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Review

New insights into physiology and metabolism of *Propionibacterium freudenreichii*

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ABSTRACT

Dairy propionibacteria are Actinobacteria, mainly isolated from dairy environments. *Propionibacterium freudenreichii* has been used for a long time as a ripening culture in Swiss-type cheese manufacture, and is more and more considered for its potent probiotic effects. This review summarises the knowledge on the main *P. freudenreichii* pathways and the main features explaining its hardiness, and focuses on recent advances concerning its applications as a cheese ripening agent and as a probiotic for human health. Propionibacteria have a peculiar metabolism, characterised by the formation of propionic acid as main fermentation end-product. They have few nutritional requirements and are able to use a variety of carbon substrates. From the sequence of *P. freudenreichii* CIRM-BIA1^T genome, many pathways were reconstituted, including the Wood–Werkman cycle, enzymes of the respiratory chain, synthesis pathways for all amino acids and many vitamins including vitamin B_{12}. *P. freudenreichii* displays features allowing its long-term survival. It accumulates inorganic polyphosphate (polyP) as energy reserve, carbon storage compounds (glycogen), and compatible solutes such as trehalose. In cheese, *P. freudenreichii* plays an essential role in the production of a variety of flavour compounds, including not only propionic acid, but also free fatty acids released via lipolysis of milk glycerides and methyl-butanoic acids resulting from amino acid degradation. *P. freudenreichii* can exert health-promoting activities, such as a bifidogenic effect in the human gut and promising immunomodulatory effects. Many *P. freudenreichii* properties involved in adaptation, cheese ripening, bio-preservation and probiotic effects are highly strain-dependent. The elucidation of the molecular mechanisms involved is now facilitated by the availability of genome sequence and molecular tools. It will help in the selection of the most appropriate strain for each application.

Contents

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1. Introduction

Propionibacteria are Gram positive, high G + C%, mesophilic, aerotolerant, pleomorphic rods. They have a peculiar metabolism leading to the formation of propionic acid as main end-product of fermentation. They display low nutritional requirements and are characterised by their long-term survival in different environments.

P. freudenreichii was first described more than one century ago in Swiss Emmental cheese by Orla Jensen and von Freudenreich (von Freudenreich and Orla-Jensen, 1906), who showed the relationship between the presence of these bacteria producing propionic acid and the formation of the characteristic round holes (eyes) in cheese.

P. freudenreichii is widely used as a ripening culture in the manufacture of Swiss-type cheeses (Dorner, 1939; Langsrud and Reinbold, 1973a; Steffen et al., 1993). It is also known for its production of vitamin B_{12} and propionic acid, and has properties as protective cultures in food and feed (Thierry et al., 2011). Moreover *P. freudenreichii* is also more and more studied for its probiotic properties (Cousin et al., 2010).

This review summarises the knowledge on the main metabolic pathways of *P. freudenreichii* and the main characteristics explaining its hardiness, and focuses on recent advances concerning its applications as a cheese ripening agent and as a probiotic for human health.

2. General features of propionibacteria

2.1. Propionibacteria taxonomy

Propionibacteria belong to the Actinobacteria class, comprising high G + C content Gram-positive bacteria (Stackebrandt et al., 1997). Actinobacteria exhibit a wide range of morphologies, lifestyles, physiological and metabolic properties and colonise various niches including soil (Ventura et al., 2007) The *Propionibacterium* genus currently comprises 12 species (http://www.bacterio.cict.fr). Two distinct groups are distinguished on the basis on their natural biotopes: "dairy" (or "classical") and "cutaneous" propionibacteria (Cummins and Johnson, 1986). Dairy propionibacteria have been traditionally isolated from milk and dairy products. Four typical dairy species were early described: *P. freudenreichii*, *P. acidipropionici*, *P. jensenii* and *P. thoenii*. Cutaneous propionibacteria are commensal of mammals including humans, the most studied species within this group being *P. acnes,* involved in acne and in post surgery infections. Four propionibacteria species, isolated from other biotopes than dairy products and human skin, were more recently described: *P. cyclohexanicum* isolated from spoiled orange juice (Kusano et al., 1997), *P. microaerophilum* from olive mill wastewater (Koussemon et al., 2001), *P. australiense* from granulomatous bovine lesions (Bernard et al., 2002), and *P. acidifaciens*, isolated from human mouth (Downes and Wade, 2009). The analysis of 16S rRNA gene sequences showed that *P. cyclohexanicum*, *P. acidifaciens* and *P. australiense* are phylogenetically related to *P. freudenreichii*, whereas *P. microaerophilum* is related to *P. acidipropionici* (Downes and Wade, 2009).

2.2. Genetic diversity of P. freudenreichii

The genome size of *P. freudenreichii* ranges around 2.6 Mb and its G + C content is 67%. The first genome of a *P. freudenreichii* strain (CIRM-BIA1^T) has recently been sequenced (Falentin et al., 2010a). Furthermore, molecular tools have newly been developed to over pass *P. freudenreichii*'s poor efficiency of transformation (from 10^3 to 10^8 colony-forming units (cfu)/μg of DNA), thus allowing the exploration of the role of specific genes of interest (Thierry et al., in 2011; Van Luijk et al., 2002).

