UPI0000054C46	COG0589	Putative universal stress protein	S. aureus RF122	NA	CYT	C	No	1	1	0	1	1	1
14247657	SAV1885	UPI00000CACC0	COG4585	Two-component sensor histidine kinase	S. aureus Mu50	vraS	TMH	CM	No	1	0	0	1	0	1
21204271	MW1103	UPI00000543A2	COG0515	Serine/threonine protein kinase	S. aureus MW20	NA	CYT	CM	No	0	0	0	1	0	0
123754573	SAB0654c	UPI00000D7882	COG0642	Histidine protein kinase	S. aureus RF122	saeS	TMH	CM	No	1	0	0	0	0	0
14247478	SAV1706	UPI00000D7756	COG0589	Conserved hypothetical protein	S. aureus Mu50	NA	CYT	U	No	1	0	0	0	0	0
14245787	SAV0020	UPI00000CAA8F	COG4863	Conserved hypothetical protein	S. aureus Mu50	NA	SpI	U	No	1	0	0	0	0	0
Cell cycle control, cell division, chromosome partitioning															
14245808	SAV0041	UPI00000DA0B7	COG0768	Penicillin binding protein 2 prime	S. aureus Mu50	mecA	CYT	CM	No	1	0	0	0	0	0
123740970	SAB1576c	UPI0000054C4F	COG4477	Conserved hypothetical protein/Septation ring formation regulator EzrA	S. aureus RF122	ezrA	CYT	CM	No	0	1	0	1	1	0
14247323	SAV1552	UPI00000CAAD1	COG0768	Penicillin-binding protein 3	S. aureus Mu50	pbp3	CYT	CM	No	1	0	0	1	0	0
14246956	SAV1186	UPI00000543C7	COG0206	Cell division protein FtsZ	S. aureus Mu50	ftsZ	CYT	C	No	1	0	0	1	0	0
269940929	SATW20_14350	UPI000197AC14	COG1196	Very large surface anchored protein	S. aureus TW20	ebh	SpI	CW	Yes	0	0	1	0	0	0
14247421	SAV1649	UPI00000D78B1	COG1792	Rod shape-determining protein MreC	S. aureus Mu50	NA	SpI	U	No	1	0	0	0	0	0
Transcription															
14246311	SAV0543	UPI00000D77B3	COG0086	RNA polymerase beta-prime chain	S. aureus Mu50	rpoC	CYT	C	No	1	1	1	1	0	1
109914399	SAB0493	UPI00000D771C	COG0085	DNA-directed RNA polymerase subunit beta	S. aureus RF122	rpoB	CYT	C	No	1	1	1	1	0	0
14246455	SAV0686	UPI00000548AE	COG1846	Transcriptional regulator	S. aureus Mu50	mgrA	CYT	C	No	1	0	0	0	0	0
119366734	SAB2097c	UPI00005FE721	COG0202	DNA-directed RNA polymerase subunit alpha	S. aureus RF122	rpoA	CYT	C	No	0	0	0	1	0	0
123740964	SAB1481c	UPI000005428C	COG0782	Transcription elongation factor GreA	S. aureus RF122	greA	CYT	C	No	1	0	0	0	0	0

Defense mechanisms															
14246827	SAV1057	UPI00000D76EA	COG1680	Autolysis and methicillin resistant-related protein	S. aureus Mu50	fmtA	CYT	CM	No	1	0	0	1	1	0
14247703	SAV1931	UPI00000CA978	COG1131	Similar to ABC transporter (ATP-binding protein)	S. aureus Mu50	NA	CYT	CM	No	1	1	1	1	1	1
323440604	SAO11_0692	UPI0001FAD62A	COG1131	ABC transporter	S. aureus O11	NA	CYT	CM	No	1	0	1	0	0	0
269942161	SATW20_27120	UPI0001BE5B83	COG2367	Beta-lactamase precursor	S. aureus TW20	blaZ	SpII	CW	Yes	0	0	0	0	0	1
14246412	SAV0643	UPI00000547C5	COG1132	ATP-binding cassette transporter A	S. aureus Mu50	NA	TMH	CM	No	0	0	0	0	1	0
Intracellular trafficking, secretion, and vesicular transport															
14246734	SAV0965	UPI000012E4AA	COG0681	Type-1 signal peptidase 1B	S. aureus Mu50	spsB	TMH	CW	No	1	1	1	1	1	1
14247409	SAV1637	UPI00000D7784	COG0341	Protein-export membrane protein SecDF	S. aureus Mu50	secF	SpI	CM	Yes	1	1	1	1	1	1
14247410	SAV1638	UPI000005426C	COG1862	Conserved hypothetical protein	S. aureus Mu50	NA	CYT	CM	No	1	1	1	1	1	1
123727402	SAB0705	UPI000005226D	COG0653	Protein translocase subunit secA	S. aureus RF122	secA	CYT	C	No	1	0	0	1	1	0
14246733	SAV0964	UPI000012E499	COG0681	Type-I signal peptidase	S. aureus Mu50	spsA	TMH	CW	No	1	0	0	0	0	0
General function prediction only															
323439847	SAO11_1262	UPI0001B70093	COG4851	Lipoprotein	S. aureus O11	NA	SpII	U	Yes	1	1	1	1	1	1
298695601	SAOV_2383c	UPI000175C51C	COG4152	Probable sodium ABC transporter ATP-binding protein	S. aureus ED133	NA	CYT	CM	No	0	0	0	1	1	0
14247913	SAV2140	UPI0000054610	COG3011	Conserved hypothetical protein	S. aureus Mu50	NA	CYT	CM	No	0	0	0	1	0	0
1119377513	SaO11_01052	UPI00093184CC	COG1288	Uncharacterized membrane protein YfcC, YfcC family protein	S. aureus O11	NA	SpI	CM	Yes	0	0	0	1	0	0
Replication, recombination and repair															
14247095	SAV1324	UPI00000D77A5	COG1525	Thermonuclease	S. aureus Mu50	nuc	SpI	Extra	No	1	0	0	1	0	0
14245772	SAV0005	UPI00000CAD47	COG0187	DNA gyrase subunit B	S. aureus Mu50	gyrB	CYT	C	No	0	0	0	0	0	1
14245769	SAV0002	UPI0000050CC9	COG0592	DNA polymerase III beta chain	S. aureus Mu50	dnaN	CYT	C	No	1	0	0	0	0	0
Mobilome: prophages, transposons															
323439071	SAO11_2070	UPI0001FAD7B0	COG4653	Capsid protein	S. aureus O11	NA	CYT	C	No	0	1	1	0	0	1
21204569	MW1400	UPI00000D9E15	COG4695	Phage major capsid protein portal protein	S. aureus MW20	NA	CYT	U	No	0	0	1	0	0	1

323439069	SAO11_2068	UPI0001FAD7AE	COG4695	Portal protein	S. aureus O11	NA	CYT	C	No	0	1	1	0	0	0
Nucleotide transport and metabolism															
14246500	SAV0731	UPI00000CAB50	COG0209	Ribonuceloside diphosphate reductase major subunit	S. aureus Mu50	nrdE	CYT	C	No	1	0	0	0	0	0
Secondary metabolites biosynthesis, transport and catabolism															
14247176	SAV1405	UPI00000D787B	COG4915	Similar to 5-bromo-4-chloroindolyl phosphate hydrolysis	S. aureus Mu50	NA	TMH	C	No	1	0	0	1	0	0
Cell motility															
14248419	SAV2644	UPI00000CACD9	COG1705	N-acetylmuramoyl-L-alanine amidase similar to autolysin precursor	S. aureus Mu50	NA	SpI	Extra	Yes	1	0	1	0	0	0
Unclassified function															
172046784	SAV2035	UPI00001110E1		Delta-hemolysin	S. aureus Mu50	hld	CYT	Extra	No	0	1	1	1	1	1
446715237	SAV2418	UPI0000358A34		Immunoglobulin-binding protein	S. aureus Mu50	sbi	SpI	U	Yes	1	1	1	1	0	0
394329555	Newbould305_1925	UPI0000505A18		Phage major capsid protein	S. aureus N305	NA	CYT	U	No	0	0	0	1	0	0
14246862	SAV1092	UPI00000CAA03		Conserved hypothetical protein	S. aureus Mu50	NA	SpII	U	Yes	1	1	1	1	1	1
21204223	MW1056	UPI00000D9D8F		Hypothetical protein	S. aureus MW20	NA	CYT	U	No	0	1	1	1	1	1
21204567	MW1398	UPI000009B7B2		Hypothetical protein	S. aureus MW20	NA	CYT	C	No	0	0	1	0	0	1
323438943	SAO11_2265	UPI00005FE322		Hypothetical protein SAO11_2265	S. aureus O11	NA	SpII	U	Yes	1	1	1	1	1	1
14247561	SAV1789	UPI00000CAABD		Hypothetical protein	S. aureus Mu50	NA	CYT	U	No	1	1	1	1	1	1
14246577	SAV0808	UPI00000CAE0D		Hypothetical protein	S. aureus Mu50	NA	SpII	U	Yes	1	1	1	0	0	1
323439299	SAO11_1892	UPI0001FAD4D7		Hypothetical protein	S. aureus O11	NA	SpII	U	Yes	1	1	1	1	1	1
123548096	SAB1806c	UPI00000D7827		Monofunctional glycosyltransferase	S. aureus RF122	mgt	CYT	CM	No	1	1	1	1	1	1
14247777	SAV2004	UPI00000CACAB		Hypothetical protein/leukocidin/hemolysin toxin family protein	S. aureus Mu50	NA	SpI	Extra	Yes	1	0	0	0	0	0
206557787	MW0406.1 PSMA4_STAAW	UPI000161A44A		Phenol-soluble modulin alpha 4 peptide	S. aureus	psmα4	CYT	U	No	0	1	1	1	1	1
446360646	SAV0892	UPI00005FE60A		Phage major capsid protein	S. aureus Mu50		CYT	C	No	0	0	0	0	1	0
206557785	MW0406.3 PSMA2_STAAW	UPI00015FD703		Phenol-soluble modulin alpha 2 peptide	S. aureus	psmα2	CYT	U	No	0	1	1	1	1	1
323440263	SAO11_1000	UPI0001FADB3A		Hypothetical protein	S. aureus O11	NA	SpI	U	No	0	0	1	1	0	1

123549205	SAB1310c	UPI0000054A3F	Conserved hypothetical protein	S. aureus RF122	gpsB	CYT	C	No	1	1	1	1	0	1
298694442	SAOV_1151	UPI0001DA236F	probable exported protein	S. aureus ED133		SpI	Extra	Yes	1	1	1	1	0	1
206557786	MW0406.2 PSMA3_STAAW	UPI000161A449	Phenol-soluble modulin alpha 3		psmα3	CYT	U	No	0	0	1	0	0	0
14246381	SAV0613	UPI00000D76AE	Hypothetical protein	S. aureus Mu50	NA	SpI	U	Yes	0	1	1	1	1	1
14248142	SAV2368	UPI00000CAB73	Hypothetical protein	S. aureus Mu50	NA	SpII	CM	Yes	1	1	0	1	1	1
298693772	SAOV_0458	UPI0001DA22B4	Staphylococcal tandem lipoprotein	S. aureus ED133	NA	CYT	U	No	0	1	1	0	0	1
14248207	SAV2433	UPI00000CAD76	Hypothetical protein	S. aureus Mu50	NA	CYT	U	No	1	1	0	0	0	0
21203920	MW0754	UPI00000D9D10	Hypothetical protein	S. aureus MW20	NA	CYT	C	No	0	0	0	0	0	1
14247778	SAV2005	UPI00000CAF1E	Hypothetical protein/leukocidin/hemolysin toxin family protein	S. aureus Mu50	NA	SpI	Extra	Yes	1	0	0	0	0	0
323439373	SAO11_1797	UPI00000CAF2B	Membrane-embedded lytic regulatory protein	S. aureus O11	NA	TMH	CM	No	0	1	1	1	1	1
21204652	MW1483	UPI00000D9E4E	Hypothetical protein	S. aureus MW20	NA	SpII	U	Yes	0	1	1	1	1	1
323439012	SAO11_2179	UPI0001FAD9B8	Hypothetical protein	S. aureus O11	NA	CYT	U	No	0	0	1	0	0	1
323439165	SAO11_2003	UPI0001FAD6FD	Hypothetical protein/Host cell surface-exposed lipoprotein	S. aureus O11	NA	SpI	U	Yes	0	0	1	0	0	0
269940284	SATW20_07820	UPI000018DB08	Putative membrane protein	S. aureus TW20	saeQ	TMH	CM	No	1	0	0	0	0	0
14247252	SAV1481	UPI00000CADE3	Elastin binding protein	S. aureus Mu50	ebpS	CYT	CM	No	0	1	0	1	0	0
14246983	SAV1213	UPI00000543AA	Hypothetical protein	S. aureus Mu50	NA	SpII	CM	Yes	1	0	0	0	0	1
394329515	Newbould305_1885	UPI00026C21D9	Hypothetical protein	S. aureus N305	NA	SpII	U	Yes	0	0	0	1	0	0
394330135	Newbould305_1721	UPI00026C23CA	Hypothetical protein	S. aureus N305	NA	SpI	CM	No	1	0	0	1	0	0
14246873	SAV1103	UPI00000522D5	Conserved hypothetical protein	S. aureus Mu50	NA	TMH	CM	No	1	0	0	1	0	0
21204562	MW1393	UPI00000541F7	Major tail protein	S. aureus MW20	NA	CYT	U	No	0	0	0	0	0	1
14247654	SAV1882	UPI000005449E	Hypothetical protein	S. aureus Mu50	NA	CYT	C	No	1	0	0	1	0	0
323438619	SAO11_2556	UPI0001FAD4FE	Putative lipoprotein	S. aureus O11	NA	SpII	U	Yes	1	0	1	0	0	0
14247957	SAV2184	UPI00000D7671	Hypothetical protein	S. aureus Mu50	NA	TMH	CM	No	0	0	0	0	0	1
298695647	SAOV_2429	UPI0001DA22FA	Conserved hypothetical protein	S. aureus ED133	NA	CYT	U	No	0	0	0	1	1	0
14248159	SAV2385	UPI0000054770	Hypothetical protein	S. aureus Mu50	NA	SpI	U	No	0	0	0	1	1	0
14247696	SAV1924	UPI000005446B	Hypothetical protein	S. aureus Mu50	NA	SpI	CM	No	0	0	1	1	1	0
14248125	SAV2351	UPI00000CAAE2	Hypothetical protein	S. aureus Mu50	NA	CYT	CM	No	1	0	0	0	0	0
323438961	SAO11_2204	UPI0001FADA58	Lipoprotein	S. aureus O11	NA	SpII	U	Yes	0	0	1	0	1	0
447090640	Newbould305_2629	UPI00005FE29C	ATP-dependent metallopeptidase	S. aureus N305	ftsH	TMH	CM	No	0	0	0	1	0	0

14246582	SAV0813	UPI00000CAD7F	Extracellular ECM and plasma binding protein	S. aureus Mu50	ssp	SpI	CW	Yes	1	0	0	0	0	0
109894865	SAB2167c	UPI00000D7855	Staphylococcal accessory regulator protein	S. aureus RF122	sarR	CYT	C	No	1	0	0	0	0	0
14247736	SAV1963	UPI00000CAC20	Hypothetical protein	S. aureus Mu50	NA	CYT	U	No	1	0	0	0	0	0
394331472	Newbould305_0088	UPI00021AE82F	Hypothetical protein	S. aureus N305	NA	SpII	U	No	0	0	0	1	0	0
323439508	SAO11_1643	UPI0001FADB44	Hypothetical protein	S. aureus O11	NA	SpII	U	Yes	0	0	1	0	0	0
446654630	SAV2627.1	UPI0000351A9B	DUF2648 domain-containing protein	S. aureus	NA	CYT	U	No	1	0	0	0	0	0
21204607	MW1438	UPI000009B60A	Hypothetical protein	S. aureus MW20	NA	SpII	Extra	Yes	0	1	0	0	0	1
269942062	SATW20_26090	UPI000069AB67	Putative lipoprotein	S. aureus TW20	NA	CYT	C	No	0	0	1	0	0	1
14248343	SAV2569	UPI00000CAC78	Immunodominant antigen A	S. aureus Mu50	isaA	SpI	Extra	Yes	1	0	1	0	0	0
14248477	SAV2702	UPI00000CAE09	vraE protein	S. aureus Mu50	vraE	TMH	CM	No	1	0	0	0	0	0
14246451	SAV0682	UPI00000548D6	Conserved hypothetical protein	S. aureus Mu50	NA	CYT	U	No	0	0	0	1	0	0
394329551	Newbould305_1921	UPI0000505A13	Phage portal protein	S. aureus N305	NA	CYT	C	No	0	0	0	1	0	0
143682514	MW0073	UPI00003B1A2C	Hypothetical protein	S. aureus MW20	NA	SpII	U	No	0	0	0	0	0	1
446276447		UPI00005FE60B	DUF4355 domain-containing protein	S. aureus	NA	CYT	C	No	0	0	0	0	1	0
21205068	MW1898	UPI0000054446	Hypothetical protein	S. aureus MW20	NA	CYT	U	No	0	1	0	0	0	1
394329561	Newbould305_1931	UPI0000505A1F	Hypothetical protein	S. aureus N305	NA	CYT	C	No	0	0	0	1	0	0
14247594	SAV1822	UPI00000CAC1D	Conserved hypothetical protein	S. aureus Mu50	NA	SpII	U	Yes	0	0	0	1	0	0
123548088	SAB1766	UPI00000B2072	Signal transduction protein	S. aureus RF122	traP	CYT	U	No	0	0	0	0	1	0
14247422	SAV1650	UPI00000D774A	Hypothetical protein	S. aureus Mu50	NA	CYT	CM	No	1	0	0	0	0	0
14247698	SAV1926	UPI0000054469	Hypothetical protein	S. aureus Mu50	NA	SpI	U	No	1	0	0	0	0	0
14246155	SAV0387	UPI00000CAB1F	Hypothetical protein	S. aureus Mu50	NA	CYT	C	No	1	0	0	0	0	0
14248456	SAV2681	UPI00000D7706	Hypothetical protein	S. aureus Mu50	NA	SpII	U	Yes	1	0	0	0	0	0

(1), Proteins are classified in Gene Ontology functional classes.
(2), Gene names are given according to annotation of *S. aureus* Mu50, *S. aureus* RF122, *S. aureus* Newbould 305 (N305), *S. aureus* O11, *S. aureus* O46, *S. aureus* MW2, *S. aureus* TW20 and *S. aureus* ED133. NA: No Available
(3), LipoP prediction: SpII, SPaseII-cleaved proteins; SpI, SPaseI-cleaved proteins; CYT, cytoplasmic; TMH, transmembrane.
(4), PsortB prediction: Extra, extracellular; CM, cytoplasmic membrane; C, cytoplasmic; U, Unknow.
(5), SigIP prediction.
* occurrence of proteins: 0, absent; 1, present.
(6), Proteins identified in *S. aureus* Mu50; (7), Proteins identified in *S. aureus* O46; (8), Proteins identified in *S. aureus* O11; (9), Proteins identified in *S. aureus* N305; (10), Proteins identified in *S. aureus* RF122; (11), Proteins identified in *S. aureus* MW2

Annex 3

Exfoliative toxin E, a new *Staphylococcus aureus* virulence factor with host-specific activity.

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ABSTRACT

Exfoliative toxins (ETs) are secreted virulence factors produced by Staphylococci. These serine proteases specifically cleave desmoglein 1 (Dsg1) in mammals and are key elements in staphylococcal skin infections. We recently identified a new *et* gene in *S. aureus* O46, a strain isolated from ovine mastitis. Its deduced amino acid sequence was similar to known ETD. During the present study, we characterized the new *et* gene at a genetic level and the enzymatic activity of the deduced protein. The *S. aureus* O46 genome was re-assembled, annotated and compared with other publicly available *S. aureus* genomes. This indicated that the new *et* gene shared the same genetic vicinity as other ovine *S. aureus* strains. A purified new *et* gene product caused skin exfoliation *in vivo* in a murine model. The new *et*-gene was thus named *ete,* encoding a new type (type E) of exfoliative toxin. We showed that ETE degraded immunofluorescence of the extracellular segments of Dsg1 in murine, ovine and caprine epidermises, as well as in ovine teat canal epithelia, but not in bovine epidermis. We further showed that it directly solubilized human and porcine Dsg1 as well as murine Dsg1α and Dsg1β, but not canine Dsg1 or murine Dsg1γ. Molecular modeling revealed a correlation between the orientation of ETE docking on its Dsg1 cleavage site and species-specific cleavage activity, suggesting that the docking step preceding cleavage accounts for the ETE species-specificity. This may contribute to the bacterial colonization of mammary duct epithelia in certain ruminants with mastitis.

INTRODUCTION

Staphylococcus aureus is a major Gram-positive pathogen and a serious concern in both human and animal health (1) because it is implicated in a broad range of diseases ranging from superficial skin infections such as staphylococcal scalded skin syndrome (SSSS) to life-threatening endocarditis or sepsis in humans. *S. aureus* produces a wide array of virulence factors, which either alone or in conjunction contribute to the type and severity of staphylococcal infections. Most *S. aureus* virulence genes are borne by mobile genetic elements (MGE) and the type and severity of *S. aureus* infections therefore depend on strain-specific traits as much as on host traits. Although humans are the primary ecological niche and reservoir of *S. aureus*, it is also encountered in a variety of animal hosts. Livestock-associated strains have evolved following human-to-animal host jumps. This adaptive evolution has led to the emergence of endemic and sometimes host-restricted clones, and can be demonstrated at the genotype, genomic and molecular levels (2, 3). In dairy ruminants, *S. aureus* is a major causal agent of mastitis, inflammation of the mammary gland that often results from a bacterial infection. Mastitis causes significant economic losses in the milk production chain. *S. aureus* strains isolated from ruminant hosts exhibit specific traits (4) that might be useful and targeted to develop strategies for the prevention or treatment of mastitis.

Exfoliative toxins belong to a family of serine proteases that display exquisite substrate specificity and recognize and hydrolyze a single peptide bond in the extracellular segment of desmoglein 1 (Dsg1), a desmosomal cadherin-type cell-cell adhesion molecule that causes a dissociation of keratinocytes in human and animal skin. To date, three different ET serotypes (ETA, ETB and ETD) whose deduced amino acid sequences are homologous to trypsin-like serine proteases have been identified in *S. aureus* and associated with SSSS in humans. Exfoliation caused by ETs is described in many phylogenetically distant hosts, although with different degrees of susceptibility, which indicates host specificity (5).

We previously characterized *S. aureus* strains isolated from severe or mild ovine mastitis at the genomic, proteomic and seroproteomic levels (6, 7). These studies led to the identification of staphylococcal secreted proteins which were specifically encountered in strains associated with mild mastitis in ewes (6). One of these proteins was similar in its amino acid primary sequence with the previously described *S. aureus* ETD. Of note was the fact that it harbored the typical catalytic site encountered in the other ET proteins described to date. This protein was tentatively referred to as an ETD-like protein. It was recently heterologously produced and its crystal structure was determined (8).

During the present study, we addressed the issue of the exfoliative activity of the new ET and its host-specificity using *in vitro* and *in vivo* experiments as well as molecular docking.

RESULTS

Similarity and genetic vicinity of the new *S. aureus* O46 *et* gene with other *et* genes. The deduced amino acid sequence of the new *et* gene was compared with those of other characterized ET proteins in order to place this new protein in an ET phylogenetic tree (Figure 1). The amino acid sequence of the new ET clustered with those of *S. aureus* ETB and ETD, *Staphylococcus hyicus* SHETB and *Staphylococcus pseudintermedius* ExpA and ExpB. A genomic analysis of strain O46 revealed eight putative genomic islands (GIs). The new *et* gene, along with 15 other genes, belongs to a 19.4 kb putative GI with a 30.8% GC content (i.e. lower than the average of 32.8% in the whole genome). This GI is not contained in any prophage regions of the chromosome. The features of the new *et*-containing GI are displayed in Figure 2 and Table 1, while those of the genome region corresponding to putative GI, in strain RF122, are shown in Table S1. Multiple copies of Insertion Sequence (IS) families are present in the O46 genome (see Table S2); however, no IS was found in the genetic vicinity of the new *et*-containing GI region (2256079 to 2275534), which suggests that the GI is not part of a mobile genetic element.

Exfoliative activity of the new ET in neonatal mice. To determine whether the new ET affects Dsg1 in the same way as classic ETs, skin sections obtained from neonatal mice injected with the purified new ET protein were subjected to histopathological and immunofluorescence analyses. Histopathological analysis revealed intra-epidermal clefts with acantholysis in the stratum granulosum at the injection site as early as 1 h after injection (Figure 3). Immunofluorescence analysis of the skin at the injection site revealed markedly diminished immunostaining for the extracellular segments of Dsg1 on the plasma membrane of keratinocytes, whereas those for the intracellular domain of Dsg1 and the extracellular segments of Dsc1 or Dsg3 were not affected. These results clearly indicated that the new ET, which had

previously been called ETD-like protein, selectively affects the extracellular segment of Dsg1, so it was renamed to ETE.

