Production of branched-chain aroma compounds by Propionibacterium freudenreichii: links with the biosynthesis of membrane fatty acids.

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Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Production of branched-chain aroma compounds by *Propionibacterium freudenreichii*: links with the biosynthesis of membrane fatty acids

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Keywords
aroma compounds, branched-chain fatty acid, dairy, membrane, *Propionibacterium freudenreichii*.

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2007/1149: received 18 July 2007, revised 19 February 2008 and accepted 24 February 2008

doi:10.1111/j.1365-2672.2008.03830.x

Abstract

Aims: Short branched-chain fatty acids (BCFAs) are cheese flavour compounds, which result from the conversion of branched-chain amino acids (BCAAs). In Swiss cheese, the production of short BCFAs is mainly performed by *Propionibacterium freudenreichii* and is strain dependent. Our aim was to investigate the possible links between the biosynthesis of short BCFAs and membrane BCFAs in *P. freudenreichii*.

Methods and Results: Short and membrane BCFAs were analysed by gas chromatography-mass spectrometry. Two strains differing in their capacities to release short BCFAs were selected. Tri-deuterated-labelled leucine was used in both strains as a precursor of short extracellular iso-BCFAs and of membrane iso-BCFAs. The proportions of anteiso : iso BCFAs synthesized varied as function of the BCAAs provided in the growth medium, from 72 : 28 to 100 : 0, with leucine and valine, and with isoleucine as sole BC precursors, respectively. The branching pattern of short BCFAs exactly matched that of membrane BCFAs, whatever the exogenous BCAAs provided.

Conclusions: The biosynthesis of short BCFAs is closely related to that of membrane BCFAs in *P. freudenreichii*.

Significance and Impact of the Study: The biosynthesis of short BCFAs in *P. freudenreichii* depends more on the strain than on the presence of exogenous BC precursors.

Introduction

Propionibacterium freudenreichii is a ripening secondary starter in Swiss-type cheeses, and has a key role in the formation of cheese flavour. *Propionibacterium freudenreichii* produces flavour compounds which originate from different pathways: propionate, acetate derived from lactate fermentation (Langsrud and Reinbold 1973), free fatty acids from lipolysis (Chamba and Perréard 2002; Thierry *et al.* 2005), and branched-chain (BC) volatile compounds from the catabolism of branched-chain amino acids (BCAAs) (Thierry *et al.* 2004a, 2005). The main BC volatile compounds produced by *P. freudenreichii* are two branched-chain fatty acids (BCFAs), 2-methylbutanoic acid (anteiso-C5) and 3-methylbutanoic acid (iso-C5). These two BCFAs are thought to be flavour-active compounds in many cheeses where they impart cheese/sweaty notes (Urbach 1997; Yvon and Rijnen 2001), and *P. freudenreichii* is essential to their formation in Swiss cheese. Moreover, the concentrations of short BCFAs produced by *P. freudenreichii* are strain-dependent in culture media and in Swiss cheese (Thierry *et al.* 2002, 2004b).

To understand the origin of strain dependency of short BCFAs production, we aimed to investigate the physiological roles of biosynthesis of short BCFAs in *P. freudenreichii*. In other bacteria, the physiological roles of short BCFA production include the formation of precursors for membrane BCFA biosynthesis in *Bacillus* sp. and *Staphylococcus* sp. (Kaneda 1973; Beck *et al.* 2004), the formation