Data on genomic biodiversity within *P. freudenreichii* species are scarce. Fingerprinting methods such as Pulsed-Field Gel Electrophoresis and Randomly Amplified Polymorphic DNA-PCR are available to characterise *P. freudenreichii* at the strain level (Meile et al., 2008). Multi-locus sequence typing (MLST) based on the sequence analysis of internal fragments of 7 genes has recently been developed for *P. freudenreichii*. It was applied to 113 strains of different phenotypes and origins (Dalmasso et al., 2011). MLST resolved 46 sequence types grouping each 1 to 11 strains. This study showed that the *P. freudenreichii* core genome possesses a low level of nucleotide polymorphism and that recombination played a significant role in the distribution of this polymorphism among isolates (Dalmasso et al., 2011).

P. freudenreichii was in the past divided into two subspecies on the basis of lactose fermentation and nitrate reductase activity. *P. freudenreichii* subsp. *freudenreichii* is unable to ferment lactose and shows nitrate reductase activity whereas *P. freudenreichii* subsp. *shermanii* exhibits the opposite properties (Cummins and Johnson, 1986). However, the existence of *P. freudenreichii* strains harbouring the two other possible phenotypes (lactose and nitrate reductase positive, and lactose and nitrate reductase negative) has also been mentioned (Dalmasso et al., 2011; de Carvalho et al., 1994; Moore and Holdeman, 1986; Vorobjeva, 1999). In the same way, annotation of the complete *P. freudenreichii* CIRM-BIA1^T genome revealed that the *lacZ* gene responsible for betagalactosidase activity is harboured by a transposon-like mobile element and that the locus responsible for nitrate reduction activity harbours some pseudogenes (Falentin et al., 2010a). Moreover, the phylogenetic analysis of *P. freudenreichii* population using MLST did not fit with the distinction of subspecies (Dalmasso et al., 2011). All these results suggest that there seems to be no justification for separation at the subspecies level according to these two phenotypic criteria.

2.3. Propionibacteria ecology

Strains of dairy origin constitute the main part (80 to 90%) of the *P. freudenreichii* strains available in the international collections (for example, in the Belgian Co-ordinated Collections of Micro-organisms (BCCM™)/LMG collection and in the CIRM-BIA (Centre International de Ressources Microbiennes — Bactéries d'Intérêt Alimentaire, INRA, Rennes, France), and in other large laboratory collections (Fessler, 1997). However, a few *P. freudenreichii* strains have also been isolated from other biotopes, like hay and straw. Its known habitats are related to cattle environment but would require further investigations, since no systematic search has been made for propionibacteria in habitats distinct from dairy and cattle environments.

In most cheeses, *P. freudenreichii* and other dairy propionibacteria are present at low population density. In Emmental and similar cheeses, however, *P. freudenreichii* reaches a high population density, with counts over 10^9 cfu/g of cheese during the whole ripening time. The high thermotolerance of *P. freudenreichii,* compared to the other dairy species, would be responsible for the prevalence of this species

in Emmental-type cheeses in which the curd is heated at 50–55 °C for about 30 min.

2.4. Safety aspects

P. freudenreichii has a long history of safe use in human diet and animal feed. It has been granted the Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration (Mogensen et al., 2002). *P. freudenreichii* also belongs, as well as *P. acidipropionici*, to the list of agents recommended for Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA, 2009). QPS is a generic risk assessment approach introduced by EFSA for harmonising the assessment of notified biological agents intended to be introduced into the food chain (Leuschner et al., 2010).

The antibioresistance of the strains intended to be used in food and feed should be investigated. Data about the antibioresistance of *P. freudenreichii* and other dairy propionibacteria species are very scarce. Dairy propionibacteria have intrinsic or "natural" resistance to several antibiotics, including sulfonamides, oxacillin, aminoglycosides, 1st and 2nd generation quinolones, colistin, metronidazole and fosfomycin (Cummins and Johnson, 1992; Meile et al., 2008; Suomalainen et al., 2008). No mobile DNA encoding antibioresistance was detected in dairy propionibacteria. The studies on antibiotic resistance of propionibacteria mainly focused on cutaneous species of clinical importance, especially on *P. acnes* isolates (Nord and Oprica, 2006). Minimal Inhibitory Concentration (MIC), defined as the lowest concentration of the antimicrobial that inhibits bacterial growth, have been recently proposed in a Technical Guidance of EFSA for some bacteria used in animal feed including *Propionibacterium*, but were mainly based on data extrapolated from cutaneous propionibacteria studies (EFSA, 2008).

3. *P. freudenreichii*, a hardy bacterium with original metabolic pathways

Propionibacteria exhibit some peculiar metabolic pathways that have been thoroughly investigated (see reviews (Hettinga and Reinbold, 1972; Piveteau, 1999; Wood, 1981)). The metabolism of propionibacteria is very complex, because several interconnected pathways are used simultaneously. Propionibacteria are anaerobic but are also aerotolerant. They have low nutritional requirements and are able to fit, survive and remain active in various environments. This section summarises the knowledge on the main pathways of *P. freudenreichii* and the main features explaining its hardiness.

3.1. Propionic fermentation via the Wood Werkman cycle: a high energy yield

P. freudenreichii ferments a variety of substrates, including carbohydrates, polyols like glycerol and erythritol and adonitol, and organic acids like lactic and gluconic acids (Cummins and Johnson, 1986). The main fermentation products are propionic, acetic, succinic acids and CO_2. Substrates are first oxidised to pyruvate *via* glycolysis or *via* the pentose phosphate pathway, generating ATP and reduced co-enzymes. Pyruvate is oxidised to acetate and CO_2 or reduced to propionate.