ETE degrades Dsg1 in certain ruminants. To determine whether ETE affects Dsg1 in ruminants, it was incubated with cryosections of ovine, caprine and bovine nasal planum and underwent an immunofluorescence study with human pemphigus foliaceus (PF) serum (Figure 4). The immunoreactivities of IgG in human PF serum against the keratinocyte cell surfaces were abolished by the ETE protein in ovine and caprine nasal planum, but not in the bovine nasal planum. ETB abolished these immunoreactivities in all three ruminants tested.

Since *S. aureus* O46 was isolated in milk from a ewe with mastitis, we investigated whether Dsg1, a target molecule for ETE, is expressed in ruminant mammary glands or ducts. We found that the human PF IgG reacted with the plasma membrane of caprine teat canal epithelia, and IgG immunoreactivity was abolished when a caprine teat canal cryosection was pre-incubated with ETE (Figure 5).

ETE solubilizes the extracellular segments of Dsg1 in a species-specific manner. We further investigated whether ETE solubilizes Dsg1 in non-ruminants. As shown in Figure 6, both ETE and ETB directly solubilized the extracellular segments of hDsg1, sDsg1, mDsg1α and mDsg1β into smaller peptides following *in vitro* incubation. The molecular weights of the degraded Dsg1 products produced by ETE were almost identical to those produced by ETB, suggesting that they recognize the same cleavage site. Conversely, neither ETE nor ETB degraded cDsg1 or mDsg1γ.

Prevalence of the *ete* gene in other *S. aureus* genomes. The ETE predicted protein sequence was found in only 33 of the 9759 *S. aureus* predicted proteomes available on the PATRIC

database (Table S3). A predicted ETE sequence was found in five out of nine *S. aureus* strains isolated from ovine samples, including one strain presenting a truncated ETE, and one out of 94 *S. aureus* strains isolated from bovine samples. Moreover, the predicted ETE protein was found in one strain isolated from a food sample, and in two strains from bulk cow's milk. The 24 remaining strains were isolated from humans or unknown hosts. Six of the latter human strains were isolated in patients suffering from Buruli ulcers.

Similarity of Dsg1 sequences and 3D structures in various mammalian species. A comparison of amino acid sequences showed that glutamate residues cleaved by the well-characterized ETA, ETB, and ETD are conserved in ovine, human, bovine and canine Dsg1, whose amino acid sequences are available in the Uniprot database (N.B. the caprine Dsg1 sequence is not yet available), but some residues around the cleavage sites are not conserved (Figure 7A). Model structures generated using SwissModel showed highly similar 3D structures in all the four mammalian species tested (Figure 7B) with Cα-atoms Root Mean Square Deviations (RMSDs) ranging from 2.1 to 2.3Å. More precisely, orientation of the glutamate residue cleaved by ETs was the same in all four species with their side chains oriented toward the calcium ion (Figure 7C).

Two types of docking orientation predicted. In order to decipher the molecular mechanisms involved in the host-specific cleavage of Dsg1, docking simulations of ETE with Dsg1 from four different species were performed using HADDOCK. Bovine docking and the first cluster of ovine docking accounted for 95% of docking solutions, the best HADDOCK scores being -131.7 +/- 3.2 and -147.1 +/- 1.3, respectively (Table 2). In addition to -103 for the second ovine cluster, other HADDOCK scores ranged from -124 to -82 for human Dsg1 and -128 to -81 for canine Dsg1. Interestingly, a comparison of the two best solutions, obtained by superimposing

the 3D structure of ETE docked to bovine and ovine Dsg1, revealed opposite docking approach orientations on Dsg1 (Figure 8A). For the sake of clarity, the orientation of bovine Dsg1 was named 1 (which corresponded to no cleavage) and that of ovine Dsg1 was named 2 (cleavage). Ovine Dsg1 in the second cluster accounted for only 5% of the solutions and was oriented in position 1 (Figure 8A).

On human Dsg1, the best cluster was in orientation 2 and accounted for 25% of docking solutions. The second-best cluster accounted for 72% of docking solutions and appears in orientation 1 (Figure 8A). It was noteworthy that orientation 1 was also energetically less favorable (Table 2). On the canine docking simulations, the two best clusters were in orientation 1 (Figure 8B).

DISCUSSION

All *S. aureus* ETs are unique serine proteases that specifically and efficiently cleave a single one peptide bond in the extracellular segment of Dsg1. The enzymatic properties of *S. aureus* ETs cause efficient and specific abolishment of a major epidermal adhesion molecule in selected mammalian species. In the present study, we were able to characterize a new *S. aureus* ET that had initially been identified in *S. aureus* strains associated with mild mastitis in ewes (6). It was previously named ETD-like protein because its predicted protein sequence determined from the O46 genome sequence displayed 79% similarity with the ETD protein (6, 8). Our work has clarified the fact that this ETD-like protein has enzymatic activity similar to that of known ETs. This new ET protein indeed specifically solubilizes and cleaves extracellular segment of Dsg1, but not that of Dsg3 or Dsc1. We are therefore proposing that this new *S. aureus* virulence factor should be renamed Exfoliative Toxin type E (ETE).

Like many other *S. aureus* toxins (e.g. staphylococcal enterotoxins), ETs are accessory proteins which are not essential for cell growth. The genes encoding these virulence factors in *S. aureus* are most often borne by mobile genetic elements (MGE) such as *S. aureus* pathogenicity islands (SaPI), prophages, transposons, and plasmids (5). The genes encoding the well-documented ETA, ETB, and ETD proteins are indeed MGE-borne and carried by a temperate phage, a large plasmid, and SaPI (9–11), respectively. For the purposes of this study, the draft genome sequence of O46 (12) was re-assembled, re-annotated (GenBank accession number CP025395) and subjected to a search for genomic islands in order to gain information on the genetic vicinity of *ete*. The *ete* gene was localized on one of the eight GIs that were predicted on the O46 genome. However, the *ete*-containing GI did not display any characteristic features of MGE. Whether this results from a horizontal transfer or recombination event remains unknown.

Genome data regarding *S. aureus* strains isolated from animal hosts remain scarce. To date only 343 *S. aureus* strains from animal hosts have been deposited on the PATRIC database whereas

there are 6619 and 2797 genomes of *S. aureus* strains isolated from human or unknown hosts, respectively. However, out of the nine ovine strains available on PATRIC, four included a full ETE protein predicted in their proteomes, apart from one truncated ETE predicted in that of *S. aureus* O11. Although there might be a bias due to the low number of ovine strains, the proportion of 55.5% of *ete*-carrying strains is high, compared to only one out of the 94 bovine *S. aureus* proteomes (~1%) that include the ETE predicted protein. This observation correlates with our previous findings showing that the ETE protein is frequently (~half of all tested strains) detected in strains isolated from ewe mastitis (6) and suggests that the *ete* gene is found more frequently in *S. aureus* strains isolated from ovine hosts than from any other hosts. Of note, the *ete* gene shared similar genetic vicinity in all the genomes of the strains isolated from ovine, bovine and bulk milk available on PATRIC. Moreover, Dsg1 expression in the teat canal epithelia of ruminants suggests that ETE facilitates the colonization of *S. aureus* in the mammary tract of certain ruminants with mastitis.

Although humans are the primary hosts for *S. aureus*, some of its lineages have evolved to adapt to a variety of animal hosts, and strains isolated from human or small (i.e. ovine or caprine) and large (i.e. bovine) ruminants have been shown to be distinct at both the genotypic and genomic levels (3). Such species-specific traits are also observed at the molecular level. Indeed, certain virulence-associated genes have evolved towards species-specific activity, as illustrated with the bovine variant of von Willebrand factor-binding protein (13), the equine variant of staphylococcal complement inhibitor (SCIN; (14)), or ovine variants of staphylococcal enterotoxin type C (15). The ETE described here displayed species-specific activity among ruminant Dsg1, with an efficient cleavage of ovine and caprine Dsg1, whereas it was not active on bovine Dsg1. Likewise, though all ETs are active against human Dsg1, some of them have evolved to develop activity against a broader array of hosts, *e.g. S. hyicus* Exhs which is active on swine Dsg1 (16).

All ETs are unique glutamate-specific serine proteases and specifically cleave Dsg1 after glutamic acid residue 381, located between the extracellular domains 3 and 4 (17). In the same way, ETA, ETB and ETD toxins cleave Dsg1, and the effects of ETA and ETB are histologically indistinguishable (18) with epidermal detachment and the formation of flaccid blisters (19). All ETs cleave human and murine Dsg1α into two segments. Mice have three isoforms of Dsg1 (Dsg1α, β and γ) (20), and we showed that ETE solubilized the extracellular segments of murine Dsg1α and -β, but not that of Dsg1γ, in which glutamic acid residue 381 is replaced by lysine (20). This is also observed for ETA activity and indicates that glutamic acid residue 381 is the site for the cleavage of Dsg1 by ETs.

Except for murine Dsg1γ, the ET cleavage site on Dsg1 was found to be highly conserved in all the animal species used for this study. The species specificity we observed here is therefore likely to rely on steps preceding the cleavage event and on the differential abilities of ETs to reach their cleavage site. Molecular docking experiments are widely used to determine the ability of a ligand to recognize and bind to its putative receptor(s). But to date it has never been applied to investigating the docking on its cleavage site of a serine protease such as ETE. The molecular docking of ETE with Dsg1 from different species offers some keys to the approach mechanisms involved in this species-specific cleavage. Depending on the species of origin of Dsg1, ETE seems to have privileged orientations for interaction. Orientation 1 is observed particularly with bovine and canine Dsg1 that are not hydrolyzed by ETE, whereas orientation 2 is observed with ovine and human best complexes. Interestingly, in our study, the dominant orientation 2 for human and ovine Dsg1 correlated with the species-specific cleavage of Dsg1 seen during *in vitro* and *ex vivo* analyses, whereas orientation 1 for canine and bovine Dsg1 corresponded to an absence of detectable ETE cleavage in these mammalian species. Although both orientations may be observed for human Dsg1, it is worth noting that orientation 1 corresponds to a less energetically favorable docking. Further investigations using flexible

docking and molecular dynamics experiments may help to clarify whether this fully determines actual cleavage specificity, or not.

In conclusion, ETE is a new *S. aureus* virulence factor that is frequently associated with ovine *S. aureus* isolates. It is also a new example of host specialization in *S. aureus*. Of note was the fact that the *ete* gene had previously been found in *S. aureus* strains associated with mild mastitis in ewes (6). ETE may therefore be a marker of *S. aureus* virulence in ewe mastitis. Whether and how it provides selective advantages for *S. aureus* colonization and persistence in the udder still needs to be clarified, but this may be of considerable value to the diagnosis and control of mastitis.

EXPERIMENTAL PROCEDURES

Genomic analysis of *Staphylococcus aureus* O46. The genome sequencing of *S. aureus* O46 and O11 was carried out by Maréchal et al. (21) using Solexa technology (Illumina, San Diego, CA, USA). DNA reads were re-evaluated in order to close all the sequence gaps previously observed in the genome and to facilitate identification of the genomic context of the new *et* gene. SPAdes version 3.9.1 (22) was used for the *de novo* assembly of O46 and O11 genomes. Scaffolding was performed using CONTIGuator version 2.7 (23) and the genome of *S. aureus* ED133 (an ovine strain of *S. aureus* (CP001996.1) retrieved from the National Center for Biotechnology Information (NCBI) databases) was used as a reference. The sequence gaps were then filled using FGAP (24) and any remaining gaps were closed based on consensus sequences found against the genome of strain ED133, using CLC Genomics Workbench 7.0 (Qiagen, USA). The genomes of strains O46 and O11 were submitted to the RAST server for automatic annotation (25). Genomic island predictions were performed using GIPSy version 1.1.2 (26) and the genome of *Staphylococcus warneri* strain SG1, retrieved from the NCBI databases, was used as a reference. Genomic island (GI) sequences and their gene products were curated manually using the UniProtKB database and Artemis (27). To verify whether the GIs localized in prophage regions, the whole genomes were assessed using PHAST (28). To identify the presence of insertion sequences (IS) in the O46 genome, it was submitted to the IS Finder online database that uses algorithms such as BLAST for IS identification (29). To locate IS families through the genome, it was submitted to ISMapper, a mapping-based tool to identify the site and orientation of IS in bacterial genomes (30). The *S. aureus* O46 genome is deposited with GenBank (accession number CP025395).

The *ete* gene sequence was searched for in other *S. aureus* genome sequences using BLASTp in the PATRIC database with a e-value threshold of 0.01 (31).

Phylogenetic tree of ETs. Phylogenetic analysis based on the amino acid sequences of ETs was performed with the CLUSTAL X program (http://www.clustal.org). A neighbor-joining tree was constructed using NJPlot software (http://doua.prabi.fr/software/njplot).

Recombinant *ete* gene product. Optimized *ete* sequence with *E. coli* preferential codons was synthesized and cloned into pD441-NH expression vector by DNA2.0 (ATUM, Newark, CA). The plasmid pD441-NH:*ete* was transformed into CD43 (DE3) pLysS *E. coli* strain according to OverExpress™ Electrocompetent Cells kit (Lucigen, Middleton, WI) instructions for protein production and purification. Overnight culture of recombinant *E. coli* strain transformed with pD441-NH:*ete* was diluted 100-fold with fresh LB broth containing kanamycin (34 mg/mL) and incubated at 30 °C until the optical density (OD_{600}) reached 0.5 and was subsequently induced with 0.2 mM IPTG for 16 hours at 20 °C. Induced cells were harvested by centrifugation and lysed by sonication and centrifuged at 15,000 g for 15 minutes. The supernatant was subjected to affinity chromatography using an immobilized nickel column (GE) under native conditions and further purified using a Superdex G75 10/300 GL column. Purity of the ETE protein was determined by SDS-PAGE gels.

Recombinant ETB. A recombinant plasmid containing the *etb* gene fused with 6X His tag at the carboxyl terminus (a gift from Dr. Motoyuki Sugai, Hiroshima University) (32) was transformed in BL21 (DE3) competent cells (Merck, Darmstadt, Germany). The recombinant ETB was harvested from the cytoplasmic soluble fraction of BL21, purified with TALON metal affinity resin (Takara Bio, Kusatsu, Japan) and dialyzed against phosphate-buffered saline (PBS).

Passive transfer of ETE to neonatal mice. Neonatal ICR mice (<12 h of age; Sankyo Labo

Service, Tokyo, Japan) were injected subcutaneously with 100 µg of the purified ETE. Skin samples were collected 1 h after the injection and subjected to histopathological and immunofluorescence studies. All the experiments using mice had been ethically approved by the Animal Research Committee at Tokyo University of Agriculture and Technology (No. 25-69) and were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

Immunofluorescence study on mouse skin. Cryosections of non-fixed neonatal mouse skin were stained using the following antibodies: a human pemphigus foliaceus serum containing IgG against the extracellular segment of Dsg1 (1:500 dilution; a generous gift from Dr. Masayuki Amagai, Keio University School of Medicine) (33), AK18 anti-Dsg3 mouse monoclonal IgG antibody that recognizes the extracellular portion of Dsg3 (1:500 dilution; a generous gift from Dr. Masayuki Amagai) (34), DG3.10 mouse monoclonal IgG antibody that reacts with the cytoplasmic domain of Dsg1+2 (Progen, Heidelberg, Germany) and a human IgA pemphigus serum containing IgA antibodies against the extracellular segment of desmocollin (Dsc) 1 (a kind gift from Dr. Masayuki Amagai) (35). Fluorescence was captured and examined under a BX51 fluorescent microscope (Olympus Corp., Tokyo, Japan).

Immunofluorescence study of ruminant skin. Cryosections of ovine, caprine and bovine nasal planum, as well as caprine mammary duct, were incubated with 100 µg/mL of either ETB or the ETE protein in TBS with 5 mM $CaCl_2$ (TBS-Ca), or TBS-Ca alone for 2 hours at 37ºC. The sections were then immunostained with a human pemphigus foliaceus (PF) serum containing IgG autoantibodies that recognize the extracellular segment of Dsg1.

In vitro **digestion of Dsg1 by ETs.** Insect culture supernatants containing the baculovirus

recombinant extracellular domains of human Dsg1 (hDsg1), swine Dsg1 (sDsg1), canine Dsg1 (cDsg1), and murine Dsg1α (mDsg1α), Dsg1β (mDsg1β) and Dsg1γ (mDsg1γ), fused with E- and His-tags on their carboxyl termini, were gifts from Masayuki Amagai (33, 36–39). The recombinant Dsg1s were incubated *in vitro* with 70 μg/mL purified ETE or ETB for 2 hours at 37ºC. Dsg1s that remained intact or were degraded by ETs were detected using an anti-E-tag monoclonal antibody (GE Healthcare, Buckinghamshire, UK).

Molecular modeling. Human, bovine, canine and ovine protein sequences of Dsg1 were retrieved from UniProtKB (Respective entry id: Q02413, Q03763, Q9GKQ8, W5P9B0). SwissModel (40–42) was used to obtain 3D structural models of the extracellular domain. Each sequence was submitted to the software, and the human PDB structure of Dsg3 (5EQX) was used as a pattern. For each species, the best model was chosen and minimized using 2000 steps of steepest descent minimization under the YASARA software.

The structure of the new ETE previously published by Mariutti et al. (8) (PDB id: 5C2Z) was also submitted to 2000 steps of steepest descent minimization utilizing YASARA software (http://www.yasara.org/).

Molecular docking. Protein-protein docking was performed using HADDOCK (High Ambiguity Driven protein DOCKing) (43). Residues involved in the active sites of both proteins were required. The active ETE residues were those defined in (8) (His96, Asp145 and Ser219) and those for Dsg1 were those defined in (44) (Glu381, Gly382 in protein sequence matching Glu332, Gly333 in structural models).

HADDOCK generates up to 200 docking solutions clustered by mean Root Mean Square Deviation (RMSD), and a global score for in combined energies is calculated. Clusters are numbered according to their size and ranked according to a lowest score for the best cluster.

Two best clusters for each species were conserved for further analyses. The visualization and image generation of the structures were performed using VMD (Visual Molecular Dynamics) (45).

Acknowledgments

We are very grateful to Dr Olivier Delalande (IGDR, Rennes) for fruitful discussions on molecular docking experiments, to Gérard Lina (CNR Staphylocoques, Lyon) for helpful discussions on staphylococcal toxins, and to Dr. Masayuki Amagai for providing us with baculovirus recombinant Dsg1s, human PF serum, human IgA pemphigus serum and AK18 anti-Dsg3 monoclonal IgG antibody. We also thank Dr. Motoyuki Sugai for kindly providing the recombinant ETB plasmid. This research was supported by a Grant-in-Aid for Scientific Research (KAKENHI) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT). This work also received support from the National Institute for Agricultural Research (INRA-France), and the Brazilian government through its National Council for Scientific and Technological Development (CNPq) and the Brazilian Federal Agency for Support, Evaluation of Graduate Education (CAPES): CAPES-COFECUB project #849/15, PVE Project 400721/2013-9 and FAPESP

FIGURES

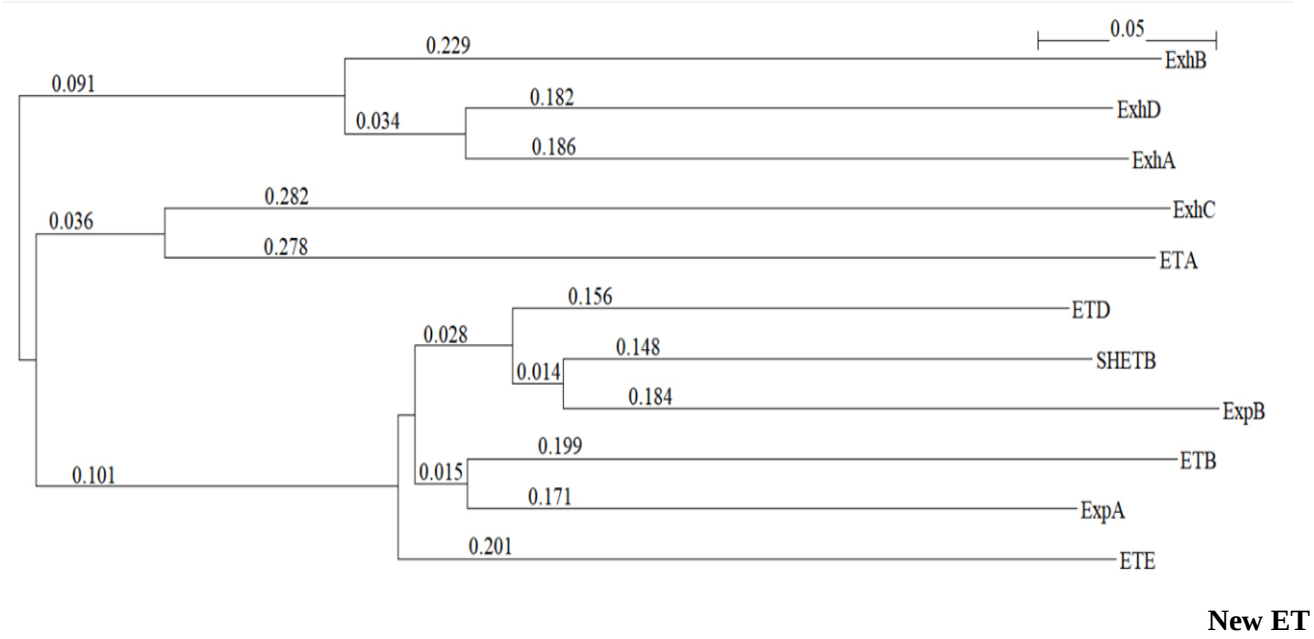

Figure 1. Phylogenetic tree of staphylococcal ETs. A phylogenetic analysis based on the overall amino acid sequences of ETs was built using a neighbor-joining method. SHETB, ExhA, ExhB, ExhC, and ExhD were ETs produced by *Staphylococcus hyicus*. ExpA and ExpB were ETs produced by *Staphylococcus pseudintermedius.*

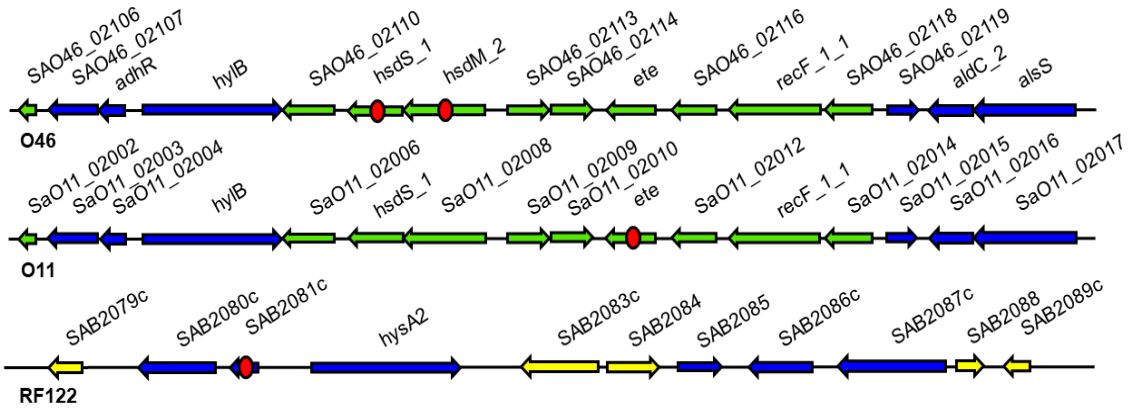

Figure 2. Putative genomic island containing the new *et* gene found in the *S. aureus* O46 genome. Comparisons with the most closely related putative genomic islands (GI) in strains O11 and RF122, isolated from ovine and bovine hosts, respectively, are shown below the upper line. Arrows represent open reading frames and their orientations. Blue: genes shared among O46, O11, and RF122 GIs. Green: genes shared between O46 and O11 GIs. Yellow: genes only present in the RF122 GI. Red circles indicate genes lacking part of their encoding sequence (*hsdM*_2 in O46, *ete* in O11) or presenting a frameshift that results in a coding sequence truncation (*hsdS*_1 in O46, *SAB2081c* in RF122).

