Propionibacterium spp. and Acidipropionibacterium spp.

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Propionibacterium spp. and *Acidipropionibacterium* spp.☆

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Introduction

Members of the *Propionibacteriaceae* family are named according to their unique metabolism with propionic acid being a major end product. The family was first described in cheese at the beginning of the 20th century by Sigurd Orla-Jensen and Eduard von Freudenreich, and propionic fermentation was studied by Louis Pasteur and Albert Fitz. Based on current genomic evidence, the family has recently been divided into three genera: *Propionibacterium* spp., *Acidipropionibacterium* spp. and *Cutibacterium* spp. Cutaneous propionic acid bacteria comprise species found on human skin and have been reclassified as cutibacteria (e.g., *Propionibacterium acnes* was reclassified as *Cutibacterium acnes*). They will not be considered here.

☆ *Change History*: September 2019 The title of the chapter has been adapted from *Propionibacterium* spp. to *Propionibacterium* spp. and *Acidipropionibacterium* spp., due to adaptions in the classification. The authorship has been expanded. Overall, the term "Emmental" has been replaced by the generic term "Swiss-type cheese". MT Fröhlich-Wyder and A Thierry revised the 'Introduction' section. U von Ah updated the 'Classification and Identification' section, described the new genus *Acidipropionibacterium* and *Propionibacteriaceae* family, added Table 1 as a new Table and updated Table 2 (from the Table 1 of the previous version). A Thierry and SM Deutsch revised and enriched the 'Morphology, Envelopes and Growth Conditions' section. M Turgay largely extended and updated the section 'Genetics', renamed into 'Genetic properties', from new complete genome sequence data, added Table 3 as a new Table, and updated Table 4 (from Table 2 of the previous version), replaced Table 3 by an extended section 'Genetic engineering' and added a section 'Immunity'. S Irmler restructured and enriched the section 'Metabolism' with short information on the biosynthesis of vitamin B12, trehalose, and pigment and additional information on the conversion of aspartate into succinate and modified Figure 1. G Jan updated and edited the section 'Adaptation to Stress'. MT Fröhlich-Wyder updated the section 'Use as Ripening Cultures in Cheese'. G Jan and H Falentin updated and edited the section 'Relation to Nutrition as Probiotics and Producers of Nutraceuticals'. HP Bachmann expanded the section 'Antimicrobial activities' into a section entitled 'Use as Protective Cultures' and updated Table 5 (from the Table 4 of the previous version). This is an update of A. Thierry, H. Falentin, S.M. Deutsch, G. Jan, Bacteria, Beneficial: *Propionibacterium* spp., Editor(s): John W. Fuquay, *Encyclopedia of Dairy Sciences* (Second Edition), Academic Press, 2011, Pages 403–411, ISBN 9780123744074, https://doi.org/10.1016/B978-0-12-374407-4.00047-9.

Dairy-related propionibacteria and acidipropionibacteria are mainly found in milk, cheese and some other fermented products, such as silage. Dairy propionibacteria are mainly used as ripening cultures for the manufacture of Swiss-type cheeses and probiotics. Their propionic acid fermentation is responsible for the characteristic holes (referred to as eyes) and the nutty, slightly sweet flavor of these cheeses. *P. freudenreichii* and *A. acidipropionici* have a long-documented history of safe use in foods, which led them to be generally recognized as safe (GRAS) in the United States and to achieve the analogous status of qualified presumption of safety (QPS) in Europe.

Classification and Identification

Propionibacteria are classified based on 16S rDNA sequences, G+C content of their DNA, and whole genome sequencing (WGS). They belong to the class of *Actinobacteria* that consists of Gram-positive bacteria with a high G+C content.

All genera of propionibacteria are included within the *Propionibacteriaceae* family. After the new genus *Acidipropionibacterium* was added to the family based on genomic evidence, the former genus *Propionibacterium* was divided into two new genera, *Propionibacterium* and *Acidipropionibacterium*, which contain 10 and 7 species, respectively, and have a G+C content of approximately 68 Mol%.

Propionibacteria that are typically present in the dairy fermentation process have been re-classified. Specifically, the separation of two subspecies—*Propionibacterium freudenreichii* subsp. *freudenreichii* and subsp. *shermanii*—has been considered irrelevant. The only difference between these subspecies was whether they had the ability to reduce nitrate (subsp. *freudenreichii*) or ferment lactose (subsp. *shermanii*). Because these phenotypic traits are not genetically coupled, they had to be classified as separate phenotypes. Table 1 lists important species for food fermentation, along with their previous and new nomenclature.

For dairy-based propionibacteria, a new tool based on 16S rDNA sequences is available today: Dairy DB is a custom-made dairy-related, manually cured database taking advantage of the today available powerful bioinformatics tools (Meola et al., 2019). Former methods of propionibacteria identification using molecular tools are still valid, but WGS has increased in importance, and it allows multi-locus sequence typing to be used to group bacteria. However, phenotypic differentiation is still valid, but is not always accurate. Diaminopimelic acid (DAP) isomer comparison allows one to distinguish the genus *Acidipropionibacterium* from *Propionibacterium*.

Morphology, Envelopes and Growth Conditions

Morphology

Dairy propionibacteria are non-motile, non-spore forming, pleomorphic rods or small cocci. They may occur singly, in pairs, short chains, or clumps. Their morphology varies markedly depending on the conditions and phase of culture.