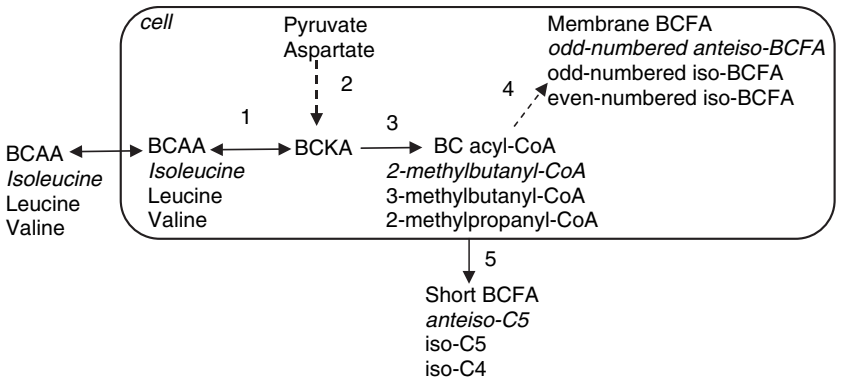

Figure 1 Proposed pathways of short and membrane BCFA (branched-chain fatty acid) biosynthesis in *Propionibacterium freudenreichii*. BCAA: branched-chain amino acid, BCKA: branched-chain keto-acid, BC acyl-CoA: branched-chain acyl coenzyme A. 1, BC aminotransferase; 2, enzymes of the BCAA biosynthetic pathways; 3, BC keto-acid dehydrogenase complex; 4, enzymes of the FA biosynthetic pathway; 5, acyl-CoA hydrolase. Anteiso-branched compounds are in italics, iso-branched in normal type.

of precursors for BCAA biosynthesis in some anaerobic bacteria species (Allison and Bryant 1963), and the production of energy in starved noncultivable lactococci (Ganesan *et al.* 2004, 2007). As *P. freudenreichii* possesses a high proportion of methyl-branched chain fatty acids (FAs) in the membrane FAs (Moss *et al.* 1969; Hofherr *et al.* 1983), the formation of short extracellular BCFAs could be related to the biosynthesis of membrane FAs in this species. Bacteria which possess BCFAs in their membrane biosynthesize them from methyl-branched-chain C4 and C5 precursors (Fig. 1), derived from carbon skeletons of the three BCAAs valine, isoleucine and leucine (Kaneda 1991) or their derivatives, e.g. keto acids and CoA esters, as seen for example in *Bacillus* sp. (Kaneda 1977, 1991; Oku *et al.* 1998). The present study aimed to determine whether the production of short extracellular BCFAs is related to the biosynthesis of membrane BCFAs in *P. freudenreichii*.

Materials and methods

Strains

Forty *P. freudenreichii* strains were screened for the ability to produce short BCFAs, 26 from the TL collection of the UMR STLO INRA Agrocampus Rennes (Rennes, France), 11 strains (ITGP5–ITGP24) from the collection of the Institut Technique Français du Fromage (ITFF, Rennes, France), and three commercial strains (coded A, B and C). The complete list of strains appears in Fig. 2.

Cultures and growth media

Bacteria were grown either in yeast extract-lactate broth (YEL), in a modified YEL medium (YEL-S), or in different chemically defined media (cdm). YEL contained, per litre, 10 g tryptone, 10 g yeast extract, 0·328 g KH_2PO_4, 56 mg $MnSO_4$ and 12·84 g D-L sodium lactate, pH adjusted to 7·0. YEL-S was prepared by reducing the