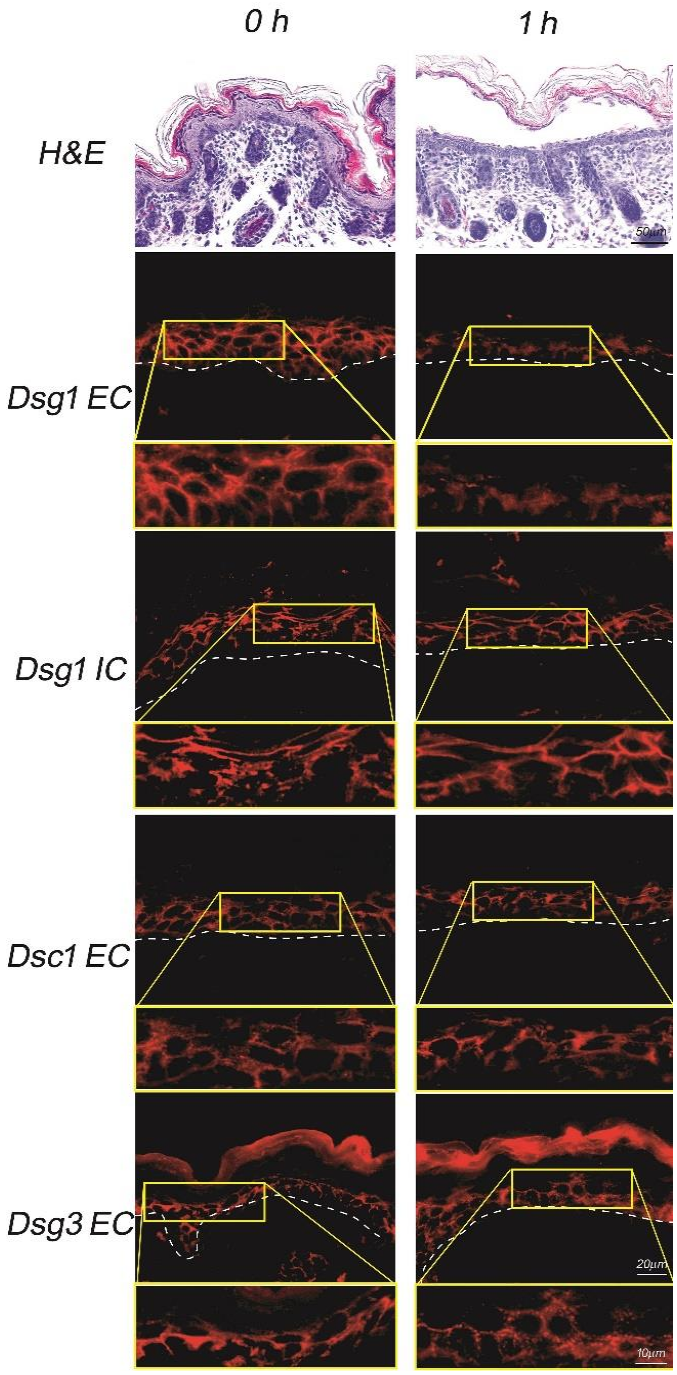

Figure 3. Exfoliative activity of the new ET in neonatal mice. Neonatal mice injected with a recombinant new ET displayed microscopic blisters 1 h after injection and immunostaining was abolished for the extracellular segment of Dsg1, but not for the intracellular domain of Dsg1 and the extracellular segments of Dsc1 or Dsg3. Dotted lines indicate the basement membrane. Bars indicate 20 μm and 10 μm at lower and higher magnifications, respectively. EC: extracellular, IC: intracellular.

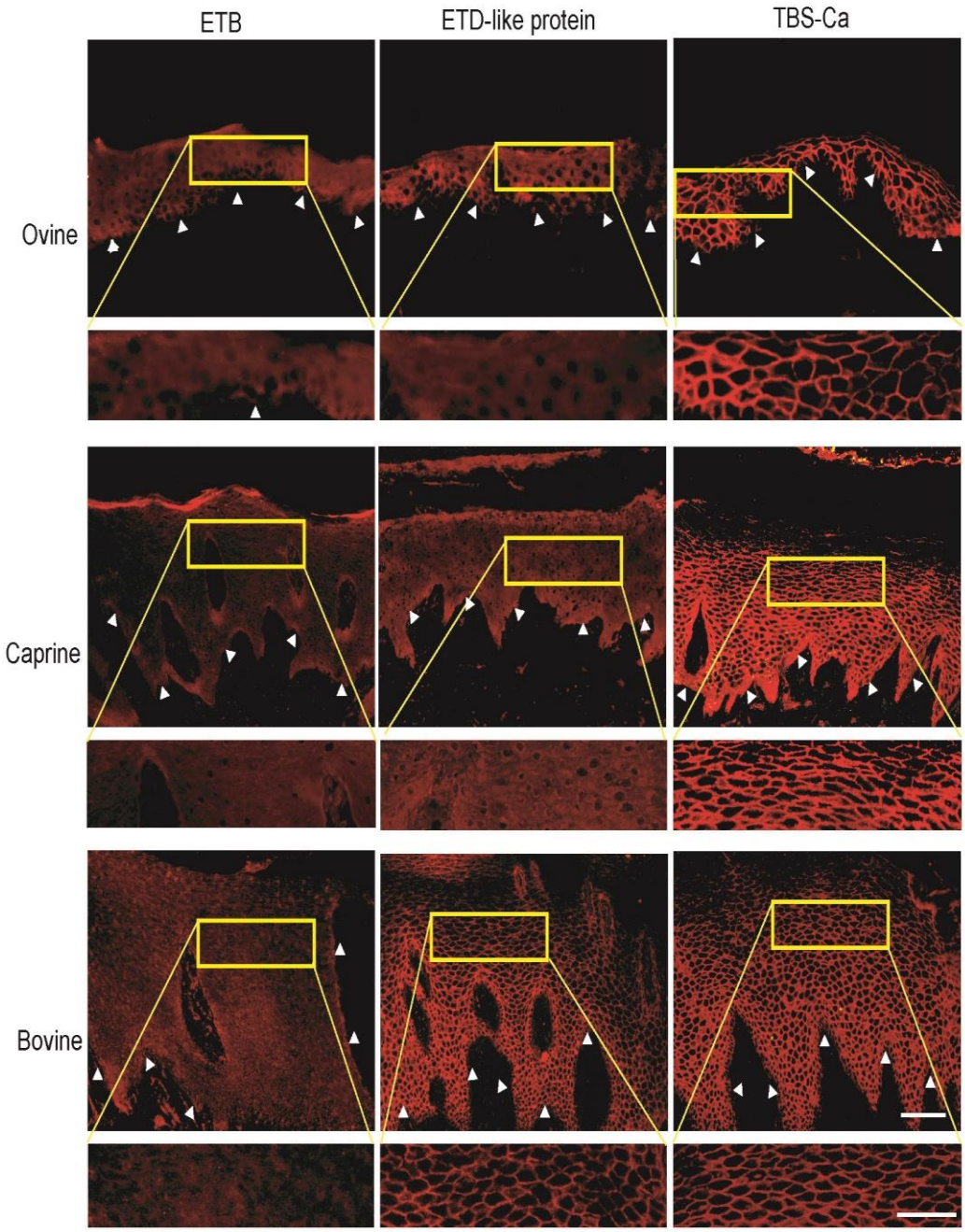

Figure 4. ETE degrades Dsg1 in ovine and caprine epidermis. Cryosectioned ovine, caprine and bovine nasal planum was incubated with ETB, the ETE protein or TBS-Ca, and subjected to immunofluorescence with human PF serum containing anti-Dsg1 IgG. Arrowheads indicate epidermal basement membranes. Bars indicate 50 μm and 20 μm at lower and higher magnifications, respectively.

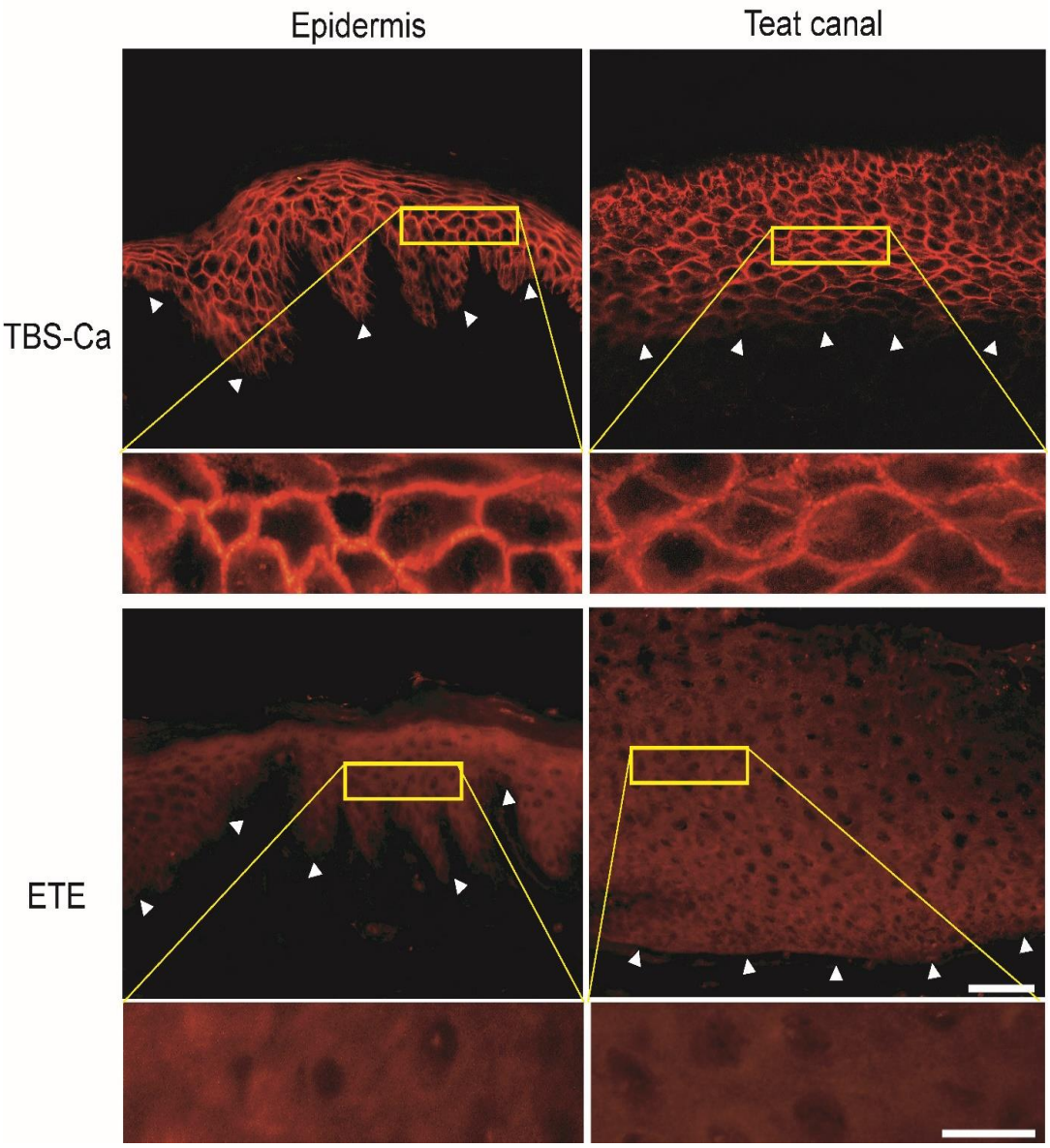

Figure 5. ETE degrades Dsg1 in caprine teat canal epithelia. Cryosections of caprine epidermis and teat canal were incubated with either TBS-Ca or ETE, and subjected to immunofluorescence with the human PF serum. Arrowheads indicate basement membranes. Bars indicate 50 μm and 20 μm at lower and higher magnifications, respectively.

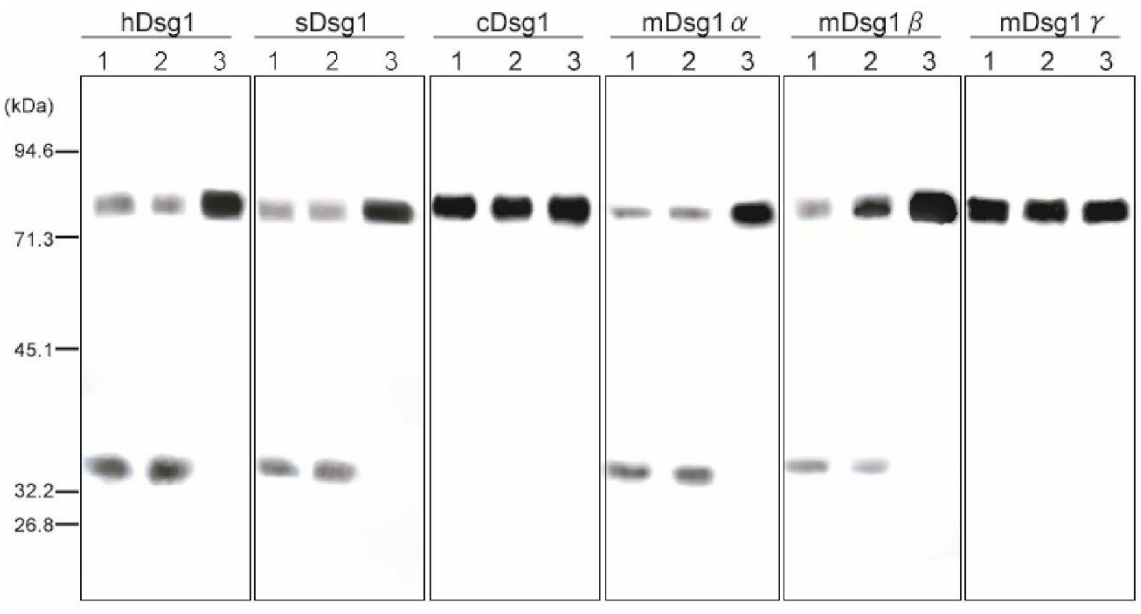

Figure 6. *In vitro* digestion of recombinant Dsg1s with ETE. Baculovirus recombinant extracellular domains of human Dsg1 (hDsg1), swine Dsg1 (sDsg1), canine Dsg1 (cDsg1), murine Dsg1α(mDsg1α), Dsg1β (mDsg1β) and Dsg1γ (mDsg1γ) were incubated with ETB (lane 1), ETE (lane 2), or TBS-Ca (lane 3), and subjected to immunoblotting with anti-E-tag monoclonal antibody.

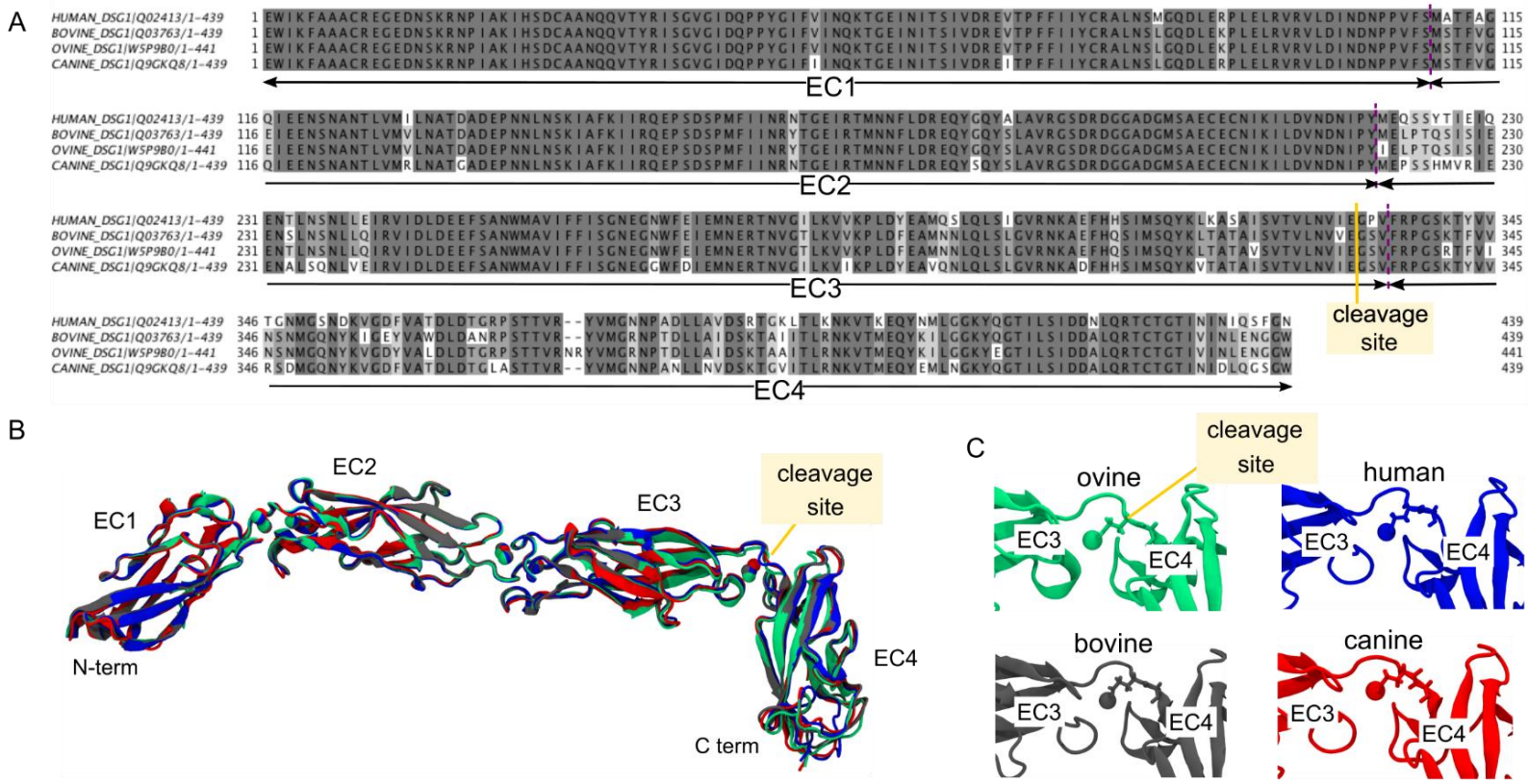

Figure 7. Protein sequence alignment and structural model of Dsg1. (A) Dsg1 protein sequence alignment of the extracellular domain. The boundaries of each cadherin repeat (EC1 to EC4) are indicated by a purple dashed line. The cleavage site is indicated by a yellow line. Residue numbers are according to model structures. (B) Superimposition of Dsg1 model structures. Ovine Dsg1 in green, human Dsg1 in blue, bovine Dsg1 in black and canine Dsg1 in red. Colored spheres are calcium ions. The cleavage site is indicated. (C) Zoom on cleavage site with the side chain of cleaved Glu332 represented and the unique calcium ion.

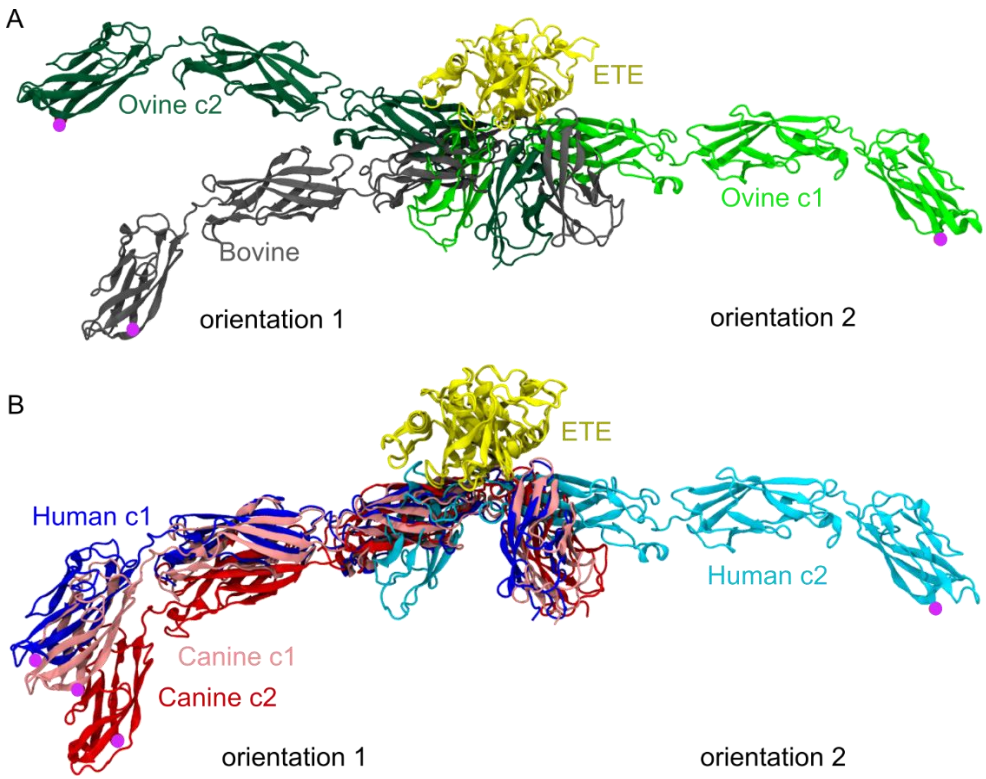

Figure 8. Best HADDOCK docking solutions. Complexes are superimposed by ETE and the same orientation is conserved in (A) and (B). Red spheres are drawn at the N-terminal end of Dsg1. (A) ETE docked with ovine and bovine Dsg1. For bovine Dsg1 (black), a single orientation is calculated using HADDOCK. The best ovine solution is ovine c1 (light green) in orientation 2, the second is ovine c2 (dark green). (B) ETD2 docked with human and canine Dsg1. The best human orientation is human c2 (light blue) in orientation 2. The second solution is human c1 (dark blue) in orientation 1. The best canine solution is canine c2 (red), the second is canine c1 (pink). Both are in orientation 1.

Table 1. Genes present in the Putative Genomic Island of strain O46 containing the new *et* gene.

Gene name	Product/ classification
sa_O462052	Hypothetical protein
sa_O462053	Oxidoreductase
adhR	Transcriptional regulator, MerR family
hylB	Hyaluronate lyase
sa_O462056	M23/M37 peptidase domain protein
***hsdS*_1**	Type I restriction modification system, DNA specificity domain protein
***hsdM*_2**	Type I restriction modification system, M subunit
sa_O462059	Serine protease
sa_O462060	Epidermal cell differentiation inhibitor
ete	Exfoliative toxin type E
sa_O462062	DNA helicase
***recF*_1_1**	Recombinational DNA repair ATPase
sa_O462064	Hydrolase
sa_O462065	Probable exported protein
***aldC*_2**	Alpha-acetolactate decarboxylase
alsS	Acetolactate synthase, catabolic

Table 2. HADDOCK scores

Simulation	Number of clusters	HADDOCK Score	RMSD	Van Der Waals	Electrostatics	Desolvatation	Buried surface	Privileged orientation
Human	3 (197 complexes)	c2: -124.4 +/- 4.9	0.8 +/- 0.5 (49 complexes = 25%)	-60.1 +/- 7.1	-212.7 +/- 21.3	-25.1 +/- 5.9	1834.2 +/- 198.4	2
	Best : c2	c1: -117,6 +/- 4,2	18.3 +/- 0.1 (142 complexes = 72%)	-68.0 +/- 2.9	-78.4 +/- 12.1	-39.4 +/- 2.2	1851.2 +/- 93.4	1
		c3: -82.4 +/- 22.4	6.3 +/- 0.2 (6 complexes = 22%)	-37.0 +/- 5.3	-104.2 +/- 49.4	-28.6 +/- 15.1	1272.9 +/- 117.0	2
Bovine	1 (197 complexes)	-131.7 +/- 3.2	1.2 +/- 0.8 (100%)	-54.6 +/- 2.9	-202.6 +/- 65.8	-26.5 +/- 6.9	1708.1 +/- 46.4	1
Ovine	2 (198 complexes)	c1: -147.1 +/- 1.3	2.5 +/- 1.8 (189 complexes = 95%)	-73.9 +/- 7.8	-202.6 +/- 27.0	-36.1 +/- 3.6	2029.4 +/- 146.0	2
	Best : c1	c2: -103.7 +/- 11.4	18.2 +/- 0.1 (9 complexes = 5%)	-59.0 +/- 7.6	-124.4 +/- 23.5	-26.4 +/- 8.5	1932.4 +/- 248.7	1
Canine		c2: -128.1 +/- 2.6	4.8 +/- 0.1 (41 complexes = 21%)	-71.9 +/- 4.1	-146.2 +/- 9.7	-31.2 +/- 5.3	1996.6 +/- 64.7	1
		c1: -127.0 +/- 4.0	0.8 +/- 0.5 (95 complexes = 48%)	-78.6 +/- 6.3	-103.9 +/- 18.5	-32.2 +/- 6.6	1959.4 +/- 108.3	1
	5 (198 complexes)	c4: -106.7 +/- 13.6	19.0 +/- 0.1 (15 complexes = 8%)	-51.2 +/- 5.9	-178.1 +/- 40.5	-23.4 +/- 8.2	1616.8 +/- 127.0	2

	Best : c2	c3: -90.5 +/- 3.5	6.0 +/- 0.5 (43 complexes = 22%)	-45.9 +/- 2.0	-49.0 +/- 10.1	-39.8 +/- 3.6	1439.4 +/- 111.3	1
		c5: -81.0 +/- 11.6	14.3 +/- 0.2 (4 complexes = 2%)	-39.1 +/- 5.7	-78.3 +/- 39.5	-30.5 +/- 8.8	1361.2 +/- 122.3	1

Supplemental data.