Envelope Composition

Cell lipids mainly contain branched-chain fatty acids (*anteiso*-C15:0 and *iso*-C15:0). They are present in all dairy propionibacteria species except *P. cyclohexanicum*, in which ω-cyclohexyl undecanoic acid is the major cellular fatty acid. The peptidoglycan of dairy propionibacteria may contain either *meso*-2,6-diaminopimelic acid (genus *Propionibacterium*) or LL-2,6-diaminopimelic acid (genus *Acidipropionibacterium*) (DAP in Table 2). The cell wall can include diverse compounds, such as polysaccharides (PSs) and proteins, in a strain-dependent manner, which strongly impacts the properties of the strains. PSs have been identified in the cell wall of both propionibacteria and acidipropionibacteria, and they may be either secreted in the environment, or not. In total, 35% of *P. freudenreichii* strains were shown to produce a surface-associated PS composed of β-D-glucan, for which only one protein is necessary for biosynthesis. Several other strains of propionibacteria and acidipropionibacteria have been shown to produce secreted

Table 1 Nomenclature of propionibacteria associated with food products (dairy-associated species in bold)

Previous nomenclature	*New nomenclature*
Propionibacterium acidipropionici	***Acidipropionibacterium acidipropionici***
Propionibacterium damnosum	*Acidipropionibacterium damnosum*
Propionibacterium jensenii	***Acidipropionibacterium jensenii***
Propionibacterium microaerophilum	*Acidipropionibacterium microaerophilum*
Propionibacterium olivae	*Acidipropionibacterium olivae*
Propionibacterium thoenii	***Acidipropionibacterium thoenii***
–	*Acidipropionibacterium virtanenii*
Propionibacterium cyclohexanicum	*Propionibacterium cyclohexanicum*
Propionibacterium freudenreichii* subsp. *freudenreichii	***Propionibacterium freudenreichii***
Propionibacterium freudenreichii* subsp. *shermanii	***Propionibacterium freudenreichii***

Table 2 Similar and distinctive features of propionibacteria and acidipropionibacteria

Species[a]	*Pf*	*Pc*	*Aj*	*At*	*Aa*	*Am*
Color[b]	Cream	White to cream	Cream or red-brown	Orange to red-brown	Cream to orange	White
Opt. growth temperature (°C)	30–32	35	30–32	30–32	30–32	30
DAP isomer	Meso	Meso	LL	LL	LL	NR
Whole cell sugars[c]	gal man rha	gal man glu rib rha	glu gal man		gal	NR
Catalase	+	–	+/–	+	+/–	–
Indole production	+/–	–	–	–	–	NR
Gelatin liquefaction	–	+	–	–	–	NR
Nitrate reduction	+/–	–	–	–	+	+[d]
Carbohydrates fermented						
Glycerol	+	+	+	+	+	+
Erythritol	+	–	+/–	+	+	+
Lactose	+/–	+	+/–	+/–	+	–
Saccharose	–	+	+	+	+	+
Maltose	–	+	+/–	+/–	+	+
Rhamnose	–	–	–	–	+	+
L-Arabinose	–	–	–	+/–	+	+
Esculin hydrolysis	+	+	+	+/–	+	–

NR, not reported.

[a]Pf, *P. freudenreichii*; Pc, *P. cyclohexanicum*; Aj, *A. jensenii*; At, *A. thoenii*; Aa, *A. acidipropionici*; Am, *A. microaerophilum*.

[b]The color may differ according to aerobic/anaerobic conditions of growth.

[c]glu, glucose; man, mannose; rha, rhamnose; gal, galactose; rib, ribose.

[d]Nitrate reduction to N_2.

hetero-PSs with different compositions. A paracrystalline protein surface layer (S-layer) was reported for one *A. jensenii* strain and one *P. freudenreichii* strain. In the latter species, highly diverse surface proteins were observed among strains. Lipoteichoic acids (LTAs), which include membrane-associated polymers (which characterize Gram-positive bacteria), are not found in propionibacteria and acidipropionibacteria. However, an unconventional LTA, lipomannan, was identified in *P. freudenreichii*. The cell wall components in propionibacteria and acidipropionibacteria are diverse and can vary between strains. They are key determinants of the surface properties of bacteria, and they must be taken into account when selecting strains for specific applications (e.g., food, probiotics).

Isolation and Growth Conditions

Dairy propionibacteria are capable to synthetize *de novo* all amino acids and nitrogen bases and have only few nutritional requirements. Hence, many strains are capable of growing in the absence of organic nitrogen sources in a basal medium containing carbon and energy sources, ammonium, minerals and two to four vitamins (at minimum, pantothenate and biotin). However, their growth can be stimulated when complex nitrogen sources, such as peptone or yeast extract, are provided.

These propionibacteria are anaerobic to aerotolerant. They grow best at ~30 °C, and their optimal pH is 6–7. Under optimal conditions, their generation time is about 5–6 h. At pH values below 5.5, growth still occurs, but at a markedly slower rate. Dairy propionibacteria are generally maintained and isolated in yeast extract-peptone-lactate (YEL) media at a neutral or slightly acidic pH and incubated at 30 °C in an air atmosphere without agitation. When placed in YEL-agar medium and incubated under strict anaerobic conditions at 30 °C, propionibacteria form lenticular colonies with a diameter of 1–4 mm within 5–6 days. Their color varies from light cream to orange or red-brown depending on the species (**Table 2**). Some inhibitory compounds, like cloxacillin, other antibiotics and/or lithium salts, can be used to increase the selectivity of culture media.

Genetic Properties

At the time of writing, 36 complete genome assemblies for dairy propionibacteria (specifically, the species *P. freudenreichii*, *A. acidipropionici* and *A. jensenii*) are publicly available in the NCBI's genome database (National Center for Biotechnology Information). The size of the genomes ranges from 2.5 to 3.7 Mb (**Table 3**). *A. acidipropionici* genome contains nearly 1000 more protein-coding genes than those of *P. freudenreichii* and *A. jensenii*. All the genes encoding transport proteins have a larger number of units in *A. acidipropionici* than in *P. freudenreichii*. As downsizing of the genome is associated with specialization, *A. acidipropionici* still has extensive metabolic potential. The most commonly reported complete genome sequences are those of *P. freudenreichii* strains. Elements, such as genomic islands, prophages, phages, plasmids, tandem repeats, transposases, and clustered, regularly interspaced short palindromic repeats (CRISPR) contribute to the strain specificity of propionibacteria/acidipropionibacteria.

Table 3 General features of complete genomes[a] of dairy-related propionibacteria and acidipropionibacteria

Species	*P. freudenreichii*	*A. acidipropionici*	*A. jensenii*
complete genome assemblies (n)	24	8	4
Genome size (Mb)	2.5–2.7	3.6–3.7	3.0–3.2
GC content (%)	67.2–67.4	68.7–68.8	68.5–68.8
No. of proteins	2111–2455	3117–3238	2511–2683
No. of rRNA operons	4	4–5	3–4

[a]October 2019.