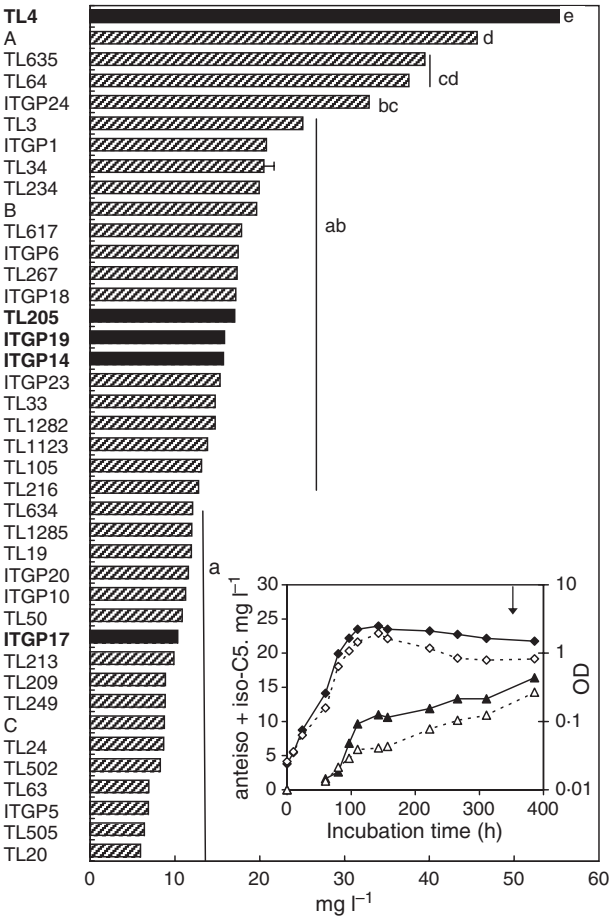

Figure 2 Concentrations of short BCFAs (iso-C5 and anteiso-C5) released in YEL-S cultures (YEL with 2·1% NaCl at pH 5·4) incubated at 24°C by forty strains of *P. freudenreichii*. Mean of duplicate analyses (and of triplicate independent cultures for strain TL34). Bars followed by the same letter did not significantly differ by SNK test ($\alpha < 0{\cdot}05$). The five strains selected for further study are in black. The inserted figure shows the growth curve (◇) and time course of accumulation of short BCFAs in culture supernatant (△) for two strains: ITGP18, filled symbols, and ITGP23, open symbols. The arrow shows the time of BCFA measurements for all strains.

pH of YEL to 5·4 and adding NaCl (21 g l^{-1}) (Thierry *et al.* 2002). The enriched medium, YEL, and YEL-S media were sterilized at 115°C for 20 min. The basal cdm (cdmB) contained lactate, amino acids, mineral salts, and vitamins, at the final concentrations given in Table 1. Other cdm contained, in addition to cdmB, different combinations of BC precursors, added at the following final concentrations: L-valine 5·29 mmol l^{-1}, L-leucine 1·52 mmol l^{-1}, L-isoleucine 1·52 mmol l^{-1}. The pH was adjusted to 7·0 and the cdm were sterilized by filtration (Minisart 0·20-μm pore size membrane; Sartorius, Aubagne, France). Labelled L-leucine (5,5,5,-D3) was purchased from Buchem bv (Apeldoorn, The Netherland).

All the media were inoculated with 1% of a culture grown in the same medium and incubated at 30°C (except YEL-S culture, incubated at 24°C) semi-anaerobically (static cultures in air atmosphere). Growth was monitored by measuring optical density at 650 nm (OD_{650}). Cultures with $OD_{650} > 0·8$ were diluted with sterile medium so as to retain good linearity between absorbance and cell mass. One test tube was used for each measurement. Final populations of propionibacteria were enumerated on YELA medium (YEL + 15 g agar per litre) incubated at 30°C anaerobically for 6 days.

Table 1 Composition of the basal chemically defined medium

	Components	Concentration
Carbon source (g l^{-1})	DL-sodium lactate	21·4
Amino acids (g l^{-1})	L-aspartic acid	1·25
	L-glutamic acid, Na, H_2O	3·84
	L-arginine	0·99
	L-cysteine HCl.H_2O	1·25
	L-histidine	0·75
	L-lysine HCl	1·06
	L-tyrosine	0·407
Minerals (g l^{-1})	$MgSO_4.7H_2O$	0·20
	$MnSO_4.H_2O$	0·02
	$FeSO_4.7H_2O$	0·01
	NaCl	0·01
	Acetate $Co.4H_2O$	0·001
	$ZnSO_4.7H_2O$	0·00006
	Na_2HPO_4	2·60
	KH_2PO_4	3·00
Vitamins (mg l^{-1})	Ca-pantothenate	1·0
	Biotin	0·004
	Thiamine.HCl	1·0
	Para-aminobenzoic acid	0·2
	Pyridoxal phosphate	2·0
	Nicotinic acid	2·0
	Riboflavin	1·0
	Folic acid	0·02
	B12 vitamin	0·002