Table S1. Genes present in the most closely related GI of strain RF122, relative to the ETE containing O46 SaPI.

Gene name	Product/ classification
SAB2079c	Hypothetical protein
SAB2080c	Oxidoreductase
SAB2181c	Transcriptional regulator, MerR family
hysA2	Hyaluronate lyase
SAB2083c	Transcriptional regulator
SAB2084	Conserved hypothetical protein
SAB2085	Exported protein
SAB2086c	Alpha-acetolactate decarboxilase
SAB2087c	Alpha- acetolactate synthase
SAB2088	Hypothetical protein
SAB2029c	Hypothetical protein

Table S2. Localization of IS families in the O46 genome.

Localization of IS families		
	Start of sequence	**End of sequence**
IS3 family	1946138	1948102
ISL3 family	768457	770315
	1747334	1749208
	1852210	1854054

Table S3: *S. aureus* harboring the *ete* gene.

Genome Name	Isolation Source	Isolation Country	Host Name
Staphylococcus aureus O46	Subclinical ewe mastitis	France	Sheep, *Ovis aries*
Staphylococcus aureus O11	Gangrenous ewe mastitis	France	Sheep, *Ovis aries*
Staphylococcus aureus strain 04Hi	Nasal	Tanzania	NA
Staphylococcus aureus strain 011Hii	Nasal	Tanzania	NA
Staphylococcus aureus strain GKP136-11	Bulk milk	United Kingdom	NA
Staphylococcus aureus strain GKP136-21	Bulk milk	United Kingdom	NA
Staphylococcus aureus strain 3688STDY6124964	Clinical sample / spesis	Thailand	Human, *Homo sapiens*
Staphylococcus aureus strain CHUV_1	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain CHUV_8	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain CHUV_5	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain CHUV_2	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain	Skin (isolated from infected Eritrean	Switzerland	Human, *Homo*

CHUV_4	and non-Eritrean patients)		*sapiens*
Staphylococcus aureus strain CHUV_6	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain CHUV_7	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain CHUV_3	Skin (isolated from infected Eritrean and non-Eritrean patients)	Switzerland	Human, *Homo sapiens*
Staphylococcus aureus strain BSAR58	Nasal	Denmark	Human, *Homo sapiens*
Staphylococcus aureus strain BSAR136_2	Wound	Denmark	Human, *Homo sapiens*
Staphylococcus aureus strain BSAR141_2	Nasal	Denmark	Cattle, *Bos sp*.
Staphylococcus aureus strain BSAR113_2	Nasal	Denmark	Sheep, *Ovis sp*.
Staphylococcus aureus strain BSAR57	Blood	Denmark	Human, *Homo sapiens*
Staphylococcus aureus strain BSAR112	Nasal	Denmark	Sheep, *Ovis sp*.
Staphylococcus aureus strain BSAR111_2	Nasal	Denmark	Sheep, *Ovis sp*.
Staphylococcus aureus strain BU_G1074_t4	Wound	Ghana	Human, *Homo sapiens*
Staphylococcus aureus strain	Wound	Ghana	Human, *Homo*

*BU*_G0301_t8			*sapiens*
Staphylococcus aureus strain BU_G1101_t2	Wound	Ghana	Human, *Homo sapiens*
Staphylococcus aureus strain BU_N17W_t2	Nose	Ghana	Human, *Homo sapiens*
Staphylococcus aureus strain BU_G0706B_t8	Wound	Ghana	Human, *Homo sapiens*
Staphylococcus aureus strain BU_G1001_t8	Wound	Ghana	Human, *Homo sapiens*
Staphylococcus aureus strain BB155	Nasal	Mali	Human, *Homo sapiens*
Staphylococcus aureus C00012787	Clinical sample	NA	Human, *Homo sapiens*
Staphylococcus aureus C00012788	Clinical sample	NA	Human, *Homo sapiens*
Staphylococcus aureus C00012789	Clinical sample	NA	Human, *Homo sapiens*
Staphylococcus aureus subsp. *aureus* strain SA-006	Food	Switzerland	NA

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Annex 4

Chapter 8

Exfoliative Toxins of *Staphylococcus aureus*

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Additional information is available at the end of the chapter

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Abstract

Virulent strains of *Staphylococcus aureus* secrete exfoliative toxins (ETs) that cause the loss of cell-cell adhesion in the superficial epidermis. *S. aureus* ETs are serine proteases, which exhibit exquisite substrate specificity, and their mechanisms of action are extremely complex. To date, four different serotypes of ETs have been identified and three of them (ETA, ETB and ETD) are associated with toxin-mediated staphylococcal syndromes related to human infections leading to diseases of medical and veterinary importance.

Keywords: epidermolytic diseases, *Staphylococcus aureus*, exfoliative toxins, Desmoglein 1, keratinocytes

1. Introduction

Staphylococcus aureus, a commensal and opportunistic microorganism, is capable of colonizing the skin and mucous of individuals and represents a global public health problem [1–3]. It has been described as the etiological agent of various diseases both in humans and animals and is the main representative bacteria of the genus Staphylococcus [4]. *S. aureus* is a versatile microorganism and is capable of quickly adapting to different environmental conditions [5, 6]. This microorganism secretes several virulence factors that are associated with its pathogenesis [2] and in facilitating access to sites in the host that are normally sterile [7]. Diseases caused by *S. aureus* do not necessarily originate only by direct tissue invasion, but may be due to the action of more than 30 exoproteins codified by the pathogen [8, 9].

The exfoliative toxins (ETs) also known as epidermolytic toxins, are serine proteases secreted by *S. aureus* that recognize and hydrolyze desmosome proteins in the skin. ETs have been

described as exotoxins produced by certain *S. aureus* strains, in the epidermis of the host, that have been associated with the loss of keratinocytes and with the cell-cell adhesion, inducing peeling of the skin and blister formation [10–13].

In 1878, Baron Gottfried Rotter Von Rittershain described the clinical features of epidermal exfoliation in newborns [14]. The relationship between skin exfoliation and *S. aureus* was established in 1967 by Lyell [14–16] and in 1972 [17] epidermal detachment at the stratum granulosum was established by electron microscopy. The pathogenic role of those toxins was demonstrated in 1970 by Melish and Glasgow by using newborn mice as experimental models [18]. However, the protein capable of causing generalized exfoliation in mice, denominated as exfoliatin, was not isolated and purified until 1971 [19] and subsequently serotypes have been identified [20, 21].

The principal isoforms of exotoxins implicated in human skin damage are exfoliative toxin A (ETA) and exfoliative toxin B (ETB) [22]. Exfoliative toxin C (ETC) isolated from a horse infection has not been associated with human disease. In 2002, a new exfoliative toxin (ETD) was identified in a clinical sample of *S. aureus* [13]. Recent crystallographic studies indicated that the ETD-like protein isolated from ewe mastitis [23] is structurally homologous to ETA and ETB [24]. ETA is codified by the *eta* gene on chromosomal DNA, carried on the genome on a temperate phage, and ETB by the *etb* gene on a large plasmid DNA [22, 25, 26]. ETD is codified by the *etd* gene which is located chromosomally on a pathogenicity island [13].

The ETA and ETB serotypes are homologous, have molecular masses of approximately 27 kDa, and contain 242 and 246 amino acids, respectively [22] and present identical dermatologic symptoms [26, 27].The ETA serotype was described as being heat stable whereas the ETB serotype has been demonstrated to be heat labile. The ETC serotype with a molecular mass of 27 kDa is also heat labile and causes exfoliation in mice and chickens [28].

2. Exfoliative toxins and associated diseases

ET-producing strains of *S. aureus* are related to localized epidermal infections such as bullous impetigo and generalized diseases like Staphylococcal scalded skin syndrome (SSSS). Approximately 5% of all *S. aureus* strains produce exfoliative toxins, with ETA being most prevalent in Europe, Africa, and America and ETB being more common in Japan [26]. Most strains of *S. aureus* associated with SSSS in Europe and the United States belong to the type II phage group, such as 71 and 55/71, however, in Japan; most of the strains belong to other groups [15, 29]. In France, based on a retrospective study conducted between 1997 and 2007 [30], the mean incidence of SSSS cases was estimated to be 0.56 cases/year/million inhabitants.

Both ETA and ETB are distinguished by the extent of the damage caused in the epidermis [29, 30]. SSSS clinical manifestations involve fever, skin hypersensitivity, and erythema followed by superficial blister formation and skin separation, leaving long areas of denuded skin [10, 31]. In the localized form, toxin production and formation of flaccid blisters with purulent fluid occur [12, 30]. SSSS occurs mainly in newborns and children with occurrences in adults being rare [11, 32]. The mortality rate in children submitted to immediate treatment is low [33].

The greater susceptibility of children has been attributed to the immature immune system, weak renal clearance of the toxin, and the fact that children are common carriers of microorganisms [30]. In the most severe cases, exfoliation may affect the entire corporal surface [33]. The quick and sensitive diagnosis of those infections may be performed using radioimmunoassays, enzyme-linked immunosorbent assays, the reverse passive latex agglutination assay [26] as well as the polymerase chain reaction (PCR) to amplify the genes that codify ETs.

When the ET serotypes and the clinical forms of the disease were correlated, the ETA toxin was found to be associated with bullous impetigo formation, whereas ETB was found to be associated with SSSS, a generalized manifestation [34]. The ETB plasmid has multiple genes that confer antibiotic resistance, which contributes to the increased resistance of *etb*-positive *S. aureus* strains [35]. The *etd*-positive strains have been isolated mainly from patients with deep pyoderma [12].

In addition to *S. aureus*, *Staphylococcus hyicus* and *Staphylococcus chromogenes* are also associated with skin infections and produce exfoliative toxins [36]. In *S. hyicus*, ET production has been associated with exudative epidermitis (EE) in pigs [37]. The toxin also has the capacity to cleave swine desmoglein [38, 39]. Clinical manifestations are characterized by exfoliation accompanied by epidermal cell separation, erythema, and serous exudation [40]. The isolated toxins of these clinical manifestations have been denominated as SHETA and SHETB [41] and ExhA, ExhB, ExhC [41], and ExhD [38, 42]. In 2007, a *Staphylococcus sciuri* strain, highly pathogenic and *ExhC*-positive, was described as the etiological agent of EE in pigs in China [43]. The ExhC recombinant protein (rExhC) has induced necrosis *in vitro* and has caused skin lesions in newborn mice [44].

Currently many phylogenetically distant hosts are described as being susceptible to exfoliation caused by the same isoforms of ET, revealing a certain specificity for various host organisms [29]. Among six different ETs (SHETA, SHETB, ExhA, ExhB, ExhC, and ExhD) codified by *S. hyicus*, SHET toxins caused exfoliation in piglets and chicks, but not in mouse, rat, guinea pig, hamster, dog, or cat [30]. All four Exh toxins cause exfoliation in pigs, but only ExhA and ExhC cause it in neonatal mice [40, 42]. SCET exfoliative toxin of *Staphylococcus chromogenes* also induces exfoliation in two different hosts; pigs and chicks. Different hosts are also susceptible to EXI exfoliative toxin of *Staphylococcus pseudintermedius* that induces exfoliation in dogs and mice.

Infections by *et*-positive *Staphylococcus intermedius* in dogs can also cause a pathology that resembles SSSS and EE [45]. A previous study described the distribution of toxin genes among phage types of animal isolates of *S. aureus* and the canine isolates of phage group II that harbored the *eta* gene [46].

In Japan, hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) strains frequently carry the *etb* gene [47] and, isolated samples of *etb*-positive *S. aureus* have been encountered in strains with mecA, contrary to the isolated samples of *eta*-positive *S. aureus* [48].

ET-producing *S. aureus* strains (*eta* and *etb*) are related to the clonal complex CC121 [49]. Infections with Staphylococci of this complex are associated with clinical features like impetigo, staphylococcal scalded skin syndrome, conjunctivitis, and exfoliative dermatitis [50].

3. Structural biology and mechanism of exfoliative toxins

The crystal structure of ETA was the first to be determined in atomic detail [51], followed by ETB [52] and by ETD [24] and currently, the atomic coordinates of six ET structures have been deposited with the Protein Data Bank (www.rcsb.org). The crystallographic structures of ETs have revealed much about their mechanisms of action, lack of hydrolytic activity against substrates in the native state, and the susceptibility of certain constituent layers of the epidermis to disruption by ETs.

4. Similarities and differences among ETs and other serino proteinases

Exfoliative toxins are glutamic-acid specific trypsin-like serine proteinases that share 50% sequence identity but display very low sequence identity with other serine proteases. The significant sequence identity of ETs is also reflected in the high structural similarity as evidenced by the low RMSD values of the superposed structures (ETA-ETB: 0.9, ETA-ETD: 1.3, and ETB-ETD: 0.6). Similar to other trypsin-like serine-proteinases, the three-dimensional structures of ETs are characterized by two six-stranded β-barrels domains, S1 and S2, whose axes lie roughly perpendicular to each other, a Greek key motif consisting of four antiparallel strands and N- and C-terminal extensions. The amino acids constituting the catalytic triad (His-Ser-Asp) and Thr190 and His213 which are characteristic of glutamate-specific serine proteinases are located at the junction of the S1 and S2 domains [51, 53].

ETs specifically cleave both mouse and human desmoglein 1 following glutamic acid 381, however only the presence of the Glu^{381}–Gly^{382} bond, highly conserved in desmogleins, does not guarantee hydrolysis. The prerequisites for the exquisite specificity exhibited by ETs involves not only the presence of this cleavage site, but, also (1) the presence of the highly charged N-terminal alpha-helix, (2) the calcium dependent conformation of its substrate Dsg-1, and (3) existence of a specific sequence 110 residues upstream of the cleavage site of the substrate Dsg-1, characteristics that differentiate them from other typical glutamic-acid-specific serine proteinases of the chymotrypsin family.

(1) The highly charged N-terminal alpha-helix of ETs: The charge profile of N-terminal alpha-helix is significantly different, principally between ETD/ETA and ETB (**Figure 1D**) and its size also varies, containing 15, 11 and 12 residues in ETA, ETB and ETD, respectively.

This N-terminal extension which is unique to ETs and its deletion results in an inactive protein [53, 54] that interacts with residues in loop 2 thereby coordinating and determining the architecture of the S1 pocket and hence contributing to substrate specificity [51–53] by modifying the pocket entrance. The amino acid sequences (**Figure 1D**) and the conformations in loop2 (**Figure 1E**) are different in the ETs. In ETA (**Figure 1E**), this loop is longer than in ETB and ETD, additionally its Trp14 and Tyr18 present in the N-terminal

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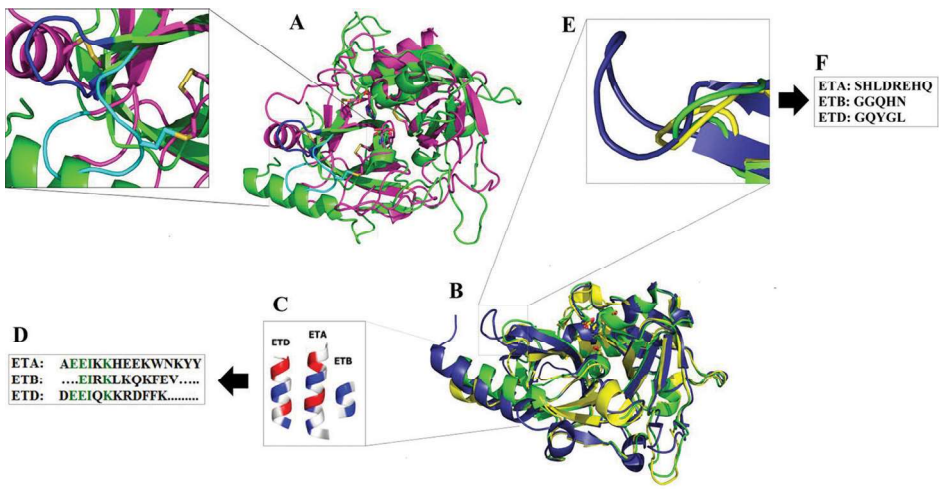

Figure 1. Results of superpositioning of the three-dimensional structures: (A) ETD in green and a serine proteinase [PDB:2AIP] of the trypsin subfamily in pink. The loop 2 is dark blue in ETD, and the equivalent loop of serine proteinase is light blue. (B) ETA (blue), ETB (yellow) and ETD (green); amino acid sequences (C) and charge profile of the N-terminal helices (D) and its variation in the amino acid sequences. (E) The lengths of loop 2 in ETA (blue), ETB (yellow) and ETD (green) and its variation in the amino acid sequences (F).

helix are buried deeper in the S1 pocket than in ETB which contains Lys and Glu and in ETD with Arg and Lys at these equivalent positions. In the other trypsin-like serine proteinases, the presence of a disulfide bridge determines the conformation of the pocket (**Figure 1A**).

(**2**) **Calcium dependent conformation of Dsg-1:** Dsg1 is a member of the cadherin superfamily [55] and most of these proteins require calcium to promote cell–cell adhesion and to ensure the proper conformation [56, 57]. Unlike trypsin, ETs are not capable of cleaving heat-denatured Dsg1 or Ca^{2+} depleted Dsg1 [58] and circular dichroism demonstrates that ET specificity is dependent on calcium-stabilized conformation of Dsg1.

(**3**) **Residues upstream of the Dsg-1 cleavage site are critical for its hydrolysis by ETA**: ETA is able to bind but is unable to cleave canine Dsg1 and a sequence of 5 amino acids 110 residues upstream of the cleavage site are essential for the hydrolysis of Dsg1 by ETA [59]. Four of five of these critical residues are identical in human and mouse Dsg-1(**Figure 2**) and when replaced in canine Dsg-1 at its same position, the cleavage by ETA becomes susceptible. The recognition of this peculiar sequence (Q271-x-x-Y274-T275-I276-E277) is one of the factors that make ETs highly specific in Dsg cleavage, even among homologous Dsg-1s. This also demonstrated that E5 and EC4 do not exert any influence on the cleavage of the substrate and the chimeric human Dsg-3 containing swapped amino acids 214-398 of Dsg-1was cleaved by ETA.

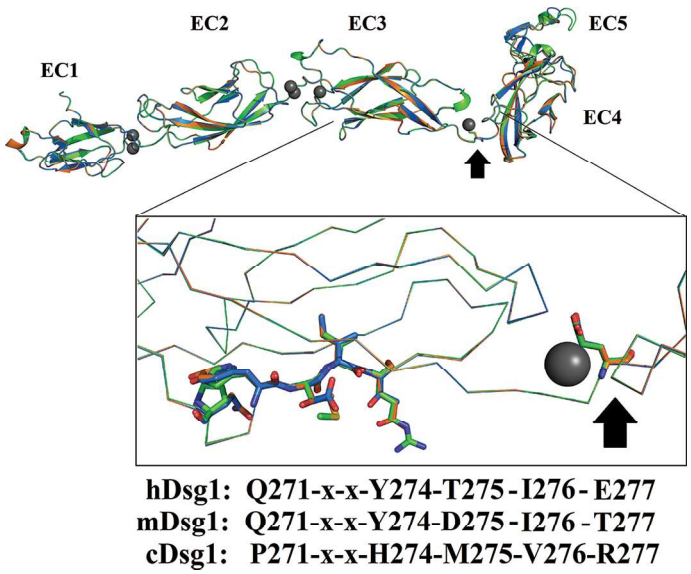

Figure 2. Results of superpositioning of the structural model of human Dsg1(orange), canine Dsg1 (green) and mouse Dsg1 (blue), purple spheres represent bound Ca2+, the susceptible glutamic acid is indicated by an arrow; amino acids sequence of the human, mouse, and canine Dsg1s upstream of the cleavage site; upstream location of the recognition sequence and the susceptible glutamic acid.

5. Tyrosines 157 and 159 are essential for ETB activity

Based on the results of site-directed mutagenesis, Sakurai et al. [60] concluded that the substitution of either Tyr 157 or 159 in ETB decreased exfoliative activity and the double mutation resulted in the complete loss of exfoliative activity and antigenicity. Interestingly, ETA does not possess either one of these tyrosines but contains Phe and His at these positions and in ETD these positions are occupied by Tyr and Thr.

6. Why are the exfoliative toxins inactive in the native states?

Gly193 is highly conserved in serine proteinases, however, in structures of ETs the peptide bond between residues 192 and 193 (chymotrypsin numbering) is flipped 180° relative to the other serine proteases. Pro192 in ETA and ETD and Val192 (ETB) form hydrogen bonds with both the amide nitrogen atoms and the hydroxyl oxygen atoms of the catalytic serine residues interrupting the charge-relay-network. These enzymes can only be functional if this bond is ruptured and the conformation is restored as in other serine proteinases.

7. Molecular mechanisms of the *S. aureus* exfoliative toxin

7.1. *S. aureus* exfoliative toxins selectively and directly solubilize mouse and human desmoglein 1

In 1970, Melish and Glasgow first investigated mechanisms of action of the exfoliative toxin (ET)-producing *S. aureus* in SSSS [11]. When the organisms isolated from SSSS patients were injected into neonatal mice, they cause epidermal blisters resembling those in the naturally occurred human disease. Several years later, two serotypes of ETs, ETA and ETB, which are capable of inducing epidermal blisters, were identified [21]. However, the exact molecular mechanisms of ET-causing epidermal blisters had long been unknown over the three decades.

In 2000, Amagai and colleagues established desmoglein 1 (Dsg1), a desmosomal cadherin-type adhesion molecule and also known as pemphigus foliaceus autoantigen, as the target of *S. aureus* ETs [61]. They advocate this hypothesis based on the fact that histopathology of superficial epidermal blisters created by ET injection in mice resembles to those created by pemphigus foliaceus IgG. They revealed that immunostaining for the extracellular domain of Dsg1, but not that for Dsg3, is abolished in ETA-injected mouse skin. Moreover, an *in vitro* analysis revealed that ETA selectively solubilize the recombinant extracellular segments of human and mouse Dsg1 (α) produced by baculovirus. The same group also identified that *S. aureus* ETB and a newly identified ETD have similar enzymatic activity to solubilize Dsg1 [10, 12].

The site of blister formation by ETs could be explained in the context of tissue distribution of desmosomal cadherins (**Figure 3**) [13, 62].

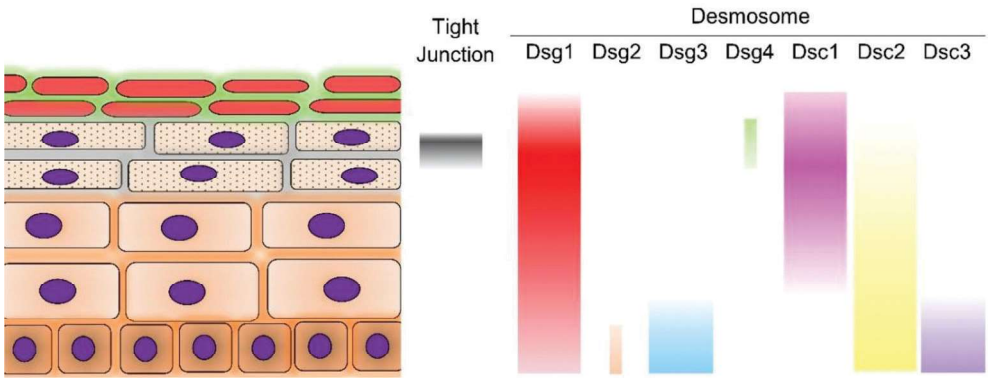

Figure 3. Distribution of functional tight junction and desmosomalcadherins in the epidermis. Dsg, desmoglein; Dsc, desmocollin. Functional tight junctions are located in the upper granular layer. Expression pattern of four isoforms of desmogleins (Dsg) and three isoforms of desmocollins (Dsc) is associated with differentiation level of keratinocytes.

In humans, there are four subclasses of Dsg with different tissue distributions. Among them, Dsg2 is expressed in all desmosome-bearing tissues, whereas Dsg1 and Dsg3 are expressed preferentially in stratified squamous epithelia [63]. Dsg1 and Dsg3 are hypothesized to have compensatory effects [64]. For example, if both Dsg1 and Dsg3 express in the same epithelial cells, and adhesive function by Dsg1 is abolished, the loss of adhesive function can be compensated by intact Dsg3. In the epidermis, Dsg1 is expressed in the whole layers, whereas Dsg3 is expressed in basal and immediate suprabasal layers [65]. In contrast, in oral mucous membrane, both Dsg1 and Dsg3 are expressed in the whole layer, but the expression level of Dsg1 is relatively low compared with that of Dsg3 [63]. As Dsg2 and Dsg4 are expressed weakly in basal and upper granular layers, respectively [65], these molecules may have less ability to compensate the loss of Dsg1 function.