Genomic islands of *P. freudenreichii* include numerous genes, such as those involved in metabolic processes (e.g., degradation of glycerol, β-D-galactose, inositol, melibiose, lactose, L-arabinose, ribose and nitrate). The cause of strain-specific properties, such as nitrate, lactose and melibiose degradation, can be explained by the presence or absence of intact genomic islands harboring all implicated genes.

Plasmids as Genetic Tools

Endogenous *Propionibacterium* plasmids are of interest in the field of genetic engineering because they can be modified into shuttle vectors for use as genetic tools (**Table 4**). Genes are incorporated into a plasmid that are propagated in *Escherichia coli* and finally transferred to *Propionibacterium*. As a result, they contain a replicative origin and an antibiotic resistance gene for *E. coli* and a replicative origin and a resistance gene for *Propionibacterium*.

Genetic Engineering

A main objective of genetic engineering has been to increase the production of propionic acid in *Propionibacterium*. Three enzymes—malate dehydrogenase (*mdh*), fumarate hydratase (*fumC*) and glycerol dehydrogenase (*gldA*)—were identified as key factors affecting propionic acid production. Due to lack of *A. jensenii* genome information, genes from *Klebsiella pneumonia* (*mdh*, *fumC* and *gldA*) were introduced into *A. jensenii* with the shuttle vector pZGX04. The co-expression of *adh* and *gldA* was most successful.

Although propionic acid synthesis in propionibacteria is hampered by organic acid accumulation, acid tolerance can be increased to overcome this issue. The genes in the arginine deaminase and glutamate decarboxylase systems significantly influence acid tolerance, and glutaminase (*ybaS*) from *E. coli* has been described as acid-resistant. Therefore, to enhance propionic acid production, acid resistance was increased by overexpressing *arcA* (arginine deiminase), *arcC* (carbamate kinase), *gadB* (glutamate decarboxylase) and *gdh* (glutamate dehydrogenase) from *A. acidipropionici* and *ybaS* from *E. coli* using shuttle vector pZGX04.

The genes encoding biotin-dependent methylmalonyl-CoA carboxytransferase (*mmc*), biotin-dependent pyruvate carboxylase (*pyc*) and biotin-dependent methylmalonyl-CoA decarboxylase (*mmd*) in *A. acidipropionici*, were cloned into the shuttle vector pKHEM04 to test their influence on propionic acid biosynthesis in *P. freudenreichii*. Mutants overexpressing *mmc* and *mmd* produce more propionic acid and less acetic and succinic acid. Mutants overexpressing *pyc* produced more succinic acid and less propionic acid. The shuttle vector pKHEM04, which was derived from pPK705 with a P4 promotor, was also applied to test the expression of *ppc* (phosphoenolpyruvate carboxylase) from *E. coli* in *P. freudenreichii* with the result of increased propionic acid production.

Table 4 Cloning shuttle vectors developed for propionibacteria and acidipropionibacteria

Name	*Size (bp)*	*Antibioresistance genes*	*Original plasmid*	*Replication type*	*References*
pZGX04	8212	CmR: *cml (A) cmx(A)* from *Corynebacterium*, AmpR: *bla* from *E. coli (pUC18)*	pZGX01	Theta	Zhuge et al. (2013)
pAMT1 and pAMT2	6250	CmR: *cml (A) cmx(A)* from *Corynebacterium*, AmpR: *bla* from *E. coli (pUC18)*	pLME108	Rolling circle	Stierli (2002)
pSL106 (or pSL104)	3316	CmR: *cml (A) cmx(A)* from *Corynebacterium*, AmpR: *bla* from *E. coli (pUC18)*	pLME108	Rolling circle	Brede et al. (2007)
pPK705	8257	HygB: *hygB* from *Streptomyces*, AmpR: *bla* from *E. coli* (pUC18)	pRG01 = pLME106	Theta	Kiatpapan and Murooka (2001)
pBRESP36A	8191	eryR: *ermE* from *Saccharopolyspora*, AmpR: *bla* from *E. coli* (pBR322)	p545	Theta	Jore et al. (2001)

Renewable biomass is preferred for propionic acid production. Lignocellulose, which contains glucose and xylose, is an abundant type of renewable biomass. However, *P. freudenreichii* cannot metabolize xylose. The shuttle vector pKHEM01 (a combination of pPK705 and a P138 promotor), which consists of *xylA* (xylose isomerase), *xylT* (xylose transporter) and *xylB* (xylulokinase) from *A. acidipropionici*, was electroporated into *P. freudenreichii* cells to allow utilization of both xylose and glucose.

To identify the function of a gene, an integrative vector can be used for genetic modification. The link between gene coding for polysaccharide synthase (*gtf*) and β-glucan capsular EPS production was proven with the integrative vector pUC:Δgtf:CmR, which disrupted *gtf* in *P. freudenreichii*. The same approach revealed the key role of surface proteins, including SlpB, in both adhesion and immunomodulation properties in this bacterium and of the secreted esterase responsible for lipolytic activity of *P. freudenreichii* in cheese.

Genetic engineering using shuttle vectors can be difficult. The efficiency of transformation is highly variable (from 10 to 10^8 [cfu] μg^{-1} of DNA) and dependent on the species, the strain, the nature of the DNA and the conditions of transformation. The natural resistance of some target strains to antibiotics that are commonly used to select transformed clones enhances the difficulties to screen transformants.

Immunity

Integral part of the genome are restriction-modification systems (RM-system) and CRISPR, which prevent the entry of foreign DNA. CRISPR, together with the CRISPR associated proteins (Cas), are present in several loci within the genome of most propionibacteria. This immune system is constantly expanding and specifying by integrating foreign DNA (spacer), separating it through direct repeat sequences. The spacers are later used in the detection and destruction of invading DNA. Up to 105 spacers are recorded in one CRISPR locus in *P. freudenreichii*. Results of investigated *P. freudenreichii* spacers indicate the immunity against the already sequenced phages: B22, Anatole, E1, Doucette, E6, G4, B3. All these phages contain a putative tyrosine integrase suggesting they are temperate. It has been proposed to investigate the practicality of this trait for the development of integration vectors, in order to increase the stock of genetic tools that can be used in *P. freudenreichii*.