Membrane fatty acid analyses

Bacteria were harvested by centrifugation (7500 ***g***, 15 min, 4°C) from 48 ml of culture. The supernatants were frozen at −20°C until analyses of short BCFAs. The cell pellets were washed using 40 ml sterile distilled water at room temperature and frozen at −20°C until analyses of membrane FAs. Membrane FAs were extracted from the cell pellets by saponification, methylation, and analysed by gas chromatography (GC), using a routine clinical analysis method (Sasser 1990), with the following adaptations. For saponification, the cell pellets were suspended in 3 ml of a solution of 3·75 mol l^{-1} NaOH in methanol:water (1 : 1, v/v) and incubated for 30 min at 100°C, with maximum speed vortex stirring for 10 s after 5 min of incubation. The tubes were then cooled down at room temperature before methylation by addition of 6 ml of a solution of 3·46 mol l^{-1}HCl in methanol : water (0·73 : 1, v/v), vortex stirring, and incubation for 10 ± 1 min at 80° ± 1°C. Tubes were then cooled down at room temperature before addition of a hexane/diethyl ether (50/50, v/v) mixture, with moderate stirring (orbital rotation table, 100 rev min¯1, 10 min). The aqueous phase (lower phase) was discarded and the organic phase (upper phase) was washed one time using 9 ml of 0·3 mol l^{-1} NaOH solution under moderate stirring (orbital rotation table, 100 rev min^{-1}, 5 min) to eliminate nonesterified FAs, and analysed by two GC systems. A Varian 3800 gas chromatograph with flame ionization (FID) detection was used for routine analyses, and a HP5890 (Agilent Technologies, Santa Clara, CA) gas chromatograph coupled to a HP5972A quadrupole mass spectrometer (GC-MS) was used to confirm peak identification. For routine analyses, the samples were on column-injected at 65°C, separated on a capillary column HP5 (60 m × 0·32 mm × 1·0 μm film thickness), with hydrogen as carrier gas. Oven temperature was 150°C, held for 4 min, then heated to 250°C at 2°C min^{-1}, held 25 min. FID temperature was 280°C. For GC-MS analyses, the samples were injected on split/splitless mode 1/50 at 200°C. Fatty acid methyl esters were separated on a BPX-5 column (SGE, 60 m × 0·25 mm × 1·0 μm film thickness) with helium as carrier gas, and ionized by electronic impact (280°C, mass range 25–350, 2·32 scan s^{-1}). Oven temperature was 150°C, held for 4 min, then heated to 250°C at 4°C min^{-1}, held 50 min. Fatty acid methyl esters were identified by comparison of their retention times and their mass spectra with the ones of authentic standards (Bacterial Acid Methyl Esters CP Mix, Supelco: 47080U). Membrane FAs were expressed as percentage of total cellular FAs. Values obtained from duplicate analyses from the same culture did not differ by more than 1% and 9%, in cells grown in YEL and in cdm, respectively.

Analyses were performed in duplicate from cells harvested from two to four independent cultures.

Calculation of approximate concentrations of FAs in cells

The approximate concentrations of FAs in cells was calculated by taking into account the population at harvest time, the levels of lipids in bacteria (10–26% of membrane in cell dry weight, consisted of 20% lipids, mainly phospholipids (Mindich 1973), and the nature of membrane lipids in propionibacteria [mainly diphosphatityl glycerol, phosphatityl glycerol and phosphatityl inositol (Sutcliffe and Shaw 1993)].