Desmocollin (Dsc) 1, another desmosomal cadherin is also expressed in superficial epidermis. It is hypothesized that Dsg1 and Dsc1 may have combinational effect on integrity of keratinocyte cell adhesion [66]: Abolishment of either Dsg1 by ETs or genetic ablation of Dsc1 causes dissociation of keratinocytes in the superficial layer of mouse epidermis [10, 13, 59, 67]. If adhesive function of Dsg1 is abolished by ETs, it may cause keratinocyte separation only in spinous-to-granular layers of epidermis, in which loss of adhesive function by Dsg1 could not be compensated by other Dsgs. This could be a reasonable explanation why ETs cause only superficial epidermal blisters in SSSS patients, although ETs produced in upper respiratory organs (e.g., tonsils), enter the circulatory system and induce toxemia [27].

7.2. *S. aureus* ETs are unique glutamate-specific serine proteases that hydrolyze a single peptide bond within the extracellular segment of Dsg1

Hanakawa et al. demonstrated that substitution of catalytic serine in ETA, ETB and ETD to alanine causes loss of their functions to solubilize Dsg1 [59]. Kinetic analysis of three ETs revealed kcat/Km values in the range of 2–6 × 104 M^{-1} s^{-1}, suggesting their efficient enzymatic activity to digest relatively large molecules. These findings indicate that three known *S. aureus* ETs are serine proteases that efficiently solubilize the extracellular segment of Dsg1.

The same group also investigated substrate-specificity of *S. aureus* ETs [58]. All three ETs cleave human and mouse Dsg1 (α) into two segments. Moreover, mouse has three isoforms of Dsg1 (Dsg1-α, -β and -γ) [65, 68], and ETA solubilize the extracellular segments of mouse Dsg1-α and -β, but not that of Dsg1-γ in which glutamic acid residue 381 is substituted by lysine [66, 68]. These findings indicate the specificity of the glutamic acid residue as the cleavage site of Dsg1 by ET.

S. aureus ETs are unique serine proteases that specifically and efficiently cleave only one peptide bond in the extracellular segment of Dsg1. The enzymatic properties of *S. aureus* ETs cause efficient and specific abolishment of a major epidermal adhesion molecule in selected mammalian species.

7.3. Possible mechanisms of ET-associated keratinocyte dissociation

Desmosomes composed of two major transmembrane cadherin-type adhesion molecules (Dsg and Dsc) and cytoplasmic plaque proteins that link between desmosomal cadherins and

intracellular cytoskeletons. It has been long debated questions whether disruption of Dsgs alone by pemphigus autoantibodies is sufficient to cause keratinocyte dissociation, or subsequent disorganization of other desmosomal consituents in plasma membrane of keratinocytes is necessary [69].

To determine whether cleavage of the extracellular segment of Dsg1 by *S. aureus* ETs is sufficient to cause keratinocyte dissociation, our group has investigated the fate of desmosomal constituents in ETA-injected mouse skin [66]. We found that the amino-termini of Dsg1 is abolished in plasma membrane of murine epidermal keratinocytes, whereas cleaved carboxyl-termini of Dsg1 and the extracellular segment of Dsc1 remained on the surface of keratinocytes faced to blister cavity in the early phase of keratinocyte dissociation. Based on these findings, we proposed a theory that removal of the amino-termini of Dsg1 by ETs is sufficient to initiate *in vivo* keratinocyte dissociation.

Meanwhile, Simpson et al. proposed another theory for ET-induced keratinocyte dissociation through sequestration of plakoglobin (PG), a member of catenin family cytoplasmic protein, by ectodomain-deleted Dsg1 [70]. When truncated Dsg1, in which amino acids 1–381 were spliced to mimic ET-cleaved carboxy-termini of Dsg1, was expressed in primary human keratinocytes, it reduced mechanical strength of keratinocyte sheets in a dose-dependent manner, implicating a dominant-negative effect by truncated Dsg1. Truncated Dsg1 localized in close to intercellular borders and reduce endogenous desmosomal cadherin Dsc3 and desmosomal plaque protein desmoplakin in intercellular borders. In the same cells, PG localized in intercellular borders and seem to be associated with truncated Dsg1. Remarkably, triple-point mutation of the PG-binding region in the truncated Dsg1 restored mechanical integrity of keratinocyte sheets, implicating that PG binding to truncated Dsg1 is essential in disruption of desmosomes and subsequent keratinocyte dissociation.

Putting all these findings together, the authors advocate a theory that cleavage of Dsg1 by ETs initiate keratinocyte dissociation, while subsequent PG sequestration may contribute to the expansion of intercellular spaces between keratinocytes (**Figure 4**). Further accumulation of *in vivo* evidences to elucidate the role of PG in ET-inducing keratinocyte dissociation will be expected.

7.4. How ET-producing *S. aureus* penetrate the epidermis through firm keratinocyte adhesion in the upper stratum granulosum?

The aforementioned theory can satisfactorily explain how ETs cause blistering in SSSS, in which ETs access to the skin from dermal side. However, this theory cannot explain the mechanisms of blistering in bullous impetigo, in which ET-producing *S. aureus* enter the epidermis from the skin surface. It has been reported that ETs do not penetrate tight junction, an occlusive adhesive structure located at the upper granular layer (**Figure 5**) [71]. Then the question arises how ET-producing *S. aureus* invade the epidermis apart from a route of microwounds on the skin.

To address this issue, we recently established a mouse model of bullous impetigo [72]. *S. aureus* harboring *etb* gene was inoculated epicutaneously to murine inner pinnae after the stratum corneum was partially removed by tape stripping. Intraepidermal neutrophilic pustules containing intercellular staphylococci were successfully created in the mouse skin by 6

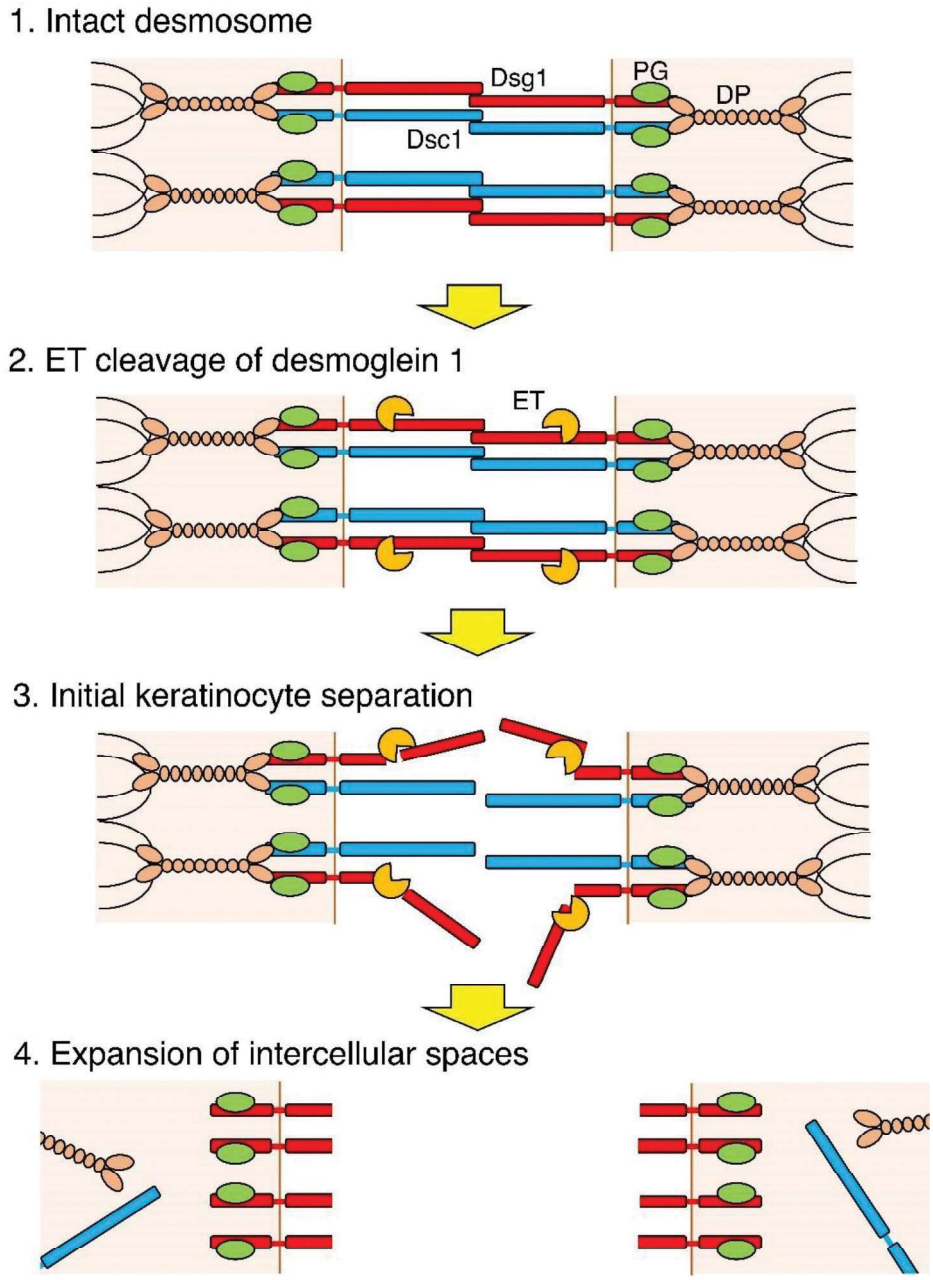

Figure 4. Possible mechanisms of ET-induced keratinocyte dissociation. PG, plakoglobin. Cleavage of the extracellular segment of Dsg1 initiates keratinocyte separation. Sequestration of PG by truncated Dsg1 and disintegrity of other desmosomal components are associated with expansion of the intercellular spaces between keratinocytes.

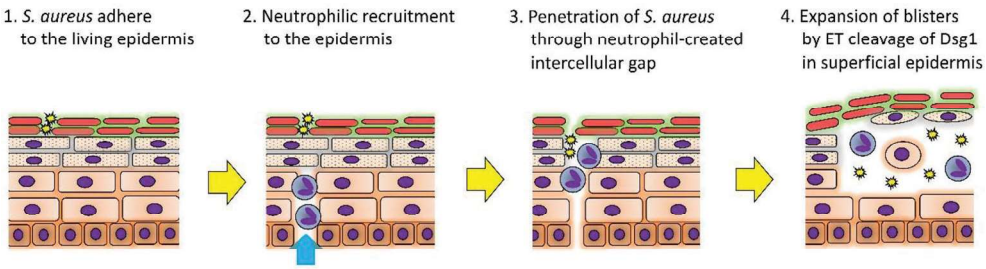

Figure 5. Proposed theory for bacterial cutaneous invasion and blistering in bullous impetigo. *S. aureus* harboring at gene adhere to the living epidermis recruits neutrophils. When neutrophils create intercellular gap between superficial keratinocytes, *S. aureus* produce ET to expand the blisters by cleavage of Dsg1.

hours after the inoculation. The size of intraepidermal pustules created by *etb* gene-harboring strains was significantly larger than those created by et gene-negative strains. Chronological study revealed that staphylococci invaded the epidermis after neutrophils infiltrated the skin. Moreover, if the neutrophilic infiltration was blocked by injection of cyclophosphamide, staphylococci in the epidermis were not recognized at all.

Based on these findings, we propose a novel hypothesis for percutaneous entry of ET-producing *S. aureus* in bullous impetigo. These strains may invade the epidermis through intercellular spaces created by skin-infiltrated neutrophils. In addition, once *S. aureus* invade the epidermis, ETs expand the interkeratinocyte spaces, which allows bacteria to skew neutrophils attack in blister cavity. Future studies to elucidate the molecular interactions that underlie neutrophilic epidermal infiltration in response to *S. aureus* adhere to living keratinocytes. In addition, the mechanisms how ET-producing penetrate the stratum corneum remains to be elucidated.

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Annex 5

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Crystal structure of *Staphylococcus aureus* exfoliative toxin D-like protein: Structural basis for the high specificity of exfoliative toxins

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ABSTRACT

Exfoliative toxins are serine proteases secreted by *Staphylococcus aureus* that are associated with toxin-mediated staphylococcal syndromes. To date, four different serotypes of exfoliative toxins have been identified and 3 of them (ETA, ETB, and ETD) are linked to human infection. Among these toxins, only the ETD structure remained unknown, limiting our understanding of the structural determinants for the functional differentiation between these toxins. We recently identified an ETD-like protein associated to *S. aureus* strains involved in mild mastitis in sheep. The crystal structure of this ETD-like protein was determined at 1.95 Å resolution and the structural analysis provide insights into the oligomerization, stability and specificity and enabled a comprehensive structural comparison with ETA and ETB. Despite the highly conserved molecular architecture, significant differences in the composition of the loops and in both the N- and C-terminal α-helices seem to define ETD-like specificity. Molecular dynamics simulations indicate that these regions defining ET specificity present different degrees of flexibility and may undergo conformational changes upon substrate recognition and binding. DLS and AUC experiments indicated that the ETD-like is monomeric in solution whereas it is present as a dimer in the asymmetric unit indicating that oligomerization is not related to functional differentiation among these toxins. Differential scanning calorimetry and circular dichroism assays demonstrated an endothermic transition centered at 52 °C, and an exothermic aggregation in temperatures up to 64 °C. All these together provide insights about the mode of action of a toxin often secreted in syndromes that are not associated with either ETA or ETB.

1. Introduction

Staphylococcus aureus, the Gram-positive bacterial pathogen, triggers a wide spectrum of infection and is the primary causative agent of pyogenic infections which can result in septicemia, osteomyelitis and meningitis, is encountered in humans and approximately 35% of the population are carriers. It is also found in warm-blooded animals [1] and is a major causative agent of mastitis in ruminants, causing thus huge economic losses in the milk production [2].

S. aureus secretes different exfoliative toxins (ETs) that result in toxin-mediated staphylococcal syndromes. These disorders range from localized bullous impetigo to staphylococcal scalded skin syndrome (SSSS) in which superficial skin blistering and exfoliation follow widespread painful erythema [3]. Thus far, four different serotypes of exfoliative toxins ETA, ETB, ETC, and ETD have been identified [4] and three of them (ETA, ETB, and ETD) are related to human infection [5]. Some ET and ET-like proteins are also found associated to skin infections in animal hosts and show cleavage specificity against human or animal desmogleins [6].

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SSSS and bullous impetigo, the major human exfoliative dermatitis caused by ETs [7], primarily affect newborns with exfoliation of 50% or more of the skin [8], are a result of the action of ETA and ETB [9]. On the other hand, ETD seems to be associated with the formation of cutaneous abscesses and furuncles [10], characterized by extensive tissue damage, which might be a result of the localized action of ETs [11].

ETA and ETB are atypical glutamic acid-specific trypsin-like serine proteinases and their accumulation in the skin causes disruption of desmosomes via proteolytic cleavage of desmoglein I [7,12]. ETD mediates intra-epidermal cleavage through the granular layer of the epidermis of neonatal mice and induces epidermal blisters in newborn mice [5]. The mechanisms underlying substrate recognition by these proteases suggest that ETs recognize their substrates *via* both the classic P1 site interactions and significant secondary interactions involving the tertiary structural features of desmoglein [11].

We recently identified an ET-like protein in *S. aureus* O46, a strain associated to mild ewe mastitis [13,14]. It showed high similarity with previously described *S. aureus* ETD in its amino acid primary sequence, including the presence of the typical catalytic site found in the other ET proteins described so far, and was thus named EDT-like. The exact role of this ETD-like variant in *S. aureus* colonization of the udder tissues or in the infection process in ruminant mastitis remains unknown.

The crystal structures of ETA and ETB [8,15–17] along with the structure of ETD-like presented here provide the structural basis for understanding the exquisite substrate specificity of these enzymes and their ability to only cleave a single bond in desmoglein 1 but not in other homologous desmogleins.

2. Materials and methods

2.1. Protein expression and purification

The gene corresponding to ETD-like was amplified from *S. aureus* O46 genomic DNA and cloned into a pD441 expression vector for further protein production and purification.

An isolated colony of *E. coli C43* (DE3) *pLysS* transformed with pD441/ETD-like was grown for 16 h at 37 °C in LB medium supplemented with kanamycin (34 μg/mL). The culture grown overnight was diluted 100-fold with fresh LB broth containing kanamycin (34 μg/mL) and incubated at 30 °C until the optical density (OD_{600}) reached 0.5 and was subsequently induced with 0.2 mM IPTG for 16 h at 20 °C. The cells were collected by centrifugation at 2600 g for 10 min at 4 °C and suspended in a 20 mM Tris–HCl buffer pH 8.0 containing 500 mM NaCl, lysed by sonication and centrifuged at 15,000 g for 15 min. The supernatant was subjected to affinity chromatography using an immobilized nickel column (GE) under native conditions and further purified using a Superdex G75 10/300 GL column and the results were analyzed by SDS-PAGE.

2.2. Crystallization, data collection, processing and structure determination

Crystals were obtained by vapor diffusion when a protein concentration of 20 mg mL^{-1} in 100 mM HEPES Sodium pH 7.5 was equilibrated against a reservoir that additionally contained 30% 2-Propanol. Diffraction data were collected from a single flash frozen crystal in a 100 K gaseous nitrogen stream at the W01B-MX2 beamline at Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil). The wavelength of the radiation source was set to 1.458 Å and a Pilatus 2M detector was used to record the diffraction intensities. The crystal was exposed for 2 s per 0.1 degree of rotation and a total of 1800 images collected. The data were indexed, integrated and scaled using the DENZO and SCALEPACK programs from the HKL-2000 package [18].

The structure was solved by molecular replacement using the atomic coordinates of ETB (PDB ID: 1DT2, 62% sequence identity) as a template and the program PHASER [19]. Model refinement was carried out using cycles of REFMAC5 [20] or phenix.refine [21] followed by visual inspection of the electron density maps and manual rebuilding with COOT [22]. Refinement cycles included secondary structure, reference-model restraints and translation/libration/screw parameters. The model quality was assessed using MolProbity [23]. Data collection and refinement statistics are presented in Table 1. The ETD-like atomic coordinates have been deposited with the RCSB Protein Data Bank under the accession code 5C2Z.

Table 1
Hydrodynamic and structural properties of ETD-like.

	DLS	AUC[a]
MW (kDa)	–	27.2
f/f_0	–	1.43
s (S)	–	2.2
Rs (nm)	2.8	–
Polydispersity (%)	30	–

[a] Calculated from sedimentation velocity data using SedFit software.

2.3. Hydrodynamic experiments

Sedimentation velocity experiments were carried out in an Optima XL-A analytical ultracentrifuge (Beckman) with the AN-60 Ti rotor set at 30,000 rpm at 15 °C. The ETD-like sedimentation data was monitored by absorbance at 230 nm and the experiments were performed with protein concentrations of 0.4 and 0.8 mg/mL prepared in 20 mM Hepes, 150 mM NaCl, pH 7.5. The SedFit software was used to process the AUC data [24] and the frictional ratio (f/f_0) was applied as a regularization parameter, which was allowed to drift freely. Buffer density (1.0039 g/mL) and viscosity (0.0102643 Poise), and the partial specific volume of the ETD-like (0.7346 mL/g) were estimated by the Sednterp program (http://www.jphilo.mailway.com/download.htm).

DLS measurements were performed using a ZETASIZER Nano series (Malvern Instruments) and the data acquisition was accomplished after the average of 14 runs at a constant temperature of 25 °C and protein concentration ranging from 1 mg/mL to 8 mg/mL in a 20 mM Hepes buffer pH 7.5 and 150 mM NaCl. The Zetasizer software was used to obtain the hydrodynamic radius (Rh) of ETD-like from the extrapolation of the translational diffusion coefficient (Dt) according to the Stokes–Einstein equation.

2.4. Differential scanning calorimetry (DSC)

DSC experiments were performed using N-DSC III (TA Instruments, USA) in the temperature ranges of 20–64 and 20–90 °C with a heating and cooling scan rate of 1 °C/min. The protein was diluted in a phosphate buffer (10 mM NaH_2PO_4, 100 mM NaF, pH 7.4) to a final concentration of 0.64 mg/mL. Both the calorimeter cells were loaded with the buffer solution, equilibrated at 20 °C for 10 min and scanned repeatedly as described above until the baseline was stable and reproducible. The sample cell was subsequently loaded with ETD-like and scanned in the same way. The baseline correction was conducted by subtracting the 'buffer vs. buffer' scan from the corresponding 'protein vs. buffer' scan and all measurements were repeated twice.

2.5. Circular dichroism spectroscopy

Circular dichroism spectroscopy was performed on a Jasco J-815 spectropolarimeter (Jasco, USA) with a Peltier-type temperature control system. The far UV–CD spectrum of ETD-like was collected from 260 to 190 nm at 20 °C in a 0.1 cm quartz cuvette. A scan speed of 50 nm/min was used with a response time of 1.0 s, spectral bandwidth of 1.0 nm and spectral resolution of 0.1 nm. The signal was averaged over 10 scans. Each spectrum was acquired independently twice. The protein was diluted to 7.35 μM in a phosphate buffer (5 mM NaH_2PO_4, 50 mM NaF, pH 7.4). In the thermal unfolding experiment, the protein sample was heated from 20 to 64 °C at a rate of 1.0 °C/min and ellipticity measurements were performed around the minimum of the ETD-like spectra (at 205 and 208 nm) every 2.0 °C. The contribution of the buffer was subtracted from the protein spectra. Percentages of secondary structure of ETD-like in solution were calculated with the CONTINLL software of the CDPro package, using the reference set of proteins SMP50 [25].

2.6. Molecular dynamics

Crystal structures from ETA and ETB were retrieved from the PDB (IDs: 1DUA and 1DT2, respectively). In order to compare the dynamical behavior of the three exfoliative proteins, 40 ns (4 × 10 ns) molecular dynamics simulations were performed using GROMACS 4.5.5 [26,27]. In each run, the protein was centered in a cubic box with edges of 1.0 nm away from any protein atom. The SPC/E water model was used and 0.1 M salt ions were added to the system in order to make it neutral. Energy minimizations were carried out with steepest descent integrator and conjugate gradient algorithm, using 1000 kJ mol^{-1} nm^{-1} as maximum force criterion. The particle mesh Ewald (PME) method for long-range electrostatic interaction [28] was used. In all simulations, a velocity rescaling thermostat [29] with a time constant of 0.1 ps was used to set the temperature to 300 K. The pressure of 1 atm was achieved by a Berendsen thermostat [30] with a time constant of 2 ps. Parallel linear constraint solver (LINCS) [31] was used to constrain all bonds. The systems were subject to 100 ps of NVT and NPT equilibration, using position constraints. Molecular dynamics simulations were carried out by 4 independent 10 ns runs with no position constraint, whatsoever. Following dynamics, the trajectories were concatenated and analyzed by different parameters, such as hydrogen bond pattern, potential energy profile, solvent area accessibility, residue mean fluctuation. Also, principal component analysis (PCA) using the *g_covar* and *g_anaeig* functions in GROMACS. The 10 first principal components (lowest frequencies) were used for comparison among the ETA, ETB and ETD-like.

3. Results and discussion

3.1. Thermodynamics and in solution behavior of ETD-like protein

The thermogram of purified ETD-like presented in Fig. 1A represents a typical protein unfolding process in the temperature range of 30–64 °C with an endothermic transition centered at 52 °C, and an exothermic aggregation in temperatures up to 64 °C. Inspection of the sample after completion of the heating and cooling scans indicated trace amounts of precipitated protein. It is likely that the observed exothermic behavior up to 64 °C is a result of protein aggregation. To confirm the thermal reversibility of ETD-like, a sample was heated to 64 °C and then cooled at the same scan rate (1 °C/min). The cooling scan of this sample indicated no transitions in the thermogram. These results indicate that the thermal unfolding of ETD-like is an irreversible process.

The CD spectrum of ETD-like presents structural characteristics of a structured protein since its minimum is located at 205 nm and the positive ellipticity is at 190 nm (Fig. 1B). The percentages of secondary structure were calculated using the CONTINLL program [25] and are in agreement with the crystallographic structure. Fig. 1C presents the temperature dependence of the average value of the ellipticity between 205 and 208 nm for the thermal unfolding of ETD-like. The melting temperature obtained by the CD measurements was 55 °C which is similar to the temperature determined by DSC, indicating significant correlation between the techniques.

Thermal denaturation studies of ETA and ETB using fluorescence spectroscopy indicated that these proteins present melting temperatures in the 57–59 and 52–54 °C ranges [32], which is very similar to the melting temperature of ETD-like (52–55 °C) determined in this work. The thermal unfolding process of ETD is similar to that of ETB, not only because of the greater similarity between the melting temperatures, but also due to the aggregation behavior that both present [33]. This may be due to the high sequence similarity of 54% between ETD-like and ETB. Secondary structure studies performed by CD spectroscopy also demonstrated that ETA and ETB present a higher percentage of β-sheet in solution [33], which is in agreement with the results obtained for ETD-like.