The RM-system recognizes and cleaves foreign DNA, while the host DNA is protected by methylation at the recognition sequence. The RM-system in *P. freudenreichii* was investigated in detail, thanks to the PacBio sequencing method, which enables the detection of the methylated bases m6A and m4C, and, thus, the identification of the recognition sequence in which they occur. The results revealed high variability in the recognition sequences and in their genomic locations.

Metabolism

Propionibacteria isolated from cheese and dairy-related environments are widely used in the cheese industry for their ability to produce metabolites that influence cheese opening and flavor. They are also able to synthetize a range of metabolites of nutritional value.

Pyruvate Metabolism

Propionibacteria can ferment many substrates, including carbohydrates, polyols, such as glycerol and erythritol, and organic acids, such as lactic acid. Carbohydrates are oxidized to produce pyruvic acid via glycolysis or the pentose phosphate pathway. Then, pyruvic acid can be temporarily exported outside of the cell or converted to lactic acid and alanine. In addition, pyruvic acid is a branching point that can be used for either NADH-generating acetic acid synthesis or NADH-consuming propionic acid synthesis (**Fig. 1**).

Propionic acid formation has been well studied in the context of *P. freudenreichii*. In Swiss-type cheeses, this species uses lactic acid as the main carbon source for propionic acid formation. Lactic acid is converted to pyruvic acid, which is then metabolized via the methylmalonyl-CoA pathway (also named the transcarboxylase cycle or WWC; **Fig. 1**). This pathway includes the dicarboxylic acids oxaloacetic, malic, fumaric, and succinic acids, and it utilizes the NADH formed during glycolysis, during the conversion of lactic acid to pyruvic acid or during the conversion of pyruvic acid to acetic acid. All reactions in the WWC are reversible. One of the key reactions is a transcarboxylation reaction without the intervention of free CO_2, in which a carboxyl group is transferred from methylmalonyl-CoA to pyruvic acid to form oxaloacetic acid and propionyl-CoA (reaction 1, **Fig. 1**). This reaction is catalyzed by a biotin-dependent methylmalonyl-CoA carboxytransferase (EC 2.1.3.1), which to date has only been found in propionibacteria. The enzyme consists of three polypeptide subunits. The expression of a gene coding for one of the subunits has been used as a specific molecular marker to investigate the probiotic abilities of *P. freudenreichii* in the human gut. Another key player in the synthesis of propionic acid is the methylmalonyl-CoA mutase (EC 5.4.99.2), also known as methylmalonyl-CoA isomerase. The enzyme is vitamin B12-dependent and catalyzes the isomerization of succinyl-CoA into methylmalonyl-CoA (reaction 2, **Fig. 1**). The second main pathway of pyruvic acid conversion is decarboxylative oxidation to acetyl-CoA via pyruvate dehydrogenase activity, which results in the formation of acetic acid and CO_2 with the concomitant production of NADH (reaction 3, **Fig. 1**).

To maintain their redox balance, cells modulate the proportion of pyruvic acid catabolized to acetic acid, CO_2 and propionic acid, as a function of the amount of reduced coenzymes produced when a substrate is oxidized to pyruvic acid. Typically, when

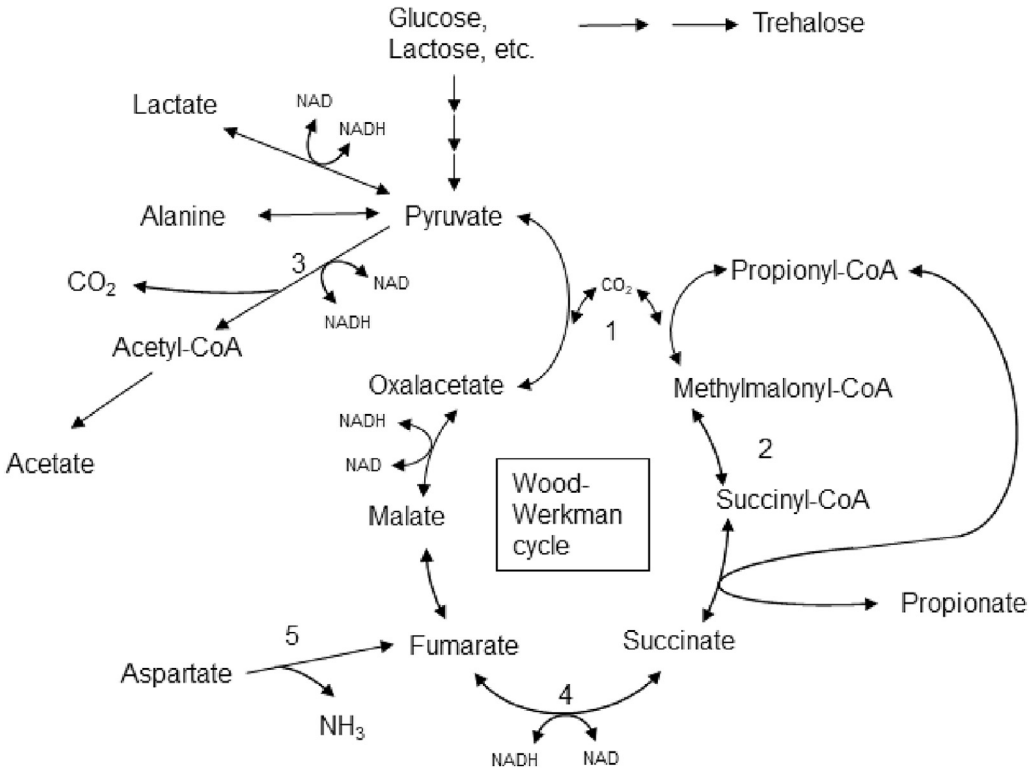

Figure 1 Proposed metabolism of pyruvate in *P. freudenreichii*. 1: Methylmalonyl-CoA carboxytransferase. 2: Methylmalonyl-CoA mutase. 3: Pyruvate dehydrogenase. 4: Fumarate reductase. 5: Aspartate ammonia-lyase.