The reduction of pyruvate to propionate occurs *via* a specific cycle referred to as transcarboxylase cycle or Wood–Werkman (Crow, 1987; Houwen et al., 1991) (Fig. 1). This pathway uses NADH formed during glycolysis and pyruvate oxidation, and produces extra-ATP. All the reactions of the transcarboxylase cycle are reversible. One of the key reactions is a transcarboxylation reaction transferring a carboxyl group from methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA, and occurring without ATP consumption. The enzyme involved is a complex, biotin-dependent carboxytransferase, composed of three subunits of known structure. Oxaloacetate is then converted to succinate, with the reactions catalysed by the enzymes of the citric acid cycle. The reduction of fumarate to succinate is catalysed by a membrane-bound succinate dehydrogenase involving anaerobic electron transport by cytochrome *b* and generation of ATP. Succinate is then converted to succinyl-CoA, concomitantly with the conversion of propionyl-CoA to propionate, by a CoAtransferase. Succinyl-CoA is finally isomerised to methylmalonyl-CoA in a reaction catalysed by methylmalonyl-CoA mutase (EC 5.4.99.2). This coenzyme B_{12}-dependent enzyme is another original enzyme of propionibacteria. In summary, the pathway of propionate production in propionibacteria is unique, functions as a cyclic process, is coupled to

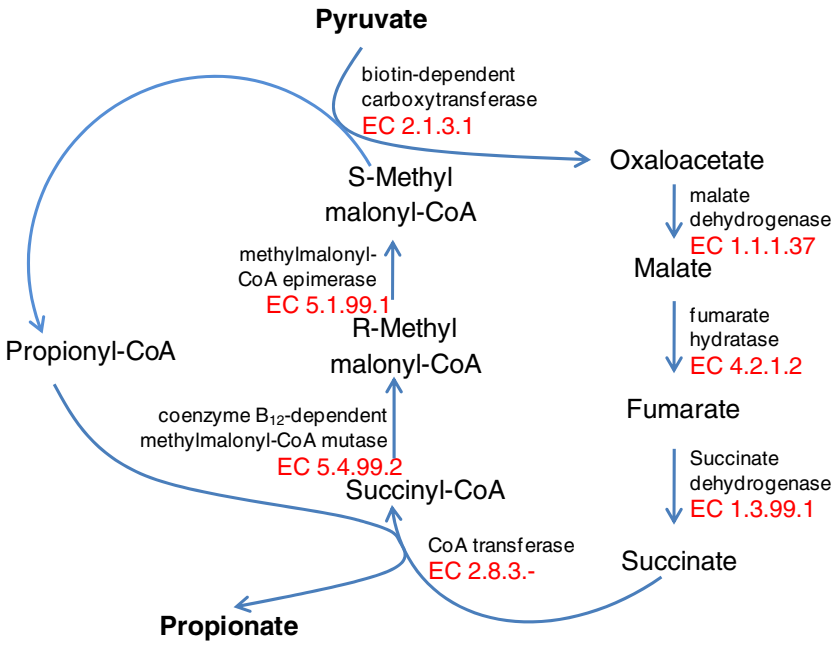

Fig. 1. Schematic representation of the Wood–Werkman cycle in *P. freudenreichii*. Reactions are directed toward propionate production, but are reversible.

oxidative phosphorylation and yields more ATP than in the other bacteria producing propionic acid.

Cells modulate the proportions of pyruvate either reduced to propionate, or oxidised to acetate and CO_2, to maintain the redox balance. The proportions of pyruvate directed to each pathway depend on the substrate used, the environmental conditions, and the properties of strains. For example, the oxidation of glucose and lactic acid leads to a molar ratio of propionate and acetate to ~2 (*i.e.* 2 moles of pyruvate are reduced to propionate while 1 mole of pyruvate is oxidised to acetate and CO_2). In contrast, the oxidation of glycerol leads to the formation of propionate only. *P. freudenreichii* is able to co-metabolise aspartate and other substrates like lactate, in a strain-dependant way. Aspartate is deaminated to fumarate that is further reduced to succinate, with a concomitant production of NAD and ATP. Strains using aspartate together with lactate convert less pyruvate to propionate and oxidised more pyruvate to acetate + CO_2, to maintain cell redox balance, compared to that metabolising lactate alone.

Acetate kinase gene disruption using integrational mutagenesis analysis on *P. acidipropionici* led to a decrease by 14% of acetate production from glucose and an increase by 13% of propionate yield (Suwannakham et al., 2006).

3.2. Respiration

P. freudenreichii is usually grown under anaerobic or microaerophilic conditions and described as an anaerobe. However, early studies also reported oxidative activity with free oxygen on a variety of substrates in propionibacteria (Vorobjeva, 1999). Accordingly, all the genes required for aerobic respiration were identified in *P. freudenreichii* genome: genes encoding NADH dehydrogenase, succinate dehydrogenase, cytochrome bd complex, ATPase and the complete pathway for heme synthesis (Falentin et al., 2010a). At high concentrations of oxygen, the synthesis of cytochrome, and thus the growth of *P. freudenreichii*, are inhibited. A switch from anaerobic to aerobic culture induces the consumption of the propionate produced in anaerobic conditions. Such a shift has been applied to improve the yield of vitamin B_{12} production (Ye et al., 1999) and 1,4-dihydroxy-2-naphthoic acid production (Furuichi et al., 2007). Under anaerobic conditions, the electron acceptor in *P. freudenreichii* can be sulphate, fumarate, nitrate, menaquinone (vitamine K_2), or a pool of ferrous iron and humic acid in soil (Benz et al., 1998).