Hydrodynamic behavior of ETD-like was investigated by DLS (Table 1) and AUC (Table 1) experiments. The results indicate that the protein is monomeric in solution, since DLS estimates the protein hydrodynamic radius as 2.8 nm and AUC estimates the molecular mass as 27 kDa (Table 1). No evidence for oligomeric behavior of ETs is available, although some ET structures contain two molecules in the crystallographic asymmetric unit.

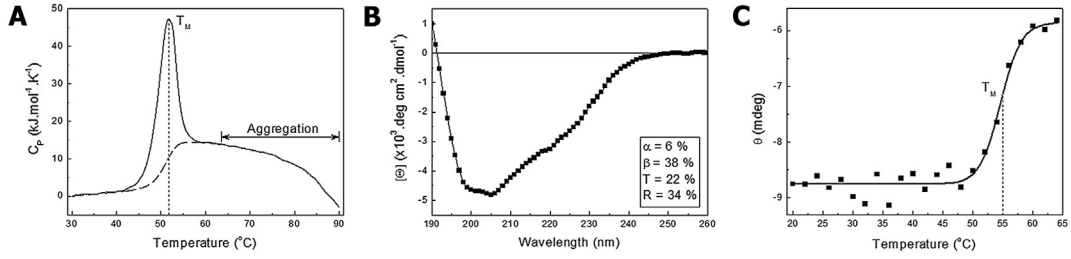

Fig. 1. Biophysical characterization of ETD-like. **(A)** DSC thermogram of ETD-like. Apparent excess heat capacity curve was recorded for ETD-like (0.64 mg/mL) in phosphate buffer (10 mM NaH_2PO_4, 100 mM NaF, pH 7.4) at a scan rate of 1 °C/min. The dotted line (- - -) indicates the melting temperature (T_M) and the dashed line (— —) represents the baseline used to correct the protein thermogram, excluding the temperature range in which the exothermic aggregation occurs. **(B)** Far UV–CD spectrum of ETD-like (7.35 μM) in phosphate buffer (5 mM NaH_2PO_4, 50 NaF, pH 7.4) at 20 °C. The letters α, β, T and R correspond to the percentage of α-helice, β-sheet, turn and random coil, respectively, calculated by the CONTINLL program. **(C)** Temperature dependence of average value of the ellipticity between 205 and 208 nm for the thermal unfolding of ETD-like obtained with scan rate of 1 °C/min. The solid line represents a sigmoidal fit and the dotted line indicates the melting temperature (T_M).

Table 2
Data collection and refinement statistics.

	ETD-like
Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	49.41, 93.14, 50.48
β (°)	91.2
Molecules per AU[a]	2
Resolution range (Å)	50.47–1.95(2.0–1.95)
R_{meas} (%)	80.4(99.2)
$I/\sigma I$	9.43 (2.15)
CC(1/2)*	0.98 (0.62)
Completeness (%)	96.0 (86.0)
Multiplicity	6.9 (5.1)
Refinement	
Resolution (Å)	35.68–1.95 (2.0–1.95)
No. reflections	31,781
R_{work}/R_{free}	0.17/0.25
No. atoms	
Protein	4215
Mean *B*-factors (Å^2)	
Protein	25.79
R.m.s. deviations	
Bond lengths (Å)	0.007
Bond angles (°)	1.071
Ramachandran Plot	
Favored (%)	94.7
Allowed (%)	5.07
Disallowed (%)	0.2

Values in parentheses are for the highest-resolution shell.
*Correlation coefficient.
[a] AU, asymmetric unit.

3.2. The crystallographic structure of ETD-like

Four structures of ETA (PDB IDs: 1EXF, 1DUA, 1DUE and 1AGJ) and two structures of ETB (PDB IDs: 1DT2 and 1QTF) have been determined and with the structural data of ETD-like we can now understand their selectivity and modes of action.

The structure of ETD-like was determined and refined at 1.95 Å resolution and resulted in a crystallographic residual of 17.7% (R_{free} 25.3%) (Table 2). The high structural similarity between exfoliative toxins ETA, ETB and ETD-like protein, which share about 50% sequence identity, is evidenced by the low RMSD values of the superposed structures (ETA–ETB: 1.27 Å, ETA–ETD-like: 1.43 Å and ETB–ETD-like: 1.00 Å). The following structural characteristics are shared between the ETs from *S. aureus*: (i) protein fold is characterized by two six-strand β-barrels whose axes lie roughly perpendicular to each other as in other trypsin-like serine proteases, (ii) the Greek key motif consists of four adjacent antiparallel strands and their linking loops, (iii) the N- and C-terminal α-helical extensions and (iv) the active site is located at the interface of the two barrels that includes an aspartic acid, a histidine, and the catalytic serine residue (Fig. 2A).

The distinct size (ETD-like, ETA, ETB are composed of 249, 242 and 246 residues, respectively) and pI values ($pI_{ETD\text{-}like}$ = 7.8 [34], pI_{ETA} = 7.0 and pI_{ETB} = 6.95 [35]) indicate that despite the high structural conservation, structural differences associated with each ET may account for the observed functional differences. Detailed sequence and structural analyses permitted the identification of regions specific for each ET (Figs. 2 and 3, and Table 3) and are described below.

3.3. Sequence and conformation of loops related to substrate binding and specificity

Loops A, B, C and D are considered the determinants for subsite preferences in these proteins [17,35,36]. Distinct conformations of loops B and D are observed between ETA and ETD-like (Fig. 2B). In ETB, structural differences between loops A and B are observed when this structure is superposed with ETD-like (Fig. 2C). Sequence

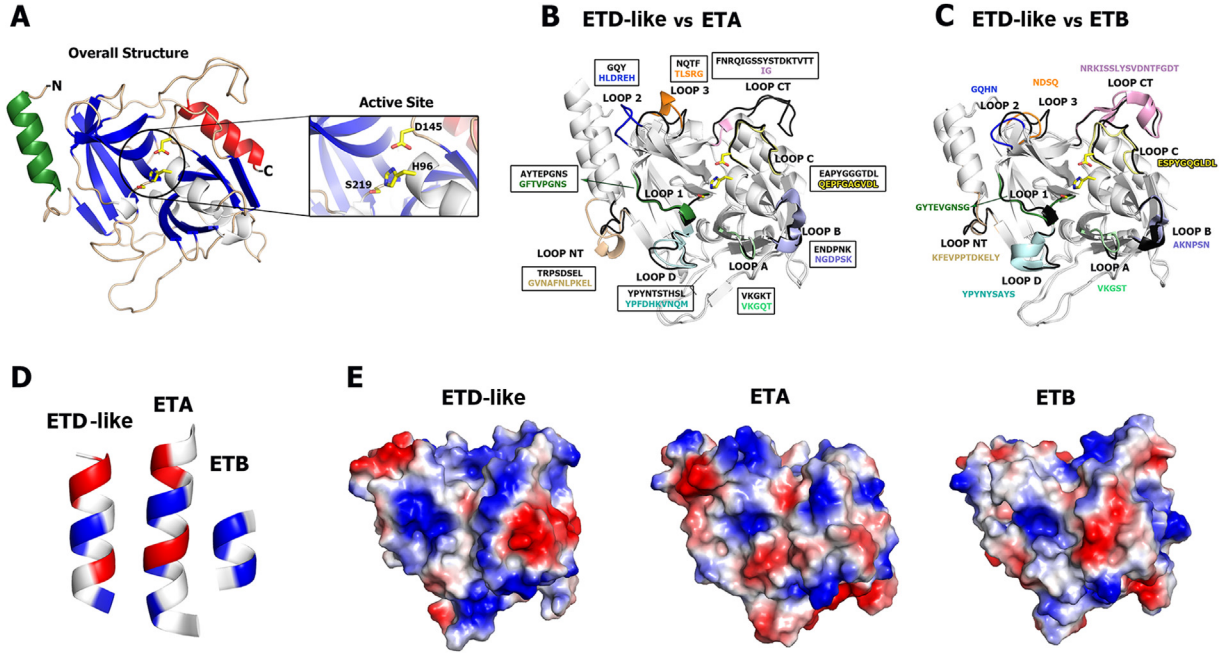

Fig. 2. Structural analysis of ETD-like. **(A)** Overall ETD-like structure. The enzyme is folded into two six-strand β-barrels (blue) whose axes that are roughly perpendicular as other trypsin-like serine proteases. The N- and C-terminal α-helices are colored in green and red, respectively. The active site of the molecule is amplified to identity the three catalytic residues. **(B and C)** Analysis of loops involved in the selectivity between ETD-like (this work), ETA (PDB ID: 1EXF) and ETB (PDB ID: 1DT2). The loops in ETD-like are colored in black and, in ETA and ETB, they are colored according to each loop. **(D)** Positive (blue) and negative (red) residues comprising the N-terminal α-helix of ETD-like, ETA and ETA. (D) Surface charge distribution around the catalytic interface of ETD-like, ETA and ETB. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

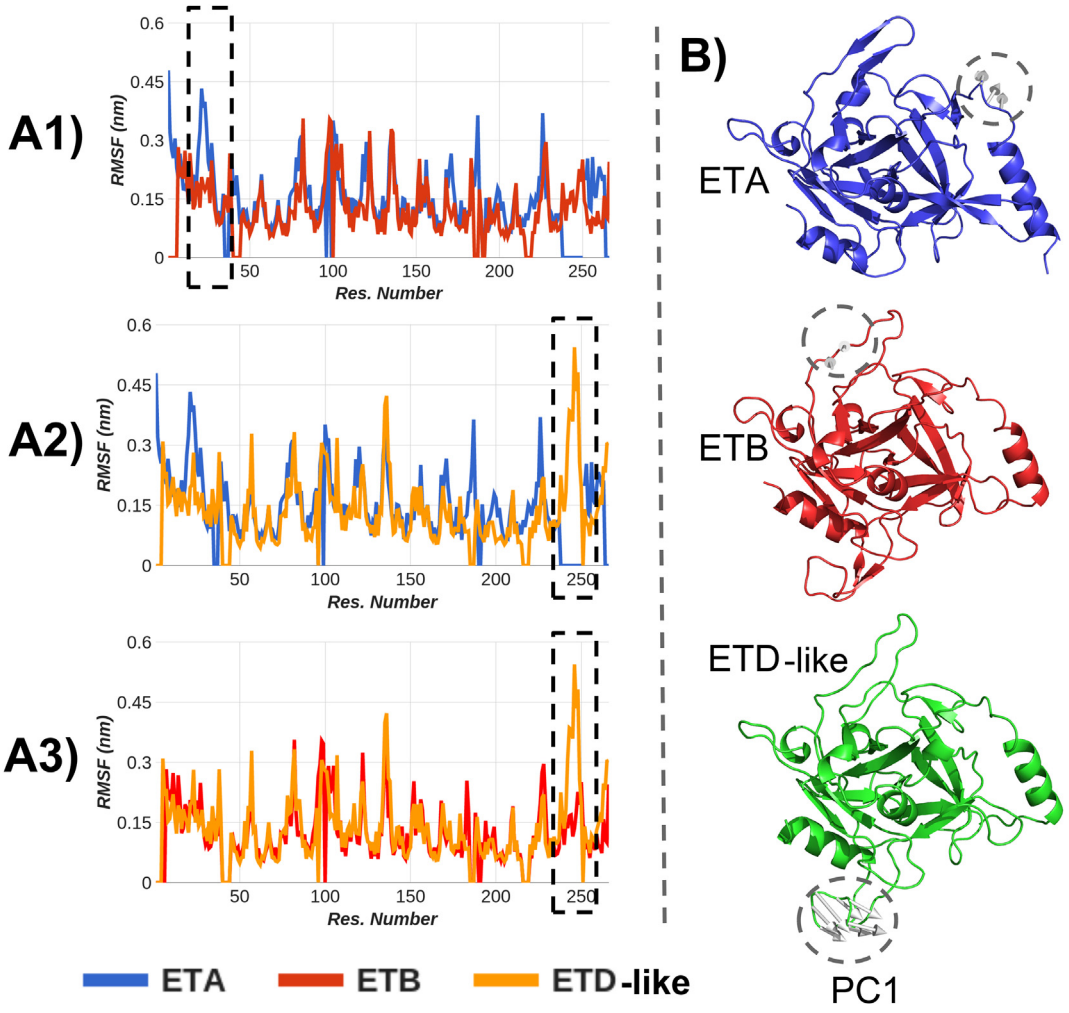

Fig. 3. Molecular dynamics simulations and structural comparisons between ETD-like, ETA and ETB. (A) Comparison of Root Mean Square Fluctuation for each amino acid residue. ETA's N-terminal is more flexible as compared to ETB and ETD-like. ETD-like C-Terminal region, absent from ETA, is more flexible as compared to ETB. (B) Projection of the molecular dynamics trajectories onto the first principal component highlighting regions found to be more flexible among ETA, ETB and ETD-like.

Table 3
Primary sequence analysis of ETs.

	ETD-like amino-acid residues
ETD-like amino-acid residues conserved with ETA	56, 57, 58, 91, 97, 104, 107, 108, 115, 119, 128, 155, 216, 231
ETD-like amino-acid residues conserved with ETB	37, 38, 39, 41, 46, 50, 52, 59, 64, 65, 69, 70, 72, 99, 109, 111, 116, 130, 132, 133, 137, 140, 148, 150, 154, 160, 163, 170, 171, 176, 177, 182, 188, 189, 191, 194, 195, 196, 199, 203, 204, 209, 211, 215, 226, 236, 238, 239, 240, 243, 244, 246, 249, 250, 251, 253, 255, 257, 258, 260, 265, 266, 269, 270, 271, 274, 275.
Amino-acid residues exclusive for ETD-like	40, 42–45, 47–49, 50, 53, 60, 62,63,68,78, 80, 84, 91, 95, 100, 102, 103, 106, 113, 114, 118, 120–125, 127, 131, 136, 138, 142, 144, 147, 156, 157, 164, 165, 168, 172, 173, 175, 181, 190, 192, 193, 197, 202, 205, 206, 207, 208, 212, 227, 241, 242, 245, 247, 248, 252, 254, 256, 259, 261, 263, 264, 267, 268, 276, 277, 278, 279.

comparisons indicate that the loop B is the most variable, whereas loops A, C and D present a fair degree of conservation (Fig. 2).

Loops 1, 2, and 3 are important in determining the specificity of the S1 subsite [17,36,37], and loop 2 is considered to be important for the proper positioning of the substrate in the active site of serine proteases, as residues along the loop form hydrogen bonds with the substrate [37]. Loop 1 is fully conserved in ETA, ETB and ETD-like (Fig. 2B,C). Conformational changes are observed in loops 2 and 3 of ETD-like when compared with the corresponding regions in ETA (Fig. 2B). The sequences in these loops are not conserved between the ETs (Fig. 2B,C). When the ETD-like and ETB structures are compared, sequence and structural differences are principally observed in loop 3 and thus likely play a role in determining P1 specificity (Fig. 2C).

3.4. Loops connected to the N- and C-terminal α-helices are characteristic for each ET

Amino acids in the loops A–D and 1–3 participate in the interactions with the substrate; however, variations are also observed

in other loops of the ETD-like structure, which do not have defined roles in recognition or specificity. These differences reside in the loop connecting the N-terminal α-helix and the β1strand of ETD-like in relation to ETA (referred to as loop NT) and in the loop in the proximal region of the C-terminal α-helix (referred to as loop CT), which is also quite different in these two toxins (Fig. 2B,C). These regions differ both in composition and conformation and are considered the most flexible zones in these toxins, which might be associated with conformational changes upon substrate binding. In comparison to ETA, ETD-like has a very long loop CT inducing a different orientation of the C-terminal helix (Fig. 2B). In relation to ETB, these two loops are relatively similar in length and composition, indicating that these regions seem to be involved in the functional differentiation between ETD-like and ETA, and not to ETB. Molecular dynamics simulations indicate that the ETA loop NT is found to be more flexible than in ETB and ETD-like. This region is more conserved between ETB and ETD-like but not in ETA. This region in ETA is an extension of the N-terminal α-helix (α1), and in both ETB and ETD-like this helix is considerable shorter and the loop connecting the C-terminal helix, is more flexible in ETD-like than in ETB (Fig. 3).

3.5. N-terminal α-helix of ETs presents differential charge profile and forms distinct interactions

Structural differences are not restricted to the interfacial loops in the ETs structures; the N-terminal α-helix is highly charged in ETA, ETB and ETD-like, but the charge profile is significantly different, principally between ETD-like/ETA and ETB (Fig. 2D). The electrostatic potential at these helices indicates that the interactions formed by the residues from the N-terminal region are also different. The first residues of ETD-like (Met^{1}–Glu^{3}, ETD-like numbering) are in contact with residues of loop 3, which differ from those of ETA whose N-terminal residues are in contact with loop 2. In ETB, Tyr^{3} (ETB numbering) forms part of a buried hydrophobic interface with residues Phe^{172}, Phe^{178} and Leu^{210} (ETB numbering) of the C-terminal barrel domain as well as Ile^{8}, Leu^{11}, and Phe^{15} (ETB numbering) of the N-terminal helix. Although the triad position of Phe^{172}, Phe^{178} and Leu^{210} (ETB numbering) is preserved in ETD-like, interactions formed by these residues are not the same. In ETD-like, the corresponding residues for Ile^{8} and Leu^{11} (ETB numbering) are Asp^{36} and Ile^{39} (ETD-like numbering).

3.6. Dynamics of ETA, ETB and ETD-like

To further compare the dynamics of the three toxins, projection of the 40 ns trajectories onto the two first principal components reveal that the intrinsic dynamics of ETA, ETB and ETD-like differ as presented in Fig. 3. The ETA dynamics indicate that the N-terminal helix and the loop comprising the segment K^{75}–K^{83} (ETA numbering) are the most mobile regions (Fig. 3). On the other hand, ETB is highly flexible in the loop consisting of Q^{82}–T^{93} (ETB numbering). In this loop, ETA has a β-strand that is not present in either ETB or ETD-like and displays low sequence identity with ETA. The ETD-like C-terminal loop is more flexible when compared to ETB. This is mainly due to G^{254} in the position of S^{220} in ETB. Also, the K^{261} in ETD-like replaces D^{226} from ETB. This negative to positive charge inversion coupled to the higher mobility of this loop in ETD-like might influence substrate recognition.

These structural deviations may influence the protein-substrate interactions. ETA, ETB and ETD are able to hydrolyze Dsg-1 both *in vitro* and *in vivo* [7,12,38,39] with hydrolysis being highly dependent on the conformation of Dsg-1 [40] and calcium ions [40–42] with cleavage occurring at exactly the same site [12]. Although, the substrate is common to all ETs, human-infecting strains of *S. aureus* produce mainly ETA and ETB, and ETD is less frequently encountered than the other two toxins [5,43]. ETD-producing strains are mainly isolated from furuncles or cutaneous abscesses and not from the same tissues as the two other toxins [5,10]. The differences identified at the structural level here might also somehow reflect an adaptation to ruminant hosts.

In conclusion, the elucidation of the crystal structure of this ETD-like protein enables detailed structural comparisons of ETD-like with ETA and ETB and is important for the identification of specific features associated with the ETs from *S. aureus* of various host origins.

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Annex 6

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RESEARCH

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Putative virulence factors of *Corynebacterium pseudotuberculosis* FRC41: vaccine potential and protein expression

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Abstract

Background: *Corynebacterium pseudotuberculosis*, a facultative intracellular bacterial pathogen, is the etiological agent of caseous lymphadenitis (CLA), an infectious disease that affects sheep and goats and it is responsible for significant economic losses. The disease is characterized mainly by bacteria-induced caseous necrosis in lymphatic glands. New vaccines are needed for reliable control and management of CLA. Thus, the putative virulence factors SpaC, SodC, NanH, and PknG from *C. pseudotuberculosis* FRC41 may represent new target proteins for vaccine development and pathogenicity studies.

Results: SpaC, PknG and NanH presented better vaccine potential than SodC after in silico analyses. A total of 136 B and T cell epitopes were predicted from the four putative virulence factors. A cluster analysis was performed to evaluate the redundancy degree among the sequences of the predicted epitopes; 57 clusters were formed, most of them (34) were single clusters. Two clusters from PknG and one from SpaC grouped epitopes for B and T-cell (MHC I and II). These epitopes can thus potentially stimulate a complete immune response (humoral and cellular) against *C. pseudotuberculosis*. Several other clusters, including two from NanH, grouped B-cell epitopes with either MHC I or II epitopes. The four target proteins were expressed in *Escherichia coli*. A purification protocol was developed for PknG expression.

Conclusions: In silico analyses show that the putative virulence factors SpaC, PknG and NanH present good potential for CLA vaccine development. Target proteins were successfully expressed in *E. coli*. A protocol for PknG purification is described.

Keywords: *Corynebacterium pseudotuberculosis*, Pathogenicity and virulence, Vaccine potential, Epitope prediction, Protein expression, Protein purification

Background

Caseous lymphadenitis (CLA) is a chronic, pyogenic, contagious disease of sheep and goat that imposes considerable economic losses for farmers in many countries [1, 2]. The disease is caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*): a gram-positive pleomorphic, non-capsulated, non-motile, fimbriated, facultative intracellular bacterium, multiplying within macrophages [1]. *Corynebacterium ulcerans and C. pseudotubercu1osis* produce phospholipase D (PLD), which is unique among corynebacteria. It promotes the hydrolysis of ester bonds in sphingomyelin in mammalian cell membranes, possibly contributing to the spread of the bacteria from the initial site of infection to the secondary sites within the host. Moreover, it provokes dermonecrotic lesions; and at higher doses it is lethal to a number of different species of laboratory and domestic animals [3–5].

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CLA disease is expressed in external and visceral forms, either separately or together [3–5]. External CLA lesions appear initially as abscesses that convert later on to pyogranulomas ranging in size from millimeters to centimeters. These external lesions are mostly located within superficial lymph nodes, but infrequently in subcutaneous tissues. Wool or hair over CLA lesions may be lost due to the weak dermonecrotic action of *C. pseudotuberculosis* exotoxins and the pressure atrophy of overlying skin by the lesions. Visceral lesions are not detectable clinically but express themselves according to their number, site and effect on the involved organ. Progressive weight loss, respiratory disorders and chronic recurrent ruminal tympany are the most prominent signs that may accompany visceral CLA lesions.

Identification/removal of infected animals is a key factor for success of disease control measures. Vaccination of healthy animals is another strategy broadly recommended for disease control. In fact, control of CLA depends on vaccination in most countries [2, 5–7]. Although bacterin, toxoid, combined, and live vaccines are available, the disease has persisted even after prolonged vaccination, indicating the suppressive nature of CLA vaccination [5, 7]. *C. pseudotuberculosis* infection of farmer animals can contaminate meat and milk, putting consumers at risk due to its zoonotic potential [7]. The ability of *C. pseudotuberculosis* to infect both animals and humans makes necessary the development of new vaccines for a reliable control and management of CLA once the currently available commercial vaccines are unable to fully protect susceptible animals against the disease [7, 8]. In this way, the study of other *C. pseudotuberculosis* virulence factors that might be involved in CLA pathogenesis can provide new vaccine targets.

The complete genome sequence of a *C. pseudotuberculois* strain (FRC41) isolated from a 12-year-old girl with necrotizing lymphadenitis allowed the identification of *spaC* and *nanH* as genes encoding proteins regarded as potential virulence factors [8]. SpaC is a putative adhesive pili tip protein. The pilus structure can probably make the initial contact with host cell receptors to enable additional ligand-receptor interactions and to facilitate the efficient delivery of virulence factors and intracellular invasion [9]. NanH, by its turn, is a putative extracellular neuraminidase [8]. Neuraminidases, or sialidases, belong to a class of glycosyl hydrolases that catalyze the removal of terminal sialic acid residues from a variety of glycoconjugates and can contribute to the recognition of sialic acids exposed on host cell surfaces. Most sialidase-producing microorganisms are pathogenic or commensal when in close contact with mammalian hosts. It has been also suggested that, in some types of pathogenic bacteria, sialidases function as potential virulence factors that contribute to the recognition of sialic acids exposed on the surface of the host cell [10]. A homologous counterpart of *C. pseudotuberculois* FRC41 NanH was characterized in *C. diphtheriae* KCTC3075 and shown to be a protein containing neuraminidase and trans-sialidase activities [11].

The *C. pseudotuberculosis* FRC41 genome also encodes a putative secreted copper,zinc-dependent superoxide dismutase (SodC) that is characterized by a lipobox motif and may be anchored in the cell membrane [8]. The extracellular location of this enzyme suggests that it may protect the surface of *C. pseudotuberculosis* cells against superoxide generated externally by the mammalian host cells. In *Mycobacterium tuberculosis*, SodC contributes to the resistance of this microorganism against the oxidative burst products generated by activated macrophages [12, 13]. The protective activity of Cu,Zn-SODs has been associated with virulence in other bacteria, such as *Neisseria meningitides* and *Hemophylus ducreyi* [8].