3 mol of lactic acid (or 1.5 mol of glucose) are oxidized to 3 mol of pyruvic acid, 1 mol of pyruvic acid is oxidized to acetic acid and CO_2 and the remaining 2 mol of pyruvic acid is reduced to propionic acid via the WWC based on the Fitz equation (with a molar ratio of propionic acid to acetic acid of 2:1). The oxidation of glycerol to pyruvic acid generates more reduced cofactors than the oxidation of lactic acid or glucose. In this case, pyruvic acid is converted into propionic acid only.

Aspartate Catabolism

Various strains of *P. freudenreichii* cometabolize aspartic acid with lactic acid. The analysis of genome data indicates that two enzymes can initiate aspartic acid catabolism: an aspartate oxidase (E.C. 1.4.3.16), which converts aspartic and fumaric acids into succinic and iminosuccinic acids, respectively, and an aspartate ammonia-lyase (E.C. 4.3.1.1), which catalyzes the deamination of aspartic acid, yielding fumaric acid and ammonia. Proteomic analysis revealed that both enzymes are produced in the *P. freudenreichii* strain CIRM-BIA1.

It is worth noting that various strains possess two neighboring genes that encode an aspartate ammonia-lyase. In the context of cheesemaking, it can be observed that such strains produce more succinic acid than those possessing only one gene. Additionally, the increased formation of succinic acid is associated with an increased formation of gas. The molecular reasons for these observations are not yet completely understood and the current research aims to gain a deeper understanding of how the genes involved in the catabolism of aspartate influence the quality of cheese.

P. freudenreichii can also grow in the absence of lactic acid when aspartic and propionic acids are provided. In this case, the WWC is reversed and propionic acid is converted into succinic acid. These reactions require the activity of aspartate ammonia-lyase (reaction 5, Fig. 1) since aspartic acid is indirectly the supplier of a CO_2 group for the methymalonyl-CoA carboxytransferase (reaction 1, Fig. 1). Whether these metabolic reactions occur in cheese, when lactic acid is exhausted, has not been studied yet.

Vitamin B12 Biosynthesis

The biosynthesis of vitamin B12 in *P. freudenreichii* starts with glutamic acid, which is converted into 5-aminolevulinic acid through a series of reactions. This acid is a requirement for the formation of porphobilinogen, which is subsequently polymerized into preuroporphyrinogen. This molecule undergoes further reactions until active, cobalt-containing vitamin B12 is produced. At least 30 genes are involved in vitamin B12 biosynthesis. It is worth noting that biosynthesis requires anaerobic and aerobic conditions and that the active form of vitamin B12 is produced in the presence of oxygen and blue light. Understanding the factors (e.g., oxygen,

pH, temperature, presence of acids, availability of precursors) that influence this complex pathway is important for selecting strains with enhanced vitamin B12 formation.

Trehalose

Propionibacteria can synthesize high amounts of trehalose, a disaccharide that consists of two (1→1) linked glucose molecules, in response to stress, since this compound exhibits protective properties when temperature, osmotic pressure, pH or water activity change. Lactose is a good source for trehalose formation. After cleavage of lactose into glucose and galactose, glucose is utilized for trehalose synthesis with glucokinase, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. The amount of trehalose produced varies between strains of *P. freudenreichii* and a screening for high producers is recommended to enrich fermented dairy products with trehalose.

Red Pigment

A. jensenii and *A. thoenii* can produce a red pigment that appears as spots in cheese, which is considered to be a cheese defect. In *A. jensenii*, the pigment was identified as an ornithine rhamno-polyene named granadaene. Currently, the metabolic pathways for granadaene biosynthesis are unknown.

Adaptation to Stress

As the predominant microflora in Swiss-type cheese and a probiotic food complement, dairy propionibacteria are exposed to technological and digestive stresses. In both applications, stress adaptation constitutes a bottleneck for propionibacteria efficacy. Propionibacteria have been isolated from diverse ecological niches, including soil, rumen and wastewater, indicating that they can adapt to various environmental parameters. Furthermore, they were recently identified as beneficial commensals of the human gut microbiota in healthy infants, meaning that they have the ability to adapt and survive in stressful environments. Surprisingly, even though they display remarkable adaptation abilities, their constitutive stress tolerance is low unless they are pre-adapted.

Evidence of osmotic adaptation, which is relevant to cheesemaking because of the salting step, was found in dairy propionibacteria. In rich media, such as YEL, or in the presence of exogenous osmoprotectants, including glycine betaine, dimethylsulfonioacetate or dimethylsulfoniopropionate, such propionibacteria grow despite NaCl concentrations of 0.5–1 M. In the absence of such osmoprotectants, propionibacteria can synthesize and accumulate large amounts of trehalose in response to osmotic or cold stress. Trehalose, as well as glycogen, can be accumulated in propionibacteria and serve as cellular carbon reserve compounds. Recently, *P. freudenreichii* was shown to adapt and grow in hyper-concentrated dairy growth media with osmolality up to six times higher than that of whey. In such conditions, *P. freudenreichii* accumulates the above-mentioned osmoprotectants, including glycine betaine, as well as trehalose, glycogen and polyphosphates. It furthermore acquires enhanced tolerance toward various kinds of stresses.

Heat stress adaptation, which enables survival at temperatures up to 55 °C, can be triggered by mild heat pre-treatment (42 °C). In *P. freudenreichii*, it involves over-expression of chaperones, ATP-dependent proteases and proteins taking part in the SOS response, metabolism of the cell wall, remediation of reactive oxygen species and nucleotide phosphorylation.