Short BCFA analyses

Culture supernatants were thawed at room temperature, and an aliquot of 100 μl was added with 100 μl of 2000 mg ml^{-1} butanoic acid aqueous solution as internal standard, and 600 μl of 0·04 mol l^{-1} oxalic acid solution to precipitate proteins, incubated for 1 h at 4°C, and centrifuged at 21 000 ***g*** for 20 min at 4°C. Aliquots of the supernatants were directly analysed by GC to prevent any loss of FAs. The samples were cold (65°C) on column injected into a Varian 3800 gas chromatograph. Short BCFAs were separated on a capillary column BP21 (SGE, 25 m × 0·53 mm × 0·5 μm film thickness) with hydrogen as the carrier gas. The FID temperature was 200°C. The initial oven temperature was 50°C, heated to 100°C at 25°C min^{-1}, heated to 135°C at 6°C min^{-1}, then to 180°C at 20°C min^{-1}. The quantification of short BCFAs was performed by linear regression of peak areas and standard solution concentrations (Thierry *et al.* 2002). It should be pointed out that iso-C5 and anteiso-C5 were not separated by this method. Iso-C5 and anteiso-C5 were quantify separately after conversion to methyl esters and analysis by head space GC-MS. Methyl esters were synthesized according to previously described method (Beck *et al.* 2002), with the following modifications: 600 μl of culture supernatant were incubated for 2 h at 90°C with 12 μl of methanol and 600 μl of a saturated $NaHSO_4$ solution. Samples were then diluted fivefold with boiled deionized water cooled down at 4°C, directly in the head-space device before each run of GC-MS analysis. Volatile compounds were trapped on a Vocarb 3000 trap (Supelco, Bella Fonte, PA, USA), thermally desorbed at 250°C, and cryofocused at −100°C before being injected into a HP5890 GC-HP5972A quadrupole MS (Agilent Technologies). Short BCFAs were separated on a BPX-5 column (SGE, 60 m × 0·25 mm × 1·0 μm film thickness) and detected by electronic impact (280°C, mass range 25–173, 4·83 scan s^{-1}). Methyl iso-C5 and methyl anteiso-C5 were identified on the basis of the comparison of their specific retention time and mass spectra with those of standards. The ion 88 is specific of methyl anteiso-C5, whereas the ion 74 is more abundant in methyl iso-C5 than in methyl anteiso-C5. The spectrum of tri-deuterated methyl iso-C5 given by Beck (Beck *et al.* 2004) was also used for identification.

Statistical analyses

The data were subjected to ANOVA using the General Linear Model procedure of STATGRAPHICS PLUS (Statistical Graphic Corp., Englewood Cliffs, NJ, USA) to determine the effects of *P. freudenreichii* strain and, when needed, of the effect of the growth medium on the formation of short and membrane FAs. Differences between the treatment means were compared using the Student–Newman–Keuls test.

Results

Screening of *P. freudenreichii* strains on the capacity to produce short BCFAs under cheese conditions

Cells were incubated in YEL-S at 24°C for 16 days. Maximal growth, induced by the exhaustion of carbon source, was achieved within 6–8 days, with means OD_{650} values of 2·9 ± 0·6 at 7 days. OD_{650} values then decreased to 1·6 ± 0·6 at 16 days (end of incubation). The concentrations of short BCFAs produced in culture supernatants at the end of incubation varied by a 10-fold factor between strains (Fig. 2). BCFA concentrations are the sum of anteiso-C5 and iso-C5, which were not separately quantified in this experiment. Iso-C4 was not detected. Four strains, TL4, A, TL634 and TL64 were distinguished by their significant higher ability to release short BCFAs (>30·0 mg l^{-1}), whereas all the others strains ranged from 6·0 to 25·1 mg l^{-1} (Fig. 2).