As part of important cell signaling mechanisms, eukaryotic-like serine/threonine protein kinases encountered in bacteria are a class of molecules that also deserves attention since they are part of complex signaling pathways and play a diversity of physiological roles in developmental processes, secondary metabolism, cell division, cell wall synthesis, essential processes, central metabolism, and virulence [14, 15]. *Mycobacterium tuberculosis* genome encodes 11 eukaryotic-like serine/threonine protein kinases (PknA to PknL, except for PknC). Protein kinase G (PknG) gained particular interest because it affects the intracellular traffic of *M. tuberculosis* in macrophages. Most microbes and nonpathogenic mycobacteria quickly find themselves in lysosomes, where they are killed. By contrast, *M. tuberculosis* stays within phagosomes; the bacterium releases PknG to block phagosome-lysosome fusion. Bacteria lacking *pknG* gene are rapidly transferred to lysosomes and eliminated [16, 17]. The genome of *C. pseudotuberculosis* FRC41 has a gene encoding for a putative PknG protein [8] but its function in the bacterium still needs to be investigated.

Therefore, *C. pseudotuberculosis* SpaC, NanH, SodC, and PknG proteins may play important roles in virulence and pathogenicity. In the present work, a characterization and evaluation of the vaccine potential of these proteins were performed in silico. The heterologous expression of these putative virulence factors in *Escherichia coli* is also described.

Methods

Protein sequences

The amino acid sequences of the target proteins were retrieved from NCBI GenBank: SpaC [gb| ADK29663.1], SodC [gb| ADK28404.1], NanH [gb| ADK28179.1], PknG [gb| ADK29622.1].

Homology searches

NCBI BLASTP [18] searches in UniProtKB database [19] were performed to identify homologues of the target proteins in the CMNR group of microorganisms (from *Corynebacterium*, *Mycobacterium*, *Nocardi,* and *Rhodococcus* genera): *Corynebacteriumn, taxid:1716; Mycobacterium, taxid:1763; Nocardia, taxid:1817; Rhodococcus, taxid:1827*. Likewise, BLASTP searches in UniprotKB database were performed to identify homologues of the target proteins in mammalian species of the *Ovis* (taxid: 9935), *Bos* (taxid: 9903), *Equus* (taxid: 9789), *Equus* (taxid: 35510), Mus (taxid: 10088), *Mus* (taxid: 862507) genera and in *Homo sapiens* (taxid: 9606). BLAST Genome [18] searches in *C. pseudotuberculosis* (taxid: 1719) complete genomes available at NCBI genome database were performed to identify the presence of the target protein genes in other *C. pseudotuberculosis* strains.

Primary and secondary structure analysis, subcellular localization and prediction of protective antigens

ProtParam [20] and Self-OPtimized prediction method with alignment—SOPMA [21] of expasy server were used to analyze different physiological and physicochemical properties of the target proteins. Molecular weight, theoretical pI, amino acid composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY) were calculated using the ProtParam preset parameters. Solvent accessibility, transmembrane helices, globular regions, bend region, random coil and coiled-coil regions were predicted using SOPMA default parameters. The amino acid sequences were evaluated by PSORTb 3.0.2 [22] to predict subcellular localization of the target proteins. SignalP 4.1 [23] was used to predict the presence and location of signal peptide cleavage sites in the amino acid sequences. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks. VaxiJen 2.0 [24] was used for alignment-independent prediction of protective antigens. The tool was developed to allow antigen classification solely based on the physicochemical properties of proteins without the need of sequence alignment.

B-cell epitope prediction

Linear B-cell epitopes were predicted from the target protein sequences using physicochemical properties [25] estimated by in silico methods available in DNASTAR Protean program (Madison, Wisconsin). The Jameson–Wolf method [26] was used to predict the potential antigenic determinants by combining existing methods for protein structural predictions. The results appear as multiple peaks in the antigenic index plot, with each peak signifying a potential antigenic determinant. The emini surface probability method [27] was used to predict the probability that a given region lies on the surface of a protein. The Kyte–Doolittle hydropathy method [28] predicts regional hydropathy of proteins from their amino acid sequences. Hydropathy values are assigned for all amino acids and are then averaged over a user defined window. The average is plotted at the midpoint of the window. The charge density method predicts regions of positive and negative charge by summing charge over a specific range of residues. DNASTAR developed this method using the pK tables of White et al. [29]. Since charged residues tend to lie on the surfaces of proteins, this method aids in predicting surface characteristics. Several wet lab experiments revealed that the antigenic portions were situated in beta turn regions of a protein [30] for these regions the Chou and Fasman beta turn prediction method was used [31, 32]. The Karplus–Schulz flexibility method [33] predicts backbone chain flexibility. The method is useful for resolving antigenic sites, as these regions tend to be among the most flexible in a polypeptide sequence. Conserved domains in the target proteins were identified by searching NCBI's conserved domain database (CDD) [34]. The results of each method were presented in a graphical frame. The peak of the amino acid residue segment above the threshold value (we used the default) is considered as predicted B-cell epitope. User can select any physicochemical property or a combination of two or more properties for epitope prediction. [35]. We selected amino acid segments in the target protein sequences where peaks above threshold overlapped in four or more methods. B-cell epitopes located in signal peptide or conserved domains were discarded.

T-cell epitope prediction

MHC I binding prediction was performed using the immune epitope database (IEDB) MHC I binding tool [36] and consensus [37] as prediction method which combines predictions from ANN aka NetMHC (3.4), SMM and comblib methods. Mouse MHC alleles (H-2-Db, H-2-Dd, H-2-Kb, H-2-Kd, H-2-Kk, H-2-Ld) and a peptide length of nine mer were selected to make the predictions from target proteins sequences. A median percentile rank of the four predictions methods was the Consensus representative percentile rank used to select the top 1 % of peptides. A small numbered percentile rank indicates high affinity.

MHC II binding predictions for target proteins were performed using NetMHCII 2.2 server [38] to predict binding of 15 mer peptides to two mouse MHC II alleles (H-2-IAb and H-2-IAd) using artificial neuron networks. The prediction values were given in nM IC50 values, and as a %-Rank to a set of 1,000,000 random natural

peptides. Strong and weak binding (SB, WB) peptides were indicated in the output. T-cell epitopes located in signal peptide or conserved domains were discarded.

Epitope clustering

Epitope clustering was performed using the IEDB Epitope cluster analysis tool [36]. Clustal omega [39] was used to group predicted B and T-cell epitopes into clusters of similarity based on multiple sequence alignment and visual inspection. Clustal omega alignments were used to double check if single-sequence clusters generated by IEDB epitope cluster analysis tool were in fact composed of unique epitopes (no pairs).

Cloning procedures

Miniprep plasmid purifications, agarose gel electrophoresis, and *E. coli* media were as described [40]. Amino acids 2–23 and amino acids 2–31 were removed from *sodC* and *nanH* ORF sequences, respectively. These regions containing signal peptide were eliminated before cloning in order to improve protein expression since they are relatively rich in hydrophobic amino acids. ORF codons of all four target proteins were replaced by *E. coli* preferential codons [41]. Optimized ORF sequences were synthesized and individually cloned into pD444-NH expression vector (T5 promoter, IPTG inducible, strong ribosome binding site, His-tag, ampicillin resistance marker, high copy origin of replication, 4027 bp size) by DNA2.0 (Menlo Park, CA). Each ORF-containing plasmid (pD444-NH;*pknG*, pD444-NH;*spaC*, pD444-NH;*sodC*, and pD444-NH;*nanH*) was transformed into BL21(DE3) *E. coli* strains according to the OverExpress™ Electrocompetent Cells kit (Lucigen, Middleton) instructions.

Protein expression in *E. coli*

Protein expression protocol was according to OverExpress™ Electrocompetent Cells kit (Lucigen, Middleton) instructions. Briefly, transformed cell cultures at OD 0.5–0.7 were induced with 1 mM IPTG for 5 h at 37 °C. SDS-PAGE of non-induced and induced cell culture samples and Coomassie blue staining was as described [42].

Purification of PknG

Bacteria transformed with pD444-NH;*pknG* was induced as described above. Cell pellet was collected by 8000 rpm centrifugation, resuspended in buffer A (10 mM NaH_2PO_4 pH7.4, 300 mM NaCl, 1 % glycerol, 5 mM imidazole), lysed on ice with ten 15-s sonication pulses using a ultrasonic processor Marconi-MA 103 (Piracicaba, São Paulo) and centrifuged at 15,000×*g* for 15 min. The supernatant containing recombinant proteins was purified under native conditions using 1 mL of immobilized Ni Sepharose (GE Healthcare). The resin was washed using buffer A with 80 mM imidazole. Recombinant PknG was eluted from the column with buffer A containing 400 mM imidazole. The eluted protein was dialyzed against buffer B (10 mM NaH_2PO_4 buffer pH 7.4 and 50 mM NaCl) and concentrated by ultrafiltration. The concentrated fraction was injected on a Superdex 75 10/300 GL (GE Healthcare) size exclusion column previously equilibrated with buffer B. The purity of the sample was assessed by SDS–PAGE.

Results and discussion

Traditional vaccination approaches are based on complete pathogen either live attenuated or inactivated. Among the major problems these vaccines brought are crucial safety concerns, because those pathogens being used for immunization may become activated and cause infection. Moreover due to genetic variation of pathogen strains around the world, vaccines are likely to lose their efficacy in different regions or for a specific population. Novel vaccine approaches like DNA vaccines and epitope based vaccines have the potential to overcome these barriers to create more effective, specific, strong, safe and long lasting immune response without all undesired effects [43]. Next-generation sequencing and proteomic techniques have enabled researchers to mine entire microbial genomes, transcriptomes and proteomes to identify novel candidate immunogens [44]. In silico techniques are the best alternative to find out which regions of a protein out of thousands possible candidates are most likely to evoke immune response [35]. This reverse vaccinology approach has enjoyed considerable success in the past decade, beginning with *Neisseria meningitides*, and continuing with *Streptococcus pneumonia*, pathogenic *E. coli*, and antibiotic resistant *Staphylococcus aureus* [44].

Homology searches

The conservation level between target proteins and proteins of the CMNR group of microorganisms was evaluated by NCBI BLASTP [18] searches in UniprotKB database [19]. This kind of analysis is important for the development of vaccines once they can be used not only for *C. pseudotuberculosis* FRC41 but for other pathogen strains and pathogens of other species. NCBI BLAST Genome searches show the presence of the target protein genes in all 37 *C. pseudotuberculosis* strains currently available in NCBI complete genomes database (data not shown). This indicates that SpaC, SodC, NanH and PknG can potentially be expressed not only in a few strains demonstrating the importance of these proteins for this pathogenic bacterium. Well conserved homologous of the target proteins were also found in microorganisms of the CMNR group (Additional files 1, 2 and 3). These

findings are a good indication that a vaccine against *C. pseudotuberculosis* made from the putative virulence factors can be effective not only against numerous strains of the pathogen but also against bacterial pathogens from other species.

The conservation degree among target proteins and mammalian (*Ovis*, *Bos*, *Equus* and *Mus* genera, *Homo sapiens*) proteins was also evaluated by BLASTP searches. The analysis was important to reveal the conservation degree among pathogen proteins and host proteins and so the possibility of undesirable immunological cross-reactions which may induce autoimmunity. The results (Additional files 1, 2 and 3) show that *C. pseudotuberculosis* FRC41 SpaC, SodC, NanH, and PknG sequences share low identity (30 % in average) with mammalian sequences. BLASTP alignments show that most of this weak homology is in conserved domains (data not shown). Thus, regions away from signal peptides and conserved domains are ideal targets for vaccine development.

Primary and secondary structure analysis

The next step was to evaluate the primary and secondary structure features of SpaC, SodC, NanH and PknG as they can predict stability and reveal functional characteristics of the proteins at some extent. Based on ProtParam instability index, SodC was considered the least stable while PknG was the most stable (Table 1). PknG was also the most hydrophilic with the highest GRAVY (−0.211). This same protein also presented the highest aliphatic (92.91) index (Table 1). SOPMA program, used to calculate secondary structure features of the target proteins, reported that SpaC, SodC and NanH were dominated by random coils, consisting in 45.35, 41.26 and 39.05 %, respectively (Table 2). Alpha helix prevailed (44.06 %) in PknG. The differences in secondary structure content and aliphatic character helps to explain the stability indexes estimated for the target proteins. [45].

Subcellular localization and prediction of protective antigens

The candidate molecules from a eukaryotic pathogen expected to induce immunity comprise proteins that are as follows: (i) present on the surface of the pathogen, (ii) excreted/secreted from the pathogen and (iii)

Table 2 Secondary structure content in the target proteins estimated using SOPMA

Secondary structure	SpaC (%)	SodC (%)	NanH (%)	PknG (%)
Alpha helix (Hh)	111 is 13.94	56 is 27.18	228 is 32.85	330 is 44.06
3_{10} helix (Gg)	0.00	0.00	0.00	0.00
Pi helix(Ii)	0.00	0.00	0.00	0.00
Beta bridge (Bb)	0.00	0.00	0.00	0.00
Extended strand (Ee)	244 is 30.65	39 is 18.93	128 is 18.44	114 is 15.22
Beta turn (Tt)	80 is 10.05	26 is 12.62	67 is 9.65	56 is 7.48
Bend region (Ss)	0.00	0.00	0.00	0.00
Random coil (Cc)	361 is 45.35	85 is 41.26	271 is 39.05	249 is 33.24
Ambiguous states (?)	0.00	0.00	0.00	0.00
Other states	0.00	0.00	0.00	0.00

Table 1 Physicochemical properties of the target proteins estimated using ProtParam

Physicochemical property	SpaC	SodC	NanH	PknG
Number of amino acids	796	206	694	749
Molecular weight	85,964.9	21,099.3	74,683.3	83,349.4
Theoretical pI	5.13	5.96	5.05	5.13
Total number of negatively charged residues (Asp + Glu)	96	23	102	101
Total number of positively charged residues (Arg + Lys)	77	17	79	76
Extinction coefficient[a]	93,085	4595[b]	77,600	81,375
Abs 0.1 % (=1 g/l), assuming all pairs of Cys residues form cystines	1.083	0.218[b]	1.039	0.976
Extinction coefficient[a]	92,710	4470[b]	77,350	81,250
Abs 0.1 % (=1 g/l), assuming all Cys residues are reduced	1.078	0.212[b]	1.036	0.975
The estimated half-life				
Mammalian reticulocytes, in vitro	30 h	30 h	30 h	30 h
Yeast, in vivo	>20 h	>20 h	>20 h	>20 h
Escherichia coli, in vivo	>10 h	>10 h	>10 h	>10 h
Instability index (II)	28.21 (stable)	19.62 (stable)	32.92 (stable)	38.18 (stable)
Aliphatic index	80.16	71.65	72.58	92.91
Grand average of hydropathicity (GRAVY)	−0.442	−0.245	−0.485	−0.211

[a] Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water

[b] This protein does not contain any Trp residues. Experience shows that this could result in more than 10 % error in the computed extinction coefficient

homologous to known proteins involved in pathogenesis and virulence [46]. Signal peptide presence and subcellular localization (Table 3) of SpaC (cell wall), SodC (cytoplasmic membrane) and NanH (extracellular) was as predicted before [8]. They were predicted as protective antigens by VaxiJen. Membrane and secreted proteins are considered potential vaccine targets once they are at the host-pathogen interface. These proteins may interact more directly with host molecules for cell adhesion, invasion, multiplication, immune response evasion, damage generation to the host, and survive to host cell defenses [8, 47, 48].

Table 3 Subcellular localization, signal peptide, and prediction of protective antigen for the target proteins

Parameter (program)	SpaC	SodC	NanH	PknG
Subcellular localization (Psortb)	Cell wall (matched LPXTG; score 9.97)	Cytoplasmic Membrane (matched 61246116: superoxide dismutase Cu–Zn precursor; score 9.68)	Extracellular (matched 585539: sialidase precursor EC 3.2.1.18 NEURAMINIDASE; score 9.70)	Cytoplasmic, (matched 54041713: probable serine/threonine-protein kinase pknG; score 9.89)
Signal peptide (signalp 4.1)[a]	No (D = 0.162 D-cutoff = 0.420)	Yes position: 1–35 (cleavage site between pos. 35 and 36: DSA-DK D = 0.631 D-cutoff = 0.450 networks = signalp-TM)	Yes position: 1–31 (cleavage site between pos. 31 and 32: APA-TL D = 0.562 D-cutoff = 0.450 networks = signalp-TM)	No (D = 0.106 D-cutoff = 0.420)
Prediction of protective antigens (VaxiJen)	Probable ANTIGEN (score 0.6912)	Probable ANTIGEN (score 0.7663)	Probable ANTIGEN (score 0.6967)	Probable NON-ANTIGEN score 0.3686)

[a] For signal peptide prediction, D-cutoff values were set as sensitive (reproduce SignalP 3.0's sensitivity)

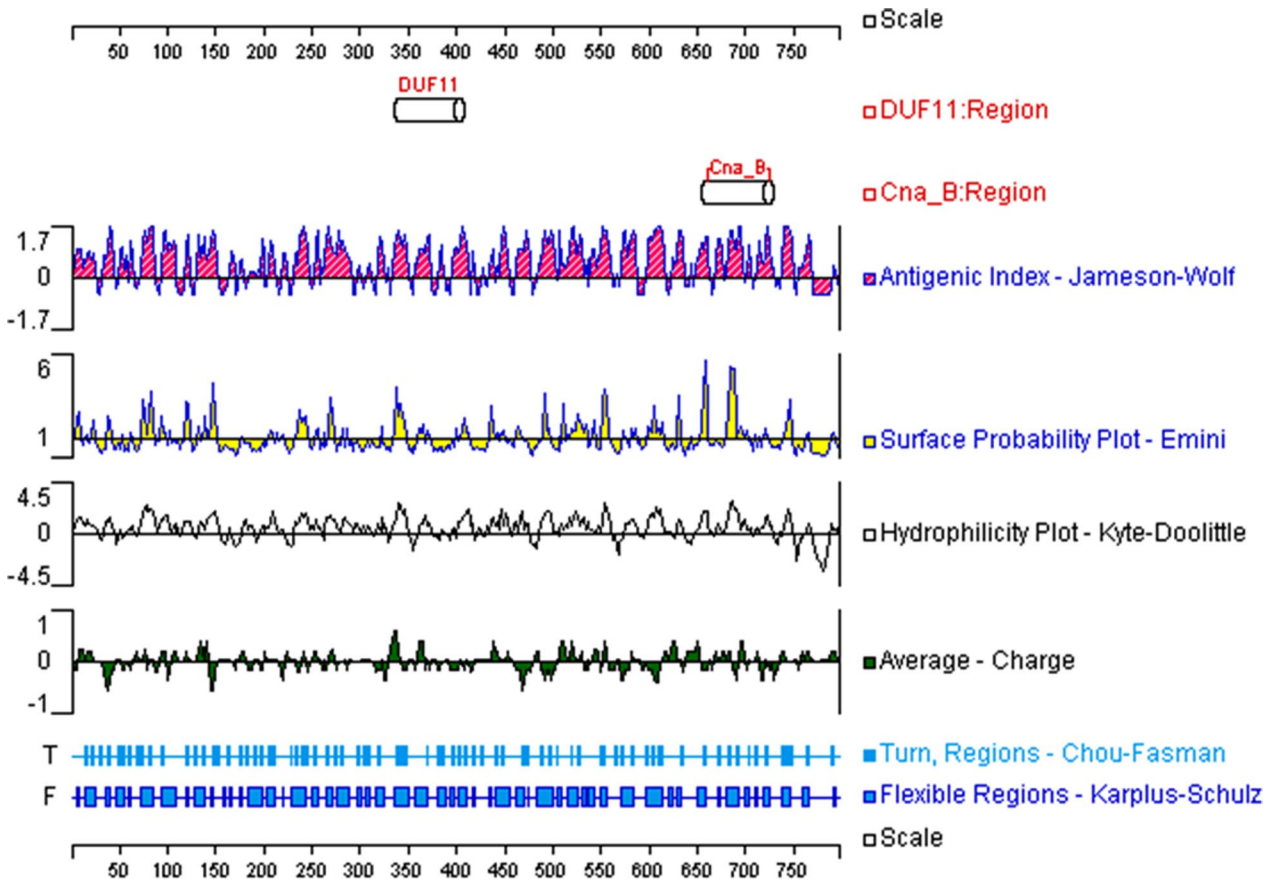

Fig. 1 Graphical outputs of the different methods used to quantitate the physicochemical properties used to predict B-cell epitopes from SpaC. On *top* are the conserved domains of the target protein identified by searching NCBI's Conserved Domain Database (CDD). The *scales* indicate the amino acid positions

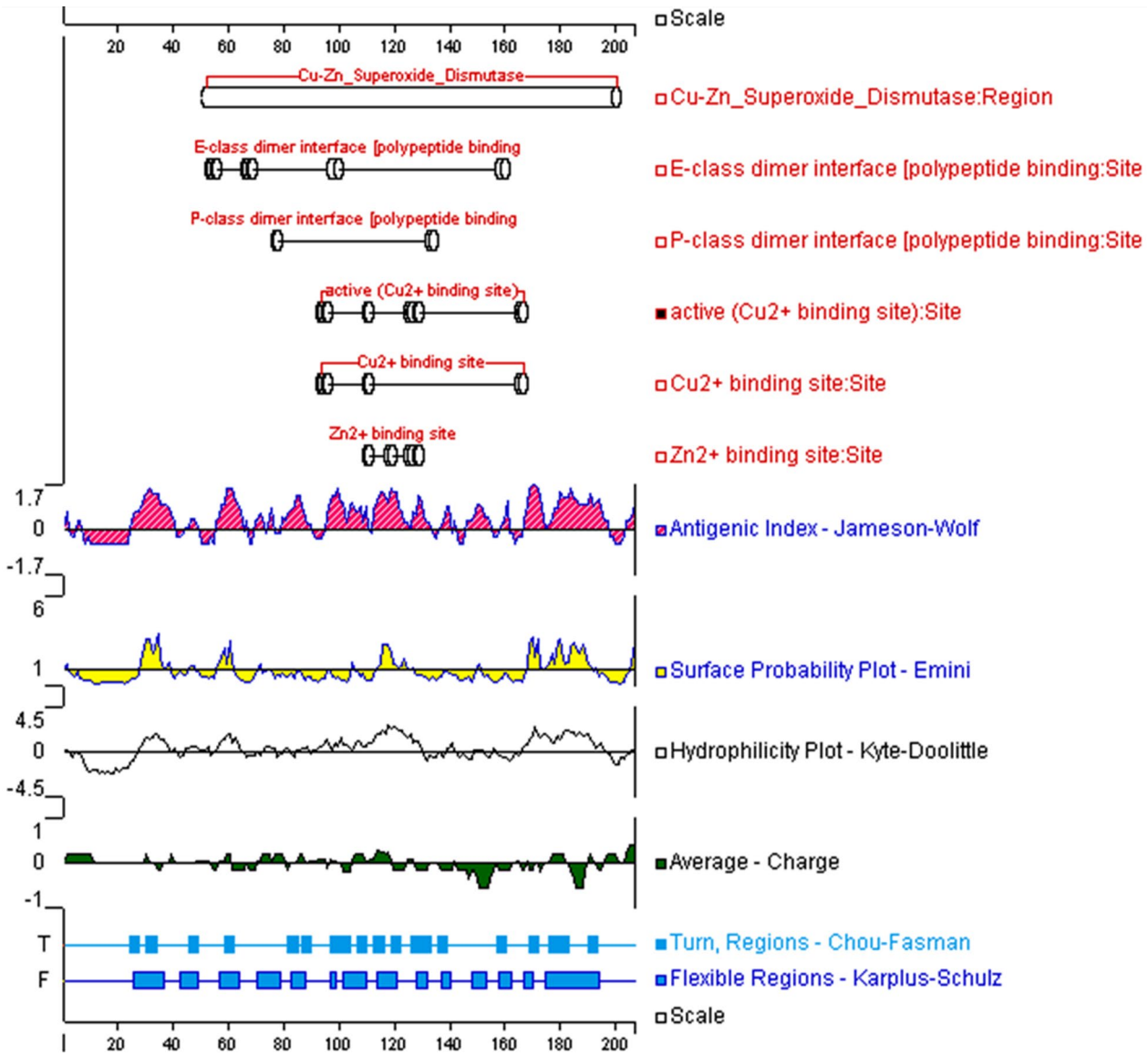

Fig. 2 Graphical outputs of the different methods used to quantitate the physicochemical properties used to predict B-cell epitopes from SodC. On *top* are the conserved domains of the target protein identified by searching NCBI's Conserved Domain Database (CDD). The *scales* indicate the amino acid positions