Dairy propionibacteria experience acid stress during both fermentation processes and transit through the digestive tract. Evidence of an acid tolerance response has been found for *P. freudenreichii*; exposure to moderate acidic conditions (pH of 4–5) led to tolerance of acid stress to a pH of 2. This acid tolerance response depends on over-expression of enzymes involved in DNA synthesis and repair, enzymes involved in the central carbon metabolism (including the transcarboxylase cycle), enzymes specific to propionic fermentation in propionibacteria, ATP-dependent proteases and chaperones.

One of the major stresses in the digestive tract is the toxic effect of bile salts. Naïve *P. freudenreichii* cells exhibit a dramatic loss of viability upon exposure to physiological concentrations of bile salts present in the colon, but pre-exposure to lesser concentrations leads to efficient adaptation. This adaptive pathway depends on over-expression of proteins involved in stress sensing and signal transduction and of enzymes involved in oxidative stress remediation and detoxification. Tolerance towards digestive stress and proteolysis is drastically enhanced by the inclusion of dairy propionibacteria within a dairy matrix, which leads to enhanced biological activity *in vivo*.

For all the considered stresses, it should be noted that there are considerable differences, in terms of susceptibility, between strains. The most tolerant strains were shown to constitutively over-express a set of stress proteins.

Use as Ripening Cultures in Cheese

Occurrence and Application

In order to achieve the characteristic eyes and nutty flavor of cheeses by propionic acid fermentation, strains of *P. freudenreichii* are indispensable. Some traditional varieties of cheese, such as PDO Comté, may undergo spontaneous propionic acid fermentation if dairy propionibacteria are present in raw milk. Propionibacteria occur naturally in the rumen and intestine of ruminants, in soil

and in silage, and therefore a low amount is also present in raw milk. The strain diversity of natural propionibacterial communities (e.g., in raw milk and cheeses) is great and has not been influenced by the use of commercially available cultures. In traditional PDO Emmentaler made from raw milk, propionibacteria are inoculated in doses of 10^3–10^4 cfu mL^{-1} of milk. In pasteurized Swiss-type cheeses, such as Jarlsberg or Maasdam, propionibacteria are added to cheese milk to guarantee that they are present in cheese post-manufacture at a rate of approximately 10^3 cfu g^{-1}. Small amounts of propionibacteria do not develop well in milk, but they do in cheese; populations ranging from 10^2–10^8 cfu g^{-1} can be detected in cheeses with spontaneous propionic acid fermentation, and populations of over 10^8–10^9 cfu g^{-1} can be detected in cheeses in which propionibacteria were inoculated.

Many factors influence the growth of *P. freudenreichii* in Swiss-type cheeses, including the temperature during manufacturing and ripening, the pH of the cheese and the NaCl concentration. This species can endure the cooking required for hard cheeses (~50–55 °C for 30 min) much better than acidipropionibacteria, and it exhibits optimal growth at temperatures of 25–35 °C. Thus, to ripen Swiss-type cheeses, it is necessary to place them in a warm room (17–24 °C). Furthermore, *P. freudenreichii* is quite sensitive to low pH values and is unable to grow at pH values below 5.0. Thus, a fundamental step in the manufacture of e.g., PDO Emmentaler, Maasdam, Grevé and Jarlsberg is the addition of water to the milk and/or curd (12%–30%) to reduce the lactose concentration. This leads to a relatively high pH value after lactic fermentation (pH of 5.20–5.35), consequently promoting propionic acid fermentation. The strain-dependent salt sensitivity of propionibacteria can be addressed by using lower salt content in cheeses, such as PDO Emmentaler, or selecting less salt-sensitive strains for cheeses, such as Jarlsberg or Maasdam. Finally, copper originating from the copper vats used in traditional manufacturing conditions is known to slow down (and therefore control) propionibacterial growth. Addressing all of these factors can guarantee that the produced cheese is of optimal quality.

Role in the Formation of Cheese Flavor

The flavor compounds produced by *P. freudenreichii* have three main origins: fermentation of lactic acid into propionic acid and acetic acid; hydrolysis of milk fat into free fatty acids; and catabolism of free amino acids into succinic acid, ammonia and short branched-chain fatty acids. The rate and extent of flavor compounds production is strain-dependent and may vary considerably.

Propionic acid and acetic acid (in their free form and/or as salts) are potent compounds producing a sweet and slightly sour taste.

Propionibacteria have very low caseinolytic activity, but they contain diverse intracellular peptidases (Pep X, Pep F), which may influence the amino acid profile of cheeses. They also have a strong strain dependent ability to convert branched-chain amino acids (i.e., leucine, isoleucine and valine) into carboxylic acids and alcohols. Isoleucine, as the amino acid that is preferentially catabolized, is mainly converted into 2-methylbutyric acid, while leucine is converted into isovaleric acid (3-methylbutyric acid) and valine into isobutyric acid (2-methylpropanoic acid). Their concentration is 3–10 times higher in Swiss-type cheeses than in cheeses without propionic acid fermentation. Thus, the presence of high levels of 2-methyl-branched-chain compounds (cheesy/sweaty notes) is a characteristic of Swiss-type cheeses. The aspartate ammonia-lyase activity of *P. freudenreichii* also varies significantly between strains, which influences the amount of succinic acid that is produced. Together with glutamic and propionic acid, succinic acid is thought to contribute to the umami taste of Swiss-type cheeses.

Lipolysis by propionibacteria is generally recognized to be necessary to achieve the typical flavor of Swiss-type cheese. The hydrolysis of milk fat results in the release of free fatty acids, which serve as precursors to other flavor compounds in cheese. The amount of free fatty acids found in Swiss-type cheeses varies from 2 to 4 g kg^{-1}; higher amounts may cause a rancid flavor. Fat hydrolysis occurs simultaneously with propionibacterial growth and is mainly due to the activity of an extracellular lipolytic esterase. The release of intracellular enzymes from lyzed cells at this early stage of growth is very unlikely.

Twelve genes encoding putative esterases were identified in the genome sequence of *P. freudenreichii*. Ten esterases were predicted to be intracellular, PF#774 was surface-exposed and PF#279—the main lipolytic esterase—was secreted into the medium. All *P. freudenreichii* strains exhibit esterase activities, but large differences have been observed between strains. In Swiss-type cheeses, ethyl esters of C2:0–C8:0, secondary alcohols and several other straight- and branched-chain alkyl esters of propionic acid and acetic acid (which produce a fruity flavor) were detected. *P. freudenreichii* is capable of synthesizing esters via both esterification and alcoholysis reactions.