3.3. Vitamin and porphyrin synthesis

P. freudenreichii exhibits few nutritional requirements. It is able to synthesise all amino acids, and all but a few vitamins (Cummins and Johnson, 1992). Hence, *P. freudenreichii* can grow on chemically defined media containing a carbon and energy source, NH_4 as nitrogen source, minerals and vitamins (pantothenate, biotin, thiamine). *P. freudenreichii* synthesises in particular vitamin B_{12} (cobalamin), a co-factor of methylmalonyl-CoA mutase, by the anaerobic pathway, regardless of the aerobic and anaerobic conditions of incubation (Iida et al., 2007; Roessner et al., 2002). Vitamin B_{12} has been industrially produced for a long time and is the most complex vitamin synthesised by bacteria. More than 20 reactions are involved in the synthesis of this cobalt-containing molecule. The pathway of vitamin B_{12} synthesis in *P. freudenreichii* has been thoroughly investigated (see reviews (Burgess et al., 2009; Murooka et al., 2005; Roessner et al., 2002). This pathway was recently completed thanks to the complete genome sequence (Falentin et al., 2010a). Genetically modified strains of *P. freudenreichii* overproducing vitamins have been developed for vitamin B_{12} (Murooka et al., 2005; Piao et al., 2004b), 5-aminolevulinic acid, the first intermediate in the synthesis of porphyrins (Kiatpapan and Murooka, 2001; Kiatpapan and Panbangred, 2008), porphyrin (Piao et al., 2004a), and riboflavin (vitamin B_2) (Burgess et al., 2009). Genetically modified strains were obtained by the electroporation of *P. freudenreichii* IFO12426 with the *E. coli* shuttle vector pPK705 containing one to three genes of the respective pathways. Productions of vitamin B_2, vitamin B_{12}, and porphyrinogen were up to 2-fold, 1.9-fold and 33-fold greater, respectively, in overproducing strains, compared to the production in the wild strain (Burgess et al., 2006; Piao et al., 2004a; Piao et al., 2004b).

3.4. Production of exopolysaccharides

P. freudenreichii produces exopolysaccharides (EPS), including both hetero- and homopolysaccharides. Hitherto two primary EPS structures have been described in different strains: an heteropolysaccharide composed of D-glucose, D-mannose, and D-glucuronic acid in molar ratios of 2:2:1 (Dobruchowska et al., 2008) and a (1→3,1→2)-β-D-glucan homopolysaccharide (Deutsch et al., 2008; Nordmark et al., 2005). A polysaccharide of the latter type, tightly associated with the cell wall, was identified in 35% of *P. freudenreichii* strains (Deutsch et al., 2010). Interestingly, a single gene, *gtf*, is responsible for the synthesis of this surface polysaccharide, as revealed by inactivation experiments and heterologous expression in *Lactococcus lactis* (Deutsch et al., 2010). By using quantitative reverse transcription PCR, it was shown that the synthesis of this surface polysaccharide was correlated with the level of *gtf* expression; a minimum level of transcription is required for synthesis of a detectable amount of the polysaccharide. The presence of an IS element in the *gtf* promoting sequence may explain the strain-dependent expression of *gtf* (Deutsch et al., 2010).

3.5. Features allowing long-term survival

Cultures of dairy propionibacteria are known to remain viable for many months at room temperature (Cummins and Johnson, 1992). Indeed *P. freudenreichii* is able to survive and to remain active in various environments, including cheese, but also in the digest tract (Hervé et al., 2007). This feature can be explained by the ability of *P. freudenreichii* to accumulate various energy and carbon storage compounds, briefly described below.

P. freudenreichii accumulates polyphosphate (polyP) as energy storage. The presence of polyP was visualised using electron microscopy, as of electron-dense granules in cells (Clark et al., 1986; Vorobjeva, 1999). PolyP are linear polymers of tens or hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds. Their synthesis is catalysed by polyphosphate kinase (PPK) that transfers the terminal phosphate of ATP to polyP. PolyPs are known to enable microorganisms to respond to and tolerate stress. They have also been proposed as the modulators of quorum sensing and biofilm development, as shown by studies of *Pseudomonas* PKK mutants (Seufferheld et al., 2008). The accumulation of polyP also regulates the activation of the RNA polymerase RpoS and the synthesis of ppGpp, a major signalling component of the stringent response (Seufferheld et al., 2008). The enzymes using polyP instead of ATP catalyse reversible reactions, as for pyrophosphate phosphofructokinase in *P. freudenreichii*, involved both in glycolysis and neoglucogenesis (Meurice et al., 2004). *P. freudenreichii* also possesses a glucokinase using polyP or ATP as the phosphate donor (Phillips et al., 1993).

Glycogen is an α-1,4 linked, α-1,6 branched glucose polymer used for long-term energy storage in animal, fungi, bacteria, and archaebacteria (Ball and Morell, 2003). A first experimental evidence of the ability of *P. freudenreichii* to synthesise glycogen was given by *in vivo* ^{13}C NMR analysis of *P. freudenreichii* type strain cells grown in the presence of ^{13}C glucose (Meurice, 2004). More recently, all the genes potentially related to glycogen metabolism were identified in the genome of the same strain (CIRM-BIA1^T = CIP103027^T) (Falentin et al., 2010a).