Membrane FA composition of five *P. freudenreichii* strains under optimal conditions

Cultures were incubated in YEL for 11 days at 30°C. Cells were harvested in early stationary phase to be compared for their membrane FA composition (65 h of incubation, mean OD_{650} values of 3·3 ± 0·2, i.e. 1200 mg dry weight l^{-1}). The single most abundant acid was anteiso-C15:0 (12-methyltetradecanoic acid, 68·1 ± 2·5%) in the five strains studied. Five other major odd-numbered FAs were identified: anteiso-C17:0 (14-methylhexadecanoic acid, 11·6 ± 1·2%), C17:0 (heptadecanoic acid, 6·9 ± 2·9%), iso-C15:0 (13-methyltetradecanoic acid, 4·5 ± 0·4%), C15:0 (pentadecanoic acid, 4·0 ± 2%), and

iso-C17:0 (15-methylhexadecanoic acid, 1·5 ± 0·5%) (Fig. 3). The remaining FAs (3·2 ± 0·8%) consisted of nine minor FAs, namely, anteiso-C13:0 (10-methyldodecanoic acid), C13:0 (tridecanoic acid), iso-C14:0 (12-methyltridecanoic acid), C14:0 (tetradecanoic = myristic acid), iso-C16:0 (14-methylpentadecanoic acid), C16:0 (hexadecanoic acid = palmitic), C16:1 (*cis*-9-hexadecenoic acid = palmitoleic), C18:0 (octadecanoic acid = stearic) and C19:0 (nonadecanoic acid). These results show that membrane lipids of *P. freudenreichii* essentially contained BCFAs (87·7 ± 4·7%), consisted of 80·5 ± 3·7% of anteiso-branched and 7·2 ± 1·1% of iso-branched, and only 12·3 ± 4·7% of straight-chain FAs. One strain, TL4, was distinguished from the four other strains tested by its very significant (SNK test, $\alpha < 0{\cdot}001$) lower percentages of odd-numbered straight-chain FAs (C15:0 + C17:0, 4·3 ± 1·2% *vs* 14·4 ± 1·2% in the four other strains), counteracted by higher percentages of BCFAs (Fig. 3).

The approximate concentration of FAs in cells was reckoned at 20 mg of membrane anteiso-BCFAs were produced per litre culture, i.e. 81 μmol equivalent of anteiso-C15.

Short BCFAs (anteiso-C5 and iso-C5) produced in the culture supernatant were quantified in early stationary phase for the five strains. The concentrations of short BCFAs were significantly (SNK test, $\alpha < 0{\cdot}001$) greater for TL4 compared with the four other strains (18·5 mg l^{-1} *vs* 2·6–4·9 mg l^{-1}, respectively, data not shown). Anteiso-C5 was the main short BCFAs, with proportions of anteiso-C5: iso-C5 of 89 : 11 and 72 : 28, respectively, for strains TL4 and ITGP17, whereas iso-C4 was not detected in supernatants.

Production of BCFA from deuterated-labelled leucine

In order to determine whether *P. freudenreichii* uses BCAA as precursors of BCFAs, the strains TL4 and ITGP17 were grown in a cdm containing tri-deuterated leucine on its methyl-residue, and harvested in early stationary phase (OD_{650} approx. 1·6) to analyse membrane FA composition and short BCFAs in culture supernatant by using GC-MS.

Regarding membrane FA analyses, the mass spectra of the compounds eluting at the retention times of iso-C15:0 and iso-C17:0 methyl esters showed that the *m/z* of several ions increased by three units compared with the corresponding ions in the unlabelled compounds. Hence, tri-deuterated iso-C15:0 methyl ester was identified from its mass spectrum characterized by a molecular ion at *m/z* of 259 and two ions derived from fragmentation at *m/z* 216 and 160 *vs* 256, 213 and 157, respectively, in the spectrum of unlabelled iso-C15:0 methyl ester. This mass spectrum was similar to the one previously described for iso-C15:0 methyl ester (Beck *et al.* 2004). Similarly, tri-deuterated iso-C17:0 methyl ester was identified from its mass spectrum showing ions at *m/z* 287 (molecular ion), 244 and 188 *vs* 284, 241 and 185 for the unlabelled compound (spectra not shown). Only the labelled forms of iso-C15:0 and iso-C17:0 methyl esters were observed, demonstrating that exogenous leucine was the main precursor of membrane odd-numbered iso-BCFAs biosynthesized by *P. freudenreichii*.