Role in the Formation of Eyes in Swiss-Type Cheeses

The entire cheese manufacturing process is aimed at creating optimal conditions for not only propionic acid fermentation but also eye formation. Propionibacteria play a key role in the formation of the typical round eyes in Swiss-type cheeses. While ripening in a warm room, *P. freudenreichii* grows actively and releases CO_2. Saturation of the cheese matrix with CO_2 (>18–36 mmol kg^{-1}) and the presence of nuclei (microscopically small openings) are required for eye formation. As soon as sufficient eyes have developed, production of CO_2 is slowed down by storing the cheeses at lower temperatures. During PDO Emmentaler ripening, up to 45 mmol kg^{-1} (~1 L kg^{-1} under normal conditions) of CO_2 can be produced, of which more than 80% originates from propionic acid fermentation. The resulting eye volume is around 4% of the cheese volume.

Aspartate ammonia-lyase activity is highly strain-dependent and may accelerate propionic acid fermentation. As a result, significantly more CO_2 will be liberated, as might be expected from aspartate ammonia-lyase activity, leading to the formation of more and larger eyes.

A soft, elastic texture is crucial for regular eye formation. The elevated temperature in the warm room during ripening ensures optimal softening of the cheese body during initial CO_2 production. The amount of casein-associated calcium in Swiss-type cheeses is higher ($\sim$10 g kg^{-1}) than in other cheese varieties due to the high pH after lactic acid fermentation (pH of 5.20–5.35). This is very important for achieving an elastic texture and, therefore, for optimal eye formation later in the ripening process.

Interactions With Lactic Acid Bacteria

Propionic acid fermentation of *P. freudenreichii* in cheese is highly affected by the type of lactic acid bacteria applied during the manufacturing process. The extent of these interactions has been studied for a long time, but the mechanisms have still not been fully elucidated.

In the production of traditional Swiss-type hard cheeses, homofermentative thermophilic lactic acid bacteria are typically used in starters, often as mixed cultures of lactobacilli (*Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis*) and streptococci (*Streptococcus salivarius* subsp. *thermophilus*). Lactose is fully hydrolyzed within 4–6 h after molding of the curd, and lactic acid fermentation is completed after 24 h, releasing L (+)- and D (-)-lactic acid in a ratio of approximately 1:1. Lactobacilli can significantly stimulate propionibacterial growth by releasing peptidases into the cheese matrix after lysis, perhaps as a result of the proteolytic activity of lactobacilli liberating the peptides and free amino acids needed by propionibacteria and/or the removal of an inhibitory peptide by *L. helveticus*.

Mesophilic lactic acid bacteria play an important role in initiating eye formation in cheese. Facultatively heterofermentative lactobacilli (e.g., *Lactobacillus paracasei/casei* and *Lactobacillus rhamnosus*), *Leuconostoc* spp. and citrate-positive *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* metabolize most of the available citric acid before warm-room ripening, releasing CO_2 and thus initiating eye formation. However, heterofermentative mesophilic lactobacilli can inhibit propionic acid fermentation. These species are present in many raw milk cheeses as natural non-starter lactic acid bacteria, or they may be deliberately added as adjunct cultures to control propionic acid fermentation and thus prevent late fermentation. Their inhibitory effect on propionibacteria is strain-dependent and is at least partly due to their ability to metabolize citric acid, which results in the formation of inhibitory products, such as diacetyl, formic and acetic acids. In traditional Swiss-type cheeses manufactured in copper vats, it also leads to the release of copper chelated by citric acid into the aqueous phase of cheese.

Relation to Nutrition as Probiotics and Producers of Nutraceuticals

Interest in Propionibacteria as Probiotics

Dairy propionibacteria do not possess any known virulence factor, although *A. thoenii* strains and some *A. jensenii* strains show β-haemolytic activity. Dairy propionibacteria exhibit natural resistance to several antibiotics, which does not appear to be plasmid-encoded. All strains of interest should be tested to determine their antibiotic resistance profile prior to use as either a dairy starter or probiotic.

Selected strains of dairy propionibacteria display remarkable robustness and efficient adaptive responses to digestive stresses, consistent with studies on their survival and metabolic activity within the human digestive tract. Moreover, the observed adhesion to human intestinal mucus is consistent with the persistence of probiotic dairy propionibacteria during the two weeks following ingestion. *In vivo*, *P. freudenreichii* adapts to the colon environment and redirects its cell machinery toward utilization of substrates available in the colon (i.e., propanediol, gluconic acid, lactic acid, nitrogen bases and amino acids).

Evidence of stimulation of bifidobacteria growth was found in humans, and the corresponding mechanisms were well established *in vitro*. Propionibacteria release 1,4-dihydroxy-2-naphthoic acid (DHNA), a bifidogenic factor favoring growth of bifidobacteria *in vitro* and *in vivo*. Alleviation of constipation was observed as a result of *P. freudenreichii* ingestion in humans. Propionibacterial metabolites, including short chain fatty acids, are thought to favor intestinal motility and absorption of divalent cations. Enhanced iron absorption from a rat colon was reported in the presence of *P. freudenreichii*.

Production of beta-galactosidase and hydrolysis of lactose, stimulated by exposure to bile, may have a role in lactose intolerance treatment, although this remains to be confirmed in humans.

A very promising—but still poorly understood—area of research is the impact of propionibacteria on the human immune system. Selected strains of *P. freudenreichii* revealed very promising immunomodulatory properties *in vitro*, which were further confirmed by the *anti*-inflammatory effect observed in animal models of colitis. Indeed, surface proteins were shown to mediate both adhesion to host cells and modulation of intestinal inflammation. This effect was further potentiated by the inclusion of propionibacteria within a cheese matrix. Accordingly, propionibacteria were identified as early healthy gut microbiota in breast-fed human infants, mitigating intestinal inflammation via regulation of Th17 cells.