Like its counterpart in *M. tuberculosis*, which is predominantly found soluble in the cytoplasm [15], PknG was predicted as a cytoplasmic protein (Table 3). However, VaxiJen predicted this *C. pseudotuberculosis* putative serine/threonine protein kinase as non-antigenic. In fact, cytoplasmic proteins have not been widely considered as potential immunogens, since they do not have a close contact to many immune systems' intermediates [49]. Regardless of this, it has been demonstrated that cytoplasmic proteins can be effectively exposed to MHC presentation and may have a key role in the development of a suitable protective immunity. In order to overcome the problem of endogenous antigen access to the MHC II compartment, lysosomal-associated membrane proteins (LAMPs), major lysosomal membrane glycoproteins that contain a cytoplasmic tail targeting sequence that directs the trafficking of the molecule through an endosome/lysosome pathway, including cellular compartments where it is co-localized with MHC II molecules, have been used to induce antigen-trafficking to MHC II compartments and increase the immune response to those antigens [50]. This strategy has shown to elicit enhanced long-term memory response against HIV-1 Gag protein. Besides, a novel mechanism of specific $CD8^+$ T cell-mediated

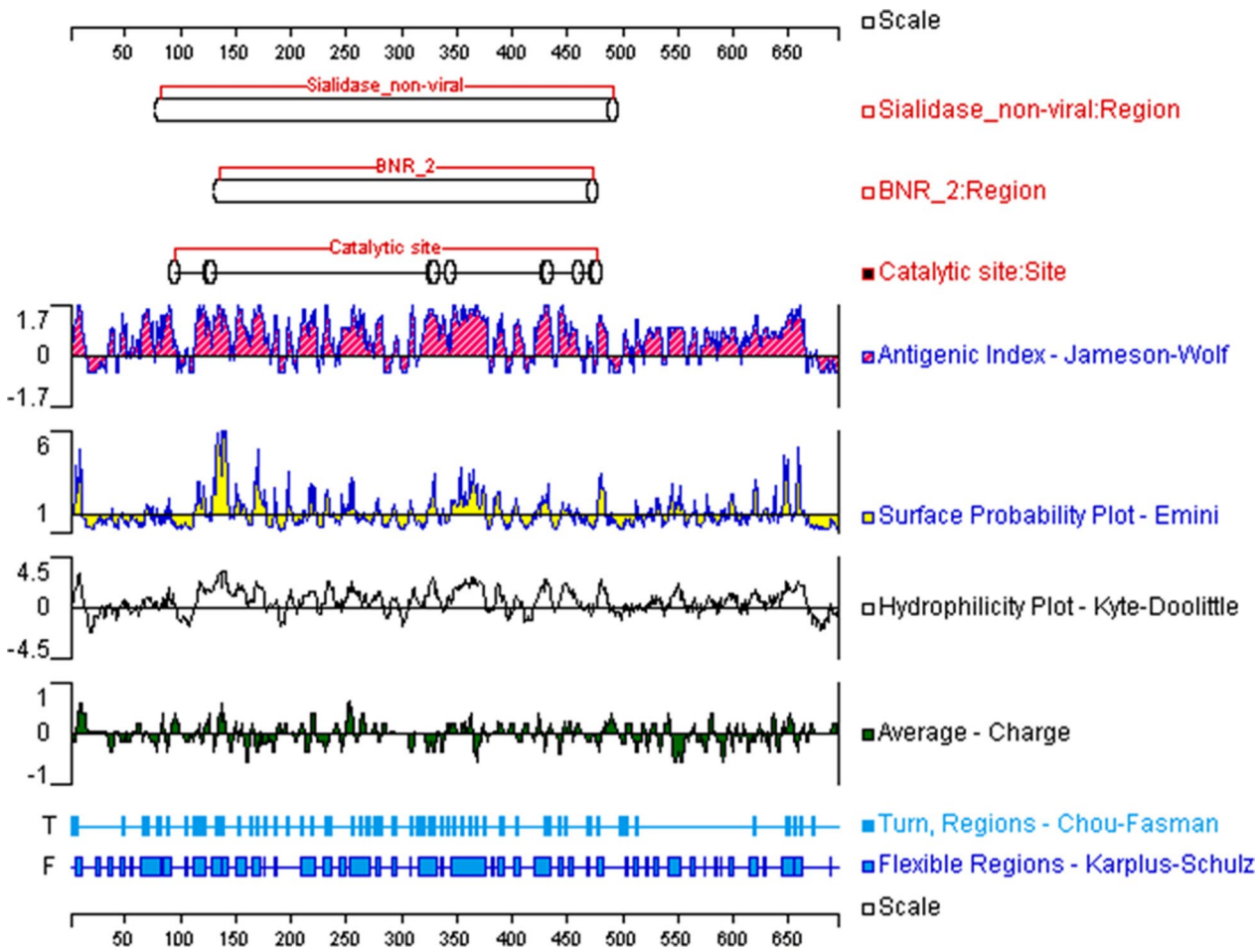

Fig. 3 Graphical outputs of the different methods used to quantitate the physicochemical properties used to predict B-cell epitopes from NanH. On *top* are the conserved domains of the target protein identified by searching NCBI's Conserved Domain Database (CDD). The *scales* indicate the amino acid positions

protective immunity can recognize malaria proteins expressed in the cytoplasm of parasites, form clusters around infected hepatocytes, and protect against parasites [51]. This strongly indicates that cellular and molecular mechanisms underlying the protective immune responses against intracellular parasites need further studies.

Linear B-cell epitope prediction

The general problem in achieving an effective treatment of *C. pseudotuberculosis* infections in animals and humans is probably related to the facultative intracellular lifestyle of this bacterium, as it can survive and multiply in macrophages [52]. The knowledge on the immunity induced by *C. pseudotuberculosis* indicates that the resistance to infection is a complex process involving components of the non-specific and specific host responses, in which humoral and cellular immune responses are both operative [7].

B-cell epitopes can induce both primary and secondary immunity. Although it is believed that the majority of B-cell epitopes are conformational epitopes, experimental determination of epitopes has focused primarily on the identification of linear (non conformational) B-cell epitopes [25]. This is mainly because predictions of conformational epitopes depend on experimentally determined protein structures or homologous protein structures for in silico modeling. So far, there is no protein structure of the target proteins or structures of highly homologous proteins available for modeling.

Most of the existing linear B-cell epitope prediction methods are based on physicochemical properties relating to surface exposure, such as flexibility or hidrophilicity [25, 35], as it is thought that epitopes must lie at the

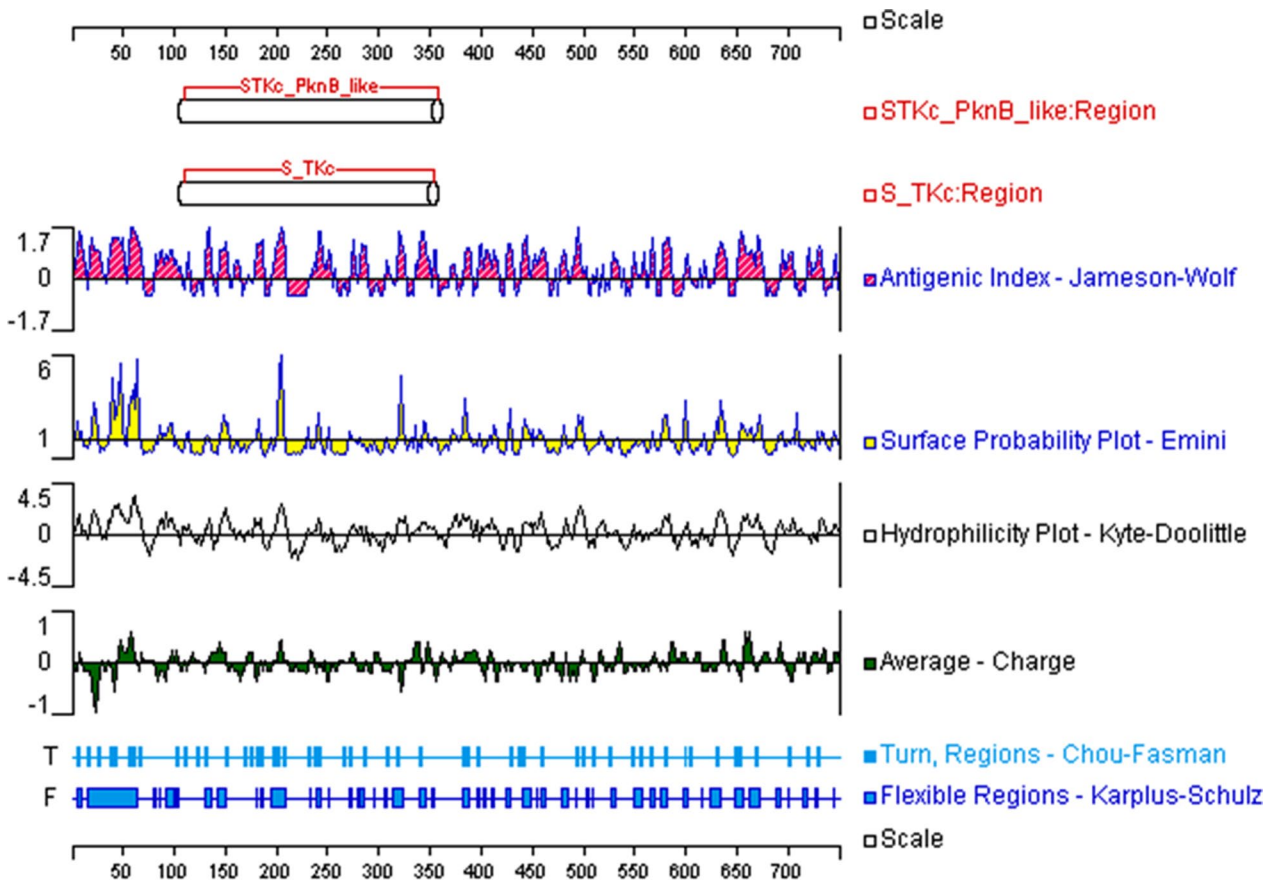

Fig. 4 Graphical outputs of the different methods used to quantitate the physicochemical properties used to predict B-cell epitopes from PknG. On *top* are the conserved domains of the target protein identified by searching NCBI's Conserved Domain Database (CDD). The *scales* indicate the amino acid positions

protein surface for antibody binding to occur. Thus, the target proteins were scanned for B-cell epitopes using several methods designed to quantitate protein physicochemical properties. Graphical outputs of the prediction methods are shown in Figs. 1, 2, 3, and 4. High values gave rise to peaks, whereas valleys correspond to negative properties of the protein. Selected B-cell linear epitopes of target proteins are shown in Table 4. The putative adhesive pili tip protein SpaC, seconded by PknG, presented the highest number of B-cell epitopes. We did pick only one B-cell epitope from SodC since the protein is short (206 aa), has a 35 aa long signal peptide (Table 3) and its highly conserved domain occupies most of the amino acid sequence (Fig. 2).

T-cell epitope prediction

A desirable vaccine preparation should present MHC I and II epitopes for the development of a protective and long lasting immune response to *C. pseudotuberculosis*. MHC I epitopes are presented to CD8+ T cells by cells infected with *C. pseudotuberculosis*, leading to the apoptosis of the host cell and interruption of the bacterial multiplication, and it was already described the injection of anti-CD4 or anti-CD8 monoclonal antibody resulted in significantly increased mortality and a marked suppression of IFN-gamma production in mice [53]. MHC II epitopes are involved in the activation of CD4+ T cells, which will drive the host immune response to a Th1 protective response, as well as to a production of IFN-gamma, that will help macrophages in the fusion of phagosomes and lysosomes, resulting in the destruction of bacteria that underwent phagocytic process [54]. Ultimately, specific high affinity binding should be the main concern since the efficiency of an epitope vaccine greatly relies on the precise interaction between epitope and HLA molecule [55]. Table 5 shows nine mer peptides from target proteins with high affinity (Consensus percentile rank <1 %) for mouse MHC I alleles. Most of them were from SpaC and PknG. SodC peptides were discarded since they were located in conserved regions. The

Table 4 B-cell epitopes predicted from target proteins

Target Protein	Epitope number	B-cell epitopes[a]
SpaC	1	1-MEVPEKTKVEIRFQTGSKISTPSTPSV-27
SpaC	2	70-SQHTNRGETFNDRNSTDLYVQ-90
SpaC	3	116-AYNPKEGYIYAISQGRLKTLQSSKLRIYDEDPNYPA-GHLL-155
SpaC	4	234-NDYTSTGKTDSNYVWGI-250
SpaC	5	251-KNSSNPAVLERIDVRDGSRKEFSLDGVKDPLGQN-VEKGIYGT-292
SpaC	6	331-IVAKRKGPTSQNNDATSNG-349
SpaC	7	434-KATYKVTANQSISNNEKCLQNTASIYAN-461
SpaC	8	504-GNGLRKVTYKIEVKNPKGFPETKYSLTDTPQ-FADSV-539
SpaC	9	540-KLERLKVISDYGKKNQEVQAADISV-564
SpaC	10	615-FGLFNSAKLKVGVSEKTSEGCAPIVR-640
SpaC	11	647-QLKKVDAENKETELQATFE-665
SpaC	12	735-PLSKSADQGKDPNLVIL-751
SpaC	13	756-VRVGTLPKTGGHGVAIYLV-774
SodC	1	26-SSSTTTKDSADKAMTS-41
NanH	1	1-MTDSHRRGTRKALVTLTA-18
NanH	2	65-GEGKLPDPVTSEFF-78
NanH	3	520-IEDAKAATAKAEEATAN-536
NanH	4	559-AEAKSAAQDAI-569
NanH	5	595-KAENEAKALAE-605
NanH	6	617-SQDQAKALAEA-627
NanH	7	645-EKEKSGKAGGTDNTENKGFWQE-666
PknG	1	1- MNDPLSRGTEAIPFDPFADDEEDDLSGLLND-31
PknG	1.1	38-DTDTDARSREKSISTFRSRRGTNRDDRTVANG-69
PknG	1.2	79-STAEEMLKDDAYIEQKGLEKPLLHPGD-105
PknG	2	381-SPQRSTFGTKHMVFRTDQLIDGIERNVRIT-SEEVNA-416
PknG	3	438-YAEPSQTLQTLRDAMAQEEFANSKEIPL-465
PknG	4	479-EARSWLDTLDATLSDDWRHQWYSGVTS-505
PknG	5	576-LTKDPETLRFKALYL-590
PknG	6	627-QVPQNSTHRRMAELTAI-643
PknG	7	651-LSESRIRRAARRLESIPTNEPRFLQIKIA-679
PknG	8	718-DSLRLLARSAPNVHHRYTLV-737

[a] Epitopes in signal peptide and conserved domains were discarded

Table 5 MHC class I epitopes predicted from target proteins

Target Protein	Mouse HLA Allele	Epitope number	Start	End	Peptide (9 mer)	Consensus rank (%)
SpaC	H-2-Db	1	615	623	FGLFNSAKL	0.3
SpaC	H-2-Kk	2	34	42	EEFENTEPI	0.3
SpaC	H-2-Kb	3	90	98	QSFNRNTGL	0.35
SpaC	H-2-Kd	4	124	132	IYAISQGRL	0.4
SpaC	H-2-Kd	5	116	124	AYNPKEGYI	0.5
SpaC	H-2-Kd	6	199	207	RYLVSNSSQ	0.5
SpaC	H-2-Kd	7	771	779	IYLVMGVLL	0.5
SpaC	H-2-Db	8	450	458	KCLQNTASI	0.6
SpaC	H-2-Db	9	208	216	SGTHNLYTL	0.7
SpaC	H-2-Dd	10	48	56	VGPSVDPTV	0.7
SpaC	H-2-Kd	11	458	466	IYANEKDLI	0.8
SpaC	H-2-Kb	12	785	793	SWSLYRNQL	0.85
SpaC	H-2-Kb	13	774	782	VMGVLLVLV	0.95
NanH	H-2-Kk	1	44	52	SEFFDSKVI	0.3
NanH	H-2-Dd	2	39	47	PDPVTSEFF	0.4
NanH	H-2-Dd	3	55	63	VDPAGQRCF	0.4
NanH	H-2-Kk	4	634	642	QELLRIFPG	0.5
NanH	H-2-Dd	5	655	663	GGMQKLLAF	0.6
NanH	H-2-Kb	6	645	653	PIFSFLASI	0.8
PknG	H-2-Kd	1	437	445	SYAEPSQTL	0.2
PknG	H-2-Kk	2	455	463	EEFANSKEI	0.2
PknG	H-2-Db	3	678	686	IAIMNAALT	0.5
PknG	H-2-Ld	4	525	533	LPGEAAPKL	0.5
PknG	H-2-Kb	5	586	594	KALYLYALV	0.55
PknG	H-2-Dd	6	665	673	SIPTNEPRF	0.6
PknG	H-2-Kb	7	685	693	LTWLRQSRL	0.6
PknG	H-2-Db	8	504	512	TSLFLDDYV	0.7
PknG	H-2-Kd	9	379	387	LYSPQRSTF	0.8
PknG	H-2-Kb	10	632	640	STHRRMAEL	0.85
PknG	H-2-Db	11	457	465	FANSKEIPL	0.9
PknG	H-2-Kk	12	21	29	EEDDLSGLL	0.9
PknG	H-2-Kk	13	353	361	LETQLFGIL	0.9

Epitopes in signal peptide and conserved domains were discarded

few strong binding peptides to MHC II were limited to mouse H-2-IAb allele and most of them were from NanH (Table 6). Only two MHC II strong binding peptides were predicted from SodC but both were discarded because they were located in conserved regions of the protein. Additional file 4 shows the MHC class II epitopes predicted from target proteins.

Epitope clustering

All B and T-cell epitopes (MHC I and II) predicted from the target proteins were grouped in clusters of sequence similarity in order to evaluate the redundancy degree among them. A total of 57 clusters were formed from a set of 136 epitopes predicted (Additional file 5). Most of them (34) were single-sequence clusters. Clusters 4 and 5 (PknG) and cluster 12 (SpaC) grouped epitopes for both B and T-cell (MHC I and II). These groups of epitopes can thus potentially stimulate a complete immune response against *C. pseudotuberculosis*. The main goal of vaccination is to induce humoral and cellular immunity by selectively stimulating antigen specific CTLs or B cells together with T_H cells [56]. Several clusters containing B-cell and either MHC I or II epitopes were also formed.

Table 6 Total numbers of MHC class II epitope prediction from target proteins

Target protein	Mouse MHC HLA allele	Number of strong binders[a]	Number of weak binders[a]	Number of peptides[b]
PknG	H-2-IAb	9	35	735
SpaC	H-2-IAb	4	48	782
SodC	H-2-IAb	0	12	192
NanH	H-2-IAb	22	64	680
PknG	H-2-IAd	0	29	735
SpaC	H-2-IAd	0	13	782
SodC	H-2-IAd	2	6	192
NanH	H-2-IAd	0	32	680

See epitope sequences in Additional file 4

[a] Strong binder threshold 50.00. Weak binder threshold 500.00

[b] Peptide length 15 mer

Among them are clusters 9 and 19 formed by epitopes from NanH (Additional file 5). Cluster 14 grouped all SodC weak binding epitopes to H-2-IAb allele.

Protein expression

Large amounts of SpaC, SodC, NanH, and PknG are necessary for future studies on the role of these proteins in *C. pseudotuberculosis* pathogenicity and virulence. *Escherichia coli* remains as one of the most attractive hosts among many systems available for heterologous protein production [57]. Thus, *pknG*, *spaC*, *sodC*, and *nanH* codon-optimized ORFs were cloned into the same expression vector system and individually transformed into BL21(DE3) *E. coli* strains. SDS-PAGE analyses show the successful expression of the target proteins (Fig. 5a). Purification of PknG using affinity and gel chromatography is shown in Fig. 5b.

From the current study we have suggested that several B and T-cell epitopes predicted from SpaC, SodC, NanH and PknG can be used for the development of a multi peptide vaccine to induce a complete immune response against *C. pseudotuberculosis*. The next step will be to evaluate experimentally these epitopes in vitro and in vivo to assess their real protective potential.

Conclusions

The in silico analyses performed show that SpaC, PknG and NanH present good potential as targets for vaccine development. Several epitopes from these proteins can potentially induce both humoral and cellular immune responses against *C. pseudotuberculosis*. The four target proteins were successfully expressed in *E. coli*. The production of these proteins in large amounts represents an important step for future studies on 3-D structure, pathogenicity, virulence, and vaccine development.

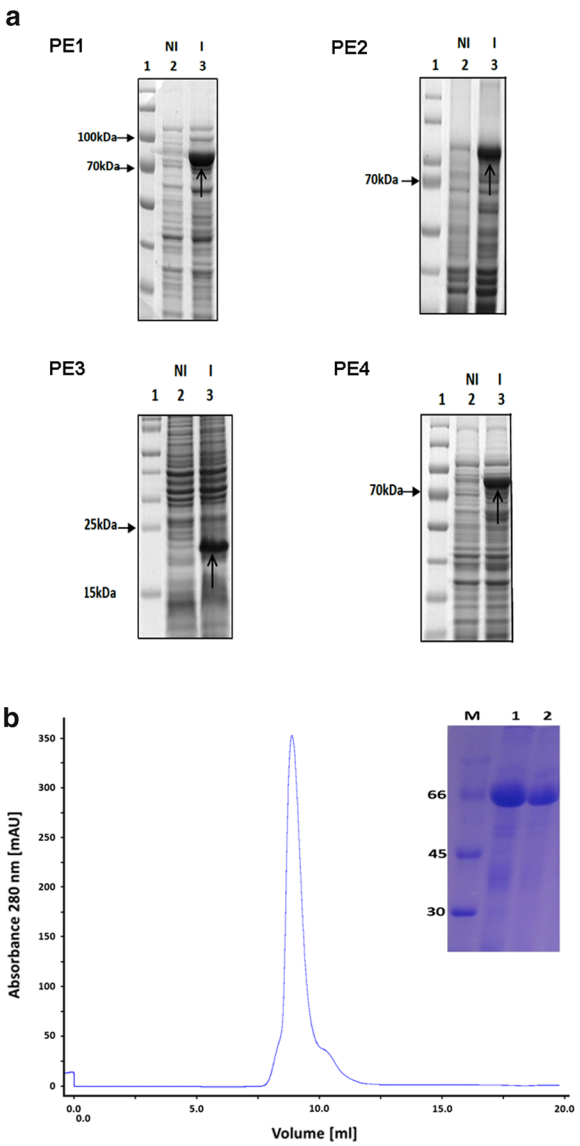

Fig. 5 Heterologous expression of the *C. pseudotuberculosis* FRC41 putative virulence factors in *E. coli* and rPknG purification. **a** Coomassie blue-stained SDS-PAGE analyses of the protein expression experiments: PE1, rPknG expression (83 kDa, 10 % gel) in *E. coli* strain BL21 Star (DE3); PE2, rSpaC expression (86 kDa, 10 % gel) in *E. coli* strain C43 (DE3); PE3, rSodC expression (18 kDa, 15 % gel) in *E. coli* strain BL21 Star (DE3); PE4, rNanH expression (71.5 kDa, 10 % gel) in *E. coli* strain C43 (DE3). 1, pre-stained protein ladder; 2 (NI), non-induced time 0; 3 (I), induced with 1 mM IPTG for 5 h at 37 °C. *Arrows* indicate the recombinant protein position in the gels. **b** Chromatogram of the rPknG purification by gel filtration. SDS–PAGE shows an analysis of the purification steps. *M* molecular-weight markers (kDa); 1, rPknG after affinity chromatography by Ni Sepharose; 2, rPknG purified by gel filtration

Additional file

Additional file 1. Homologous proteins of *C. pseudotuberculosis* FRC41 putative virulence factor PknG in CMNR microorganism and mammalians.

Additional file 2. Homologous proteins of *C. pseudotuberculosis* FRC41 putative virulence factors SpaC and NanH in CMNR microorganism and mammalians.

Additional file 3. Homologous proteins of *C. pseudotuberculosis* FRC41 putative virulence factor SodC in CMNR microorganism and mammalians.

Additional file 4. MHC class II epitopes§ predicted from target proteins.

Additional file 5. Clusters of B and T-cell epitopes predicted from target proteins.

Abbreviations

CLA: caseous lymphadenitis; CMNR: microorganisms from *Corynebacterium*, *Mycobacterium*, *Nocardi,* and *Rhodococcus* genera; PLD: phospholipase D; SOD: superoxide dismutase; ORF: open reading frame; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Authors' contributions

TMS, KTOSJ and ELA performed the bioinformatic analyses. TMS and RWP made the immunological approaches for the epitopes prediction study. KTOSJ, NRT, RFSS and RBM carried out the protein expression and purification experiments. TMS and KTOSJ drafted the manuscript. VA, TMS, RM and RKA participated in the design and coordination of the study. All authors have read and approved the manuscript.

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Competing interests

The authors declare that they have no competing interests.

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