Regarding short BCFAs in culture supernatants, tri-deuterated iso-C5:0 methyl ester was identified for the two strains, characterized by a molecular ion at *m/z* 119 and fragmentation ions at *m/z* 88 and 104 *vs* 116, 85 and

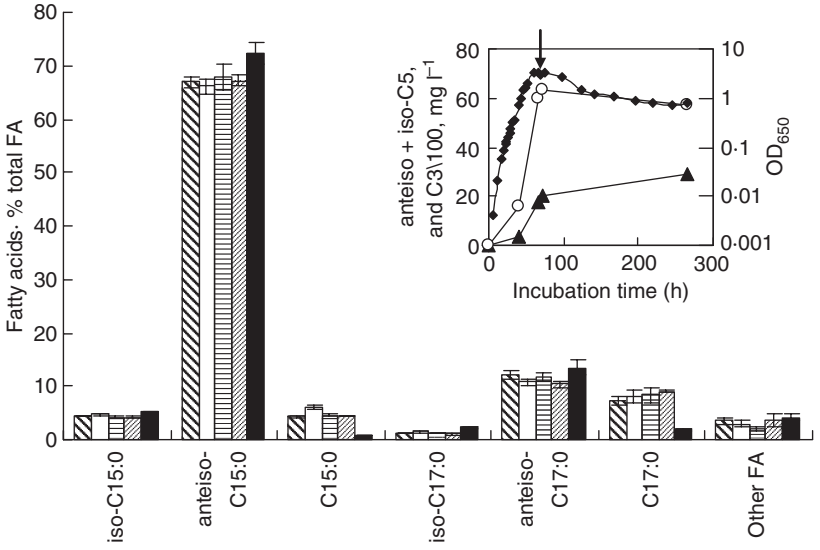

Figure 3 Membrane fatty acid compositions of five *P. freudenreichii* strains grown in YEL. Mean and standard deviations of triplicate analyses per strain. Other FAs: anteiso-C13:0, C13:0, iso-C14:0, C14:0, iso-C16:0, C16:0, C16:1, C18:0 and C19:0. The inserted figure shows the growth curve (◆) and the time course of accumulation of anteiso-C5 + iso-C5 (▲) and propionic acid (○, C3/100) in culture supernatant for strains TL4. The arrow shows the time of BCFA analyses for all strains. (▨), ITGP14; (□), ITGP17; (▤), ITGP19; (▨), TL205 (■), TL4.

101, respectively, in the mass spectrum of the methyl ester of unlabelled iso-C5:0 methyl ester.

Effect of the branching pattern of exogenous BCAAs on BCFA branching patterns

The strains ITGP17 and TL4 were grown in four cdm that contained either the two iso-branched amino acids (valine and leucine, cdmVL), either anteiso-branched amino acid (isoleucine, cdmI), either a mixture of the three BCAA (cdmVLI), or without any BC potential precursor (cdmB). Both strains grew on all these cdm within 5–7 days with maximal populations of approx. $1{\cdot}5 \times 10^9$ CFU ml^{-1} (OD_{650} of $1{\cdot}7 \pm 0{\cdot}2$). Membrane and short BCFAs were analysed at early stationary phase.