Regarding carcinogenesis, ingestion of dairy propionibacteria resulted in lowered bioavailability and absorption of aflatoxin B1 in humans who consumed food contaminated with this human hepatocarcinogen. Furthermore, a mixture of *P. freudenreichii* and *Lactobacillus rhamnosus* was shown to lower the fecal activity of azoreductases, which are enzymes involved in carcinogen biosynthesis. Finally, induction of colon cancer cell apoptosis by dairy propionibacteria, both *in vitro* in cultured adenocarcinoma cells and *in vivo* in mutagenized rats, opens new perspectives for further research on probiotics in this field.

Production of Nutraceuticals

The transcarboxylase cycle, or WWC, involved in propionic acid fermentation and used by dairy propionibacteria, requires enzymes, which in turn require cofactors, including vitamin B12 (cobalamin), B9 (folic acid) and biotin, for functioning. Dairy propionibacteria have been used for industrial production of food-grade vitamin B12, and selected strains excrete vitamin B9, suggesting that dairy products containing these strains may be a source of folate. One strain of *P. freudenreichii* was shown to produce large amounts of vitamin B2 (riboflavin) in fermented milk, which was proven to efficiently balance a riboflavin-deficient diet. Furthermore, some strains of *P. freudenreichii* convert free linoleic acid into conjugated linoleic acid, and the main isomer that is produced is rumenic acid (*cis*-9, *trans*-11-octadecadienoic acid), which is considered beneficial in the prevention of inflammatory and cardiovascular diseases. Finally, the large amounts of trehalose accumulated in propionibacteria may prove useful as low-calorie dietetic sugar.

Use as Protective Cultures

Propionibacterium spp. and *Acidipropionibacterium* spp. strains are widely used as food biopreservatives for their antimicrobial activity and production of organic acids, particularly propionic acid, diacetyl and bacteriocins. In addition, they suppress spoilage by yeast, molds and *Bacillus* spp. in many foods, including fresh fermented dairy products and sourdough.

Propionic acid is an antifungal agent, and food-grade propionic acid can be produced (rather than chemically synthesized) by propionibacteria or acidipropionibacteria. Skim milk fermented by *P. freudenreichii* is used to create a commercially available product, Microgard™ (DuPont Danisco), which exhibits inhibitory activity in relation to fungi and some Gram-negative bacteria. In addition, controlled fermentation of whey with *P. freudenreichii* produces Inhibit 3600 Dairy™ (Mezzoni Foods), a natural shelf life extender.

Propionibacterium and *Acidipropionibacterium* produce other compounds with synergistic inhibitory effects, like acetic acid, succinic acid, diacetyl, and other organic acids, including 2-pyrrolidone-5-carboxylic, 3-phenyllactic and 4-hydroxyphenyllactic acids. These organic compounds are part of the inhibitory mechanism induced by *P. freudenreichii* and facultative heterofermentative lactobacilli in the protective cultures Holdbac™ YM-B (formerly BioProfit), Holdbac™ YM-C (DuPont Danisco) and Befresh™ (Handary). In general, co-cultures of propionibacteria and lactobacilli exhibit stronger antimicrobial effects than either culture alone.

Different bacteriocins produced by dairy propionibacteria have been reported and characterized (**Table 5**). They have proven to be useful for food preservation due to their high stability in a wide range of pH values and temperatures. Some are produced by different species; for example, the propionicins SM1 and SM2 were first isolated from *A. jensenii* DF1 cultures but were later detected

Table 5 Bacteriocins of dairy-related propionibacteria and acidipropionibacteria

Name	*Size (Da)*	*Producer strain*	*Target microorganisms*	*References*
Jenseniin G	> 12000	*A. jensenii* P126 (ATCC 4872), reclassified as *A. thoenii*	*A. acidipropionici, A. jensenii*, lactococci, lactobacilli, clostridial spores	Grinstead and Barefoot (1992), Ekinci and Barefoot (1999), and Holo et al. (2002)
Propionicin PLG-1 9328	9328	*A. thoenii* P127	*A. thoenii, A. jensenii, A. acidipropionici*, lactic acid bacteria, yeasts and molds, Listeria, Pseudomonas, Vibrio, Yersinia	Lyon and Glatz (1993) and Lyon et al. (1993)
Propionicin SM1	22 300	*A. jensenii* DF1	*A. jensenii*, yeasts and molds	Miescher (1999) and Miescher et al. (2000)
Propionicin SM2	13 600			
Propionicin T1	7130	*A. thoenii* 419, *A. thoenii* LMG2792	*A. thoenii, A. jensenii, A. acidipropionici*, and *C. acnes*	Faye et al. (2000) and Faye et al. (2004)
Jenseniin P	6000–9000	*A. jensenii* B1264	Cutibacteria, lactobacilli	Barefoot and Ratnam (2001)
PAMP[a]	6383	*A. jensenii* LMG3032	*A. acidipropionici, A. jensenii, A. thoenii, P. freudenreichii*, lactobacilli	Faye et al. (2002) and Faye et al. (2004)
Propionicin F	4397	*P. freudenreichii* LMGT2946, *P. freudenreichii* LMGT2956	*P. freudenreichii*	Brede et al. (2004)
Thoeniicin 447	7130	*A. thoenii* 447	*C. acnes*, lactobacilli	Van der Merwe et al. (2004)
BLIS[b]		*A. jensenii* B1264	*C. acnes*	Wang et al. (2014)

[a]PAMP, protease-activated antimicrobial peptide.
[b]BLIS, bacteriocin-like substance.

in *A. acidipropionici* and *P. freudenreichii* cultures. Interestingly, jenseniin P and thoeniicin 447 are active against cutibacteria such as *C. acnes* and may be useful for combating opportunistic pathogens. More research is needed to assess the potential application of bacteriocin-producing strains of *Propionibacterium* and *Acidipropionibacterium* as food biopreservatives or probiotics to inhibit pathogens. New bioinformatics strategies for metadata integration could identify and prioritize antimicrobial biosynthetic gene clusters for further study.

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