Trehalose is a non-reducing disaccharide found in many organisms, from bacteria to mammals, in which it exerts various functions related to stress adaptation due to its particular physical features. In bacteria, trehalose can be used as a carbon and energy source and is also accumulated as compatible solute (Arguelles, 2000). The detection of trehalose in propionibacteria has been early described. It is synthesised in all dairy propionibacteria species and is accumulated under various stress-inducing conditions (Cardoso et al., 2004; Rolin et al., 1995). The genes involved in trehalose synthesis in *P. freudenreichii* were recently described (Cardoso et al., 2007). The amounts of trehalose synthesised are highly strain-dependant in *P. freudenreichii* (Table 1).

P. freudenreichii has the ability to adapt to the various stresses that it encounters during fermentation processes and in variable environments. Industrial starters added to milk for cheese manufacture are generally proposed as lyophilisates, and have thus to adapt to osmotic stress. During Emmental cheese manufacture, *P. freudenreichii* has to withstand different successive stresses: heating over 50 °C, acidification of the curd to pH 5.2, osmotic stress due the NaCl addition at the brining step, and low temperature (4 to 12 °C) during cheese ripening. Some strains also bear the acid- and bile-related stresses encountered in the digestive tract, which is a prerequisite of their use as probiotics. Interestingly, the cell machinery involved in stress adaptation in *P. freudenreichii* (Leverrier et al., 2004) was shown to be encoded by redundant multicopy genes (Falentin et al., 2010a). This is in agreement with the ability shown by *P. freudenreichii* to adapt efficiently to various conditions.

4. Recent advances in knowledge on *P. freudenreichii*

4.1. Evolution of published data about dairy propionibacteria

About 40 scientific publications about dairy propionibacteria have been published per year over the past two decades. Papers related to *P. freudenreichii* constitute more than 50% of these publications. Propionibacteria were mainly studied in the past for their particular metabolism and their application in cheese ripening, but the fields of research have evolved over the time and they are nowadays mainly studied for their health properties. The main research fields covered over the past 3 years are depicted in Fig. 2. Only 10% of publications are currently related to cheese-related properties of propionibacteria. In contrast, publications related to human health have increased in number over the last decade and now constitute more than 40% of publications about dairy propionibacteria. They include studies about the various probiotic potentials, the synthesis of bifidogenic growth factor, vitamins, conjugated linoleic acid (CLA) and the potential of propionibacteria as protective cultures. Papers related to the use of propionibacteria as silage bacterial additives and as probiotics for animals account for about 20% of papers.

4.2. Role in aroma formation in cheese

P. freudenreichii is the main *Propionibacterium* species used in cheese manufacture. It is used in Swiss-type cheeses, in which it is responsible for the characteristic eyes due to the explosive production of CO_2 occurring when *P. freudenreichii* grows (for reviews see for example (Fröhlich-Wyder and Bachmann, 2004; Langsrud and Reinbold, 1973b; Thierry et al., 2010). Propionibacteria can also be used in the manufacture of various cheeses without eyes to enhance flavour formation (Ben Lawlor et al., 2003; Fernandez-Espla and Fox, 1998; Thierry et al., 2005a).

P. freudenreichii has a main role in the formation of Swiss-type cheese flavour. It produces flavour compounds from three main pathways: lactate and aspartate fermentation, amino acid catabolism, and fat hydrolysis (Thierry et al., 2004a). Many of these properties are strain-dependent. Table 1 gives some examples of phenotypic biodiversity related to the use of *P. freudenreichii* in cheese ripening. The ability to grow and propionic fermentation rate in cheese have long been known to be highly variable (Richoux and Kerjean, 1995). The ability to remain active in the presence of increased amounts of salt, in particular, depends on *P. freudenreichii* strains (Richoux et al., 1998).

Lactate fermentation by *P. freudenreichii* results in the formation of propionate, acetate (and CO_2), both acids being considered as flavour compounds in cheese. As described above, the metabolism of aspartate by propionibacteria during lactate fermentation results in

Table 1
Examples of phenotypic biodiversity related to the use of *P. freudenreichii* in cheese ripening and as a probiotic.

Target	Experimental conditions	Factor of variation	Reference
Trehalose accumulation	Chemically defined medium, pH 7.0 and 30 °C, cells harvested at mid-exponential growth phase	0 to 132 mg trehalose/g cell protein	(Cardoso et al., 2004)
Salt resistance in cheese	Mini Swiss-type cheeses, with 1, 2 and 3%salt in moisture (S/M)	Lactate consumed after 14 d ripening in the warm room: 12 to 13 g/kg at 1% S/M; 7 to 13 g/kg at 2% S/M	(Richoux et al., 1998)
Lysis	Potassium phosphate buffer, pH 6.2 for 24 h at 37 °C	Decrease of optical density: 24 to 96%	(Lemée et al., 1994)
	Potassium phosphate buffer, pH 7.0 for 48 h at 33 °C	Decrease of optical density: 24 to 62%	(Ostlie et al., 1995)
	Pattern of autolytic enzymes after renaturing PAGE	Similar enzyme pattern	(Ostlie et al., 2007)
Methylbutanoic acids	Emmental cheese, 8 strains	19 to 114 mg/kg cheese	(Thierry et al., 2004c)
	40 strains grown in lactate broth, pH 5.4, 21 g/L NaCl, at 24 °C	6 to 57 mg/L culture supernatant	(Dherbécourt et al., 2008b)
Free fatty acids (FFA) from lipolysis	Emmental cheese, 5 strains	1.8 to 5.2 mg FFA/kg cheese	(Chamba and Perréard, 2002)
	Lactate broth in the presence of an emulsion of milk fat	Production of 0.8 to 3 mg FFA/kg fat	(Dherbécourt et al., 2010b) for culture conditions; Thierry, unpublished data
Aspartase activity	Incubation of cell-free extract (12 strains) in the presence of aspartate — quantification of fumarate produced	<0.5 to 35 mmol/min/mg protein	(Turgay et al., 2010)
Growth at low temperature	Lactate broth, pH 5.5, incubated at 11 °C	<0.1 to 2.0 units of optical density (OD_{578}) after 24 days	(Turgay et al., 2010)
Tolerance to acid and bile	Acid + bile stress	cfu decrease 0.5 to 4 log	(Lan et al., 2007a)
Propionate production	Medium designed to mimic the content of the human colon	0.4 to 1.4 g/L	(Lan et al., 2007a)
Surface polysaccharide	Agglutination tests (anti-*S. pneumoniae* ser 37 antiserum)	Positive/negative	(Deutsch et al., 2008; Deutsch et al., 2010)
IL-10 induction	Elisa on stimulated peripheral blood mononuclear cells	1500 to 5500 pg/mL	(Foligné et al., 2010)

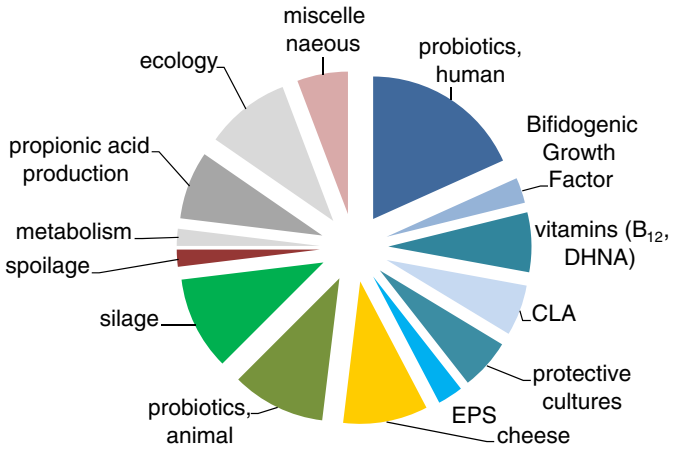

Fig. 2. Fields of research covered by publications about dairy propionibacteria since 2007 (from search in ISI Web Of Science database). DHNA, 4-dihydroxy-2-naphtoic acid; CLA, conjugated linoleic acid; EPS, exopolysaccharides.

additional CO_2 production. The balance of fermentation products in *P. freudenreichii* has important consequences on cheese ripening. The intensity of aspartate metabolism in *P. freudenreichii* is highly strain-dependent. Strains with a high ability to metabolise aspartate exhibit a higher fermentation rate and produce a higher proportion of CO_2 per mole of lactate consumed (Wyder et al., 2001). They can also be associated with an undesirable late fermentation in Emmental cheese, resulting in the formation of slits and cracks. This opening defect can occur, for example, during the ripening of Swiss Emmental cheese, which lasts for several months at 10–12 °C (Fröhlich-Wyder and Bachmann, 2004). The specific aspartase activity (L-aspartate ammonia-lyase), compared in the cell-free extracts of 12 *P. freudenreichii* strains, varied from less than 0.5 to 35 mmol/min/mg protein (Turgay et al., 2010) (Table 1).

P. freudenreichii catabolises branched-chain amino acids to branched-chain volatile (flavour) compounds. The main branched-chain compounds produced by *P. freudenreichii* are 2-methylbutanoic acid and 3-methylbutanoic acid (actual isovaleric acid), which derive from isoleucine and leucine degradation, respectively (Thierry and Maillard, 2002). These two acids are important flavour compounds in many cheeses and are associated with typical “old cheese”, “sweaty socks” notes. *P. freudenreichii* is by far the main agent of the formation of methylbutanoic acids (comprising about 80% of 2-methylbutanoic acid and about 20% of 3-methylbutanoic acid in Swiss cheese) (Thierry et al., 2004b).The production of methylbutanoic acids is constitutive in *P. freudenreichii* and is related to the synthesis of long branched-chain fatty acids which constitute more than 80% of cell membrane fatty acids (Dherbécourt et al., 2008b). The synthesis of methylbutanoic acids occurs *via* transamination of branched-chain amino acids, leading to ketoacids that are further converted into acids by oxidative decarboxylation (Thierry and Maillard, 2002). Methylbutanoic acids are produced in broth cultures and in cheese by *P. freudenreichii*. The screening of *P. freudenreichii* strains ability to produce methylbutanoic acids gave similar results *in vitro* and in Swiss-type cheese (Thierry et al., 2004c). The concentrations of methylbutanoic acids produced in a modified lactate broth incubated at 24 °C varied by a factor of 8 within a pool of 40 strains (*cf.* Table 1). Four strains were distinguished by their significant higher ability to release methylbutanoic acids (>30 mg/L) (Dherbécourt et al., 2008b).

P. freudenreichii has also a prominent role in Emmental cheese lipolysis. It is responsible for almost the whole free fatty acids released from cheese fat during Swiss cheese ripening (Dherbécourt et al., 2010a). Free fatty acids are important flavour compounds in many cheeses, including Swiss cheese. The lipolytic activity of *P. freudenreichii* is strain-dependent (Chamba and Perréard, 2002) (Thierry, unpublished results). The most probable esterases potentially involved in cheese lipolysis were identified from the complete genome sequence of *P. freudenreichii* (Dherbécourt et al., 2008a). One of them was identified as the sole secreted esterase in *P. freudenreichii* by *in silico* and biochemical approaches. Transformed clones over-expressing this enzyme showed 5 to 8 times more lipolytic activity on milk fat than the wild-type strain mutant (Dherbécourt et al., 2010b). This result strongly indicates that this enzyme plays a major role in cheese lipolysis.

In contrast, *P. freudenreichii* has very low caseinolytic activity. It possesses diverse intracellular peptidases, including several enzymes specific of proline-containing peptides. However, *P. freudenreichii* has a limited role in secondary proteolysis of cheese, compared to lactic acid bacteria, because it does not lyse in cheese or only lately (Valence et al., 1998), even if the ability of strains to lyse *in vitro* in buffer varies over large ranges (Table 1) (Ostlie et al., 1995).

The production of flavour compounds occurs during *P. freudenreichii* growth in cheeses at about 24 °C, and continues during further cold storage of cheeses, while propionic fermentation is markedly slowed down at low temperatures. For example, about 60% of methylbutanoic acids from amino acid catabolism and 40% of fatty acids from lipolysis were produced during cold storage of experimental Swiss cheeses, while only 20% of propionic acid were produced during the same period (Thierry et al., 2005b). New insights are now made possible by the use of molecular methods to evaluate the activity of micro-organisms in cheese. It was recently shown that *P. freudenreichii* maintained metabolic activity up to the end of ripening, as shown by using quantitative reverse transcription PCR (RT-qPCR) specifically targeting different housekeeping genes (*16S*, *groL1*, *groL2* and *tuf*) (Falentin et al., 2010b). The ability to grow at low temperature is also highly strain-dependent (Table 1). This property, along with a high aspartase activity, would be important in the ability of *P. freudenreichii* strains added as cultures in Swiss cheese to maintain as the dominant population over long ripening periods of time (Turgay et al., 2010).

4.3. Probiotic properties of *P. freudenreichii*

P. freudenreichii and *P. acidipropionici* were considered for their potent probiotic effects on animals and on humans later than, for instance, lactobacilli and bifidobacteria, and scientific reports began in the 90s. Their probiotic potential was early reviewed in 1999 by A. Perez-Chaia et al., 1999 and more recently by Ouwehand, 2004 and Cousin et al., 2010. Briefly, dairy propionibacteria are considered for their beneficial modulation of the gut microbiota and of the corresponding metabolic activities. *P. freudenreichii* has also more recently been studied for its modulation of the gut function and physiology. We will focus here on the reports published during the last 5 years on *P. freudenreichii* and giving new mechanistic highlights on dairy propionibacteria probiotic effects with a potential application to the human health. Clinical studies only based on the use of complex probiotic mixtures including propionibacteria as probiotic are not covered by this paper, neither are probiotic applications in animals.

The bifidogenic effect of selected strains of *P. freudenreichii* was observed in independent studies performed on human volunteers (Bouglé et al., 1999; Hojo et al., 2002; Satomi et al., 1999). Volunteers consuming propionibacteria displayed enhanced colic bifidobacterial populations. The active compound was present in propionibacterial supernatants and identified as 1,4-dihydroxy-2-naphtoic acid (DHNA) (Isawa et al., 2002). This molecule is the penultimate intermediate in the biosynthesis pathway of vitamin K_2 (menaquinone). *P. freudenreichii* synthesises vitamin K_2 and the whole pathway was reconstructed from the genome data (Falentin et al., 2010a). DHNA is synthesised from 2-succinylbenzoyl-CoA by a naphtoate synthase and

then leaks out of propionibacteria without cell lysis. It has been proposed that DHNA and its derivative 2-amino-3-carboxy-1,4-naphtoquinone (ACNQ) serve as electron transfer mediator for NADP regeneration in bifidobacteria (Yamazaki et al., 1998; Yamazaki et al., 1999), thus favouring bifidobacterial growth. Besides the microbiota, Propionibacteria are also proposed to modulate metabolic activity in the favour of beneficial metabolites.

Production of beneficial conjugated linoleic acids (CLAs) is also documented in *P. freudenreichii*. Among the biological effects described for CLAs, the anticarcinogenic properties of rumenic acid, the cis-9,trans-11 stereoisomer of CLA, are highly promising. *P. freudenreichii* has been shown to convert free linoleic acid into rumenic acid (Rainio et al., 2002; Wang et al., 2007). The corresponding mechanisms of CLA formation by *P. freudenreichii* were identified by deuterium labelling and mass spectrometry (McIntosh et al., 2009). However, the genes encoding enzymes involved in this pathway are still not elucidated and the ability of *P. freudenreichii* to produce substantial amounts of rumenic acid *in situ* in fermented products or in the colic content remains largely to be studied. Several processes of enrichment of CLA, based on a lipid hydrolysis followed by a *P. freudenreichii*-catalysed isomerisation of free linoleic acid to CLA, have been developed in various products like oats (Vahvaselka et al., 2006) and by-products of plant oil processing (Vahvaselka and Laakso, 2010).

Beneficial metabolic activity is mainly limited by the ability of dairy propionibacteria to adapt stressing environments. *P. freudenreichii* can adapt to very unfavourable conditions encountered either during industrial processes or within the digestive tract (Leverrier et al., 2004; Suomalainen et al., 2008). The corresponding adaptation proteins were particularly investigated in *P. freudenreichii* and its genome revealed a remarkable redundancy in the cell machinery involved in stress perception, adaptation, detoxification and macromolecules repair (Falentin et al., 2010a). We have shown these proteins to be differentially expressed, depending on the strain (Anastasiou et al., 2006). Accordingly, the ability to adapt and maintain an active metabolism within the colon of human microbiota-associated rats and of human volunteers was shown to be highly strain dependent (Hervé et al., 2007; Lan et al., 2007a) (Table 1). This illustrates a notable phenotypic biodiversity among strains. In the colon, selected strains were showed to express the transcarboxylase operon specific of propionic fermentation, and to produce propionate. Propionate is known for its role in the modulation of the proliferation/apoptosis balance. Accordingly, the most promising strain selected in *in vivo* and *in vitro* screening was shown to reduce proliferation yet enhance apoptosis of colon epithelial cells in rats mutagenized with the carcinogen dimethylhydrazine (Lan et al., 2008). Interestingly, propionibacteria had no effect on these parameters in healthy rats. The corresponding pro-apoptotic mechanisms were well described *in vitro* on cultured human colon cancer cells and were shown to be enhanced by an acidic environment (Lan et al., 2007b). This pro-apoptotic property may prove useful in the context of colon cancer prevention and/or treatment. In the same context, one should notice that the ability of *P. freudenreichii* to bind the food born carcinogen aflatoxin B-1 *in vitro* (El-Nezami et al., 2000) was confirmed in a human study (El-Nezami et al., 2006). Human volunteers chronically exposed to this carcinogen showed reduced bioavailability and urinary excretion of aflatoxin, as a result of probiotic consumption. However, this probiotic containing both *Lactobacillus rhamnosus* and *P. freudenreichii*, the specific role of this last is difficult to specify.

Finally, immunomodulation by dairy propionibacteria was confirmed in humans, as volunteers consuming *P. freudenreichii* JS showed reduced serum levels of CRP, indicating an anti-inflammatory effect (Kekkonen et al., 2008). Indeed, immunomodulation was evidenced in a highly strain dependent manner in dairy propionibacteria (Foligné et al., 2010). Selected strains induced high levels of regulatory anti-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs) and were effective in two colitis models in mice. The surface antigens of propionibacteria were shown to play a key role in such a modulation (Foligné et al., 2010). This is consistent with a previous study showing immunomodulatory properties of *P. freudenreichii* strain JS (Kekkonen et al., 2008).

The activity of *P. freudenreichii* against the pathogen bacterium *Helicobacter pylori* was also recently investigated. *In vitro*, *P. freudenreichii* reduced adhesion of *H. pylori* on intestinal cells and *H. pylori*-induced cell membrane leakage (Myllyluoma et al., 2008). Besides, it inhibited *H. pylori*-induced IL-8 and PGE2 release by intestinal cells. In clinical studies, the supplementation of a mixture of probiotics containing *P. freudenreichii* improved the tolerance to the treatment of *H. pylori* infection (Myllyluoma et al., 2005; Myllyluoma et al., 2007a) and led to a beneficial effect on gastric mucosa in *H. pylori*-infected patients (Myllyluoma et al., 2007b). Moreover, the bifidogenic growth stimulator DHNA produced by *P. freudenreichii* inhibited the growth of *H. pylori*, *via* the inhibition of cellular respiration (Nagata et al., 2010). Taken altogether, these studies suggest that *P. freudenreichii* may be useful to eradicate *H. pylori*.

4.4. Protective cultures

Some dairy propionibacteria can also synthesise anti-fungal and anti-yeast compounds and have a potential as food-protective cultures (Ho et al., 2009; Lind et al., 2007; Schwenninger et al., 2008; Tharmaraj and Shah, 2009). *P. freudenreichii* secretes bacteriocins. A recent review reports the literature on three antimicrobial peptides (PAMP antimicrobial peptide and propionicins T1 and F) from propionibacteria, relating the genetics, biochemistry, biosynthesis, and biological activities of these compounds (Faye et al., 2010). In *P. jensenii*, several antimicrobial organic acids acting in synergy have recently been identified, including not only acetic, propionic, succinic and lactic acids, but also 2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyl lactic acid 3-phenyllactic acid (Schwenninger et al., 2008).

5. Concluding remarks

As a conclusion, *P. freudenreichii* just entered the so-called post-genomic era and this will favour its use in various domains. Indeed, this bacterium has long attracted attention because of its peculiar properties and of its several useful applications. However, molecular tools and knowledge were lacking to deepen the general understanding of its action. The newly available genome sequence, together with the developed molecular tools, is opening new perspectives in the context of its use. The molecular mechanisms involved in key pathways for adaptation, cheese ripening, bio-preservation and probiotic effects can now be elucidated. This will in turn allow selection of the most appropriate strain for each application, taking advantage of *P. freudenreichii* remarkable biodiversity.

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