The percentages of membrane FAs were significantly influenced by the strain and/or the growth medium. No interactions between the factors 'strain' and 'medium' were shown (Table 2). The two strains mainly differed by their membrane content in odd-numbered straight chain FAs (C13:0, C15:0, C17:0 and C19:0). As observed in cells grown in YEL medium, TL4 contained lower percentages of straight chain FAs, compared with ITGP17 ($10{\cdot}2 \pm 4{\cdot}8\%$ and $28{\cdot}1 \pm 10{\cdot}8\%$, respectively, in strains TL4 and ITGP17). Regarding the effect of the growth medium, the branching pattern of BCAAs provided very significantly influenced the branching pattern of membrane BCFAs (Table 2). The percentages of anteiso-BCFAs were the greatest in cells grown in cdmI (containing isoleucine as sole source of BCAA) (94·2% and 82·6%, respectively, in strains TL4 and ITGP17) whereas, concomitantly, the percentages of iso-BCFAs were the smallest (1·1% and 0·4%, respectively, in strains TL4 and ITGP17, Table 2). In contrast, the percentages of both

Table 2 Membrane fatty acid composition and concentrations of short BCFAs in the growth medium in *Propionibacterium freudenreichii* TL4 and ITGP17 at the end of growth, in four chemically defined media containing different combinations of BCAA*

	TL4				ITGP17				ANOVA‡		
	cdmVLI†	cdmB†	cdmVL†	cdmI†	cdmVLI	cdmB	cdmVL	cdmI	Str	Med	Str × med
Anteiso-BCFAs, % of total membrane fatty acids§											
Anteiso-C13:0	0·6 (0·7)	0·3 (0·3)	0·3 (0·3)	0·2 (0·3)	0·6 (0·4)	0·3 (0·6)	0·5 (0·2)	0·3 (0·2)	–	–	–
Anteiso-C15:0	70·9 (2·2)	69·2 (2·4)	58·3 (4·8)	72·2 (3·3)	60·6 (5·8)	46·7 (5·3)	46·8 (11·7)	63·8 (4·5)	xxx	xx	–
Anteiso-C17:0	14·5 (1·7)	14·9 (4·7)	7·6 (3·4)	21·9 (0·5)	13·2 (8·5)	8·8 (4·2)	8·8 (4·2)	20·6 (0·3)	–	xxx	–
Total	86·0 (1·2)	84·5 (6·8)	66·2 (7·2)	94·1 (3·7)	74·5 (13·9)	55·9 (8·9)	55·9 (15·7)	84·7 (4·7)	xx	xxx	–
Iso-BCFAs, % of total membrane fatty acids§											
Iso-C14:0	0·4 (0·4)	0·9 (0·8)	0·9 (0·5)	0·1 (0·2)	0·2 (0·1)	0·5 (0·6)	0·8 (0·6)	ND	–	x	–
Iso-C15:0	3·0 (1·7)	2·7 (0·4)	10·0 (4·9)	0·6 (0·2)	2·1 (0·2)	7·9 (5·6)	12·4 (2·8)	0·1 (0·1)	–	xxx	–
Iso-C16:0	1·8 (0·6)	2·3 (2·0)	5·7 (0·7)	0·2 (0·2)	1·1 (0·4)	3·3 (0·5)	4·9 (1·1)	0·2 (0·1)	–	xxx	–
Iso-C17:0	1·8 (1·5)	1·0 (0·3)	2·1 (0·9)	0·3 (0·1)	1·0 (0·7)	4·0 (4·5)	4·1 (1·3)	1·0 (0·7)	–	x	–
Total	7·0 (3·0)	6·8 (1·3)	18·7 (6·0)	1·2 (0·4)	4·4 (0·7)	15·8 (11·2)	22·2 (2·6)	0·3 (0·2)	–	xxx	–
Straight-chain fatty acids, % of total membrane fatty acids§											
C13:0	ND	0·1 (0·2)	0·1 (0·2)	0·1 (0·1)	0·9 (0·7)	1·6 (1·4)	0·6 (0·7)	0·5 (0·2)	xx	–	–
C14:0	0·1 (0·1)	0·4 (0·4)	0·3 (0·0)	0·0 (0·1)	0·1 (0·1)	0·5 (0·7)	0·1 (0·1)	0·1 (0·1)	–	–	–
C15:0	1·5 (1·2)	1·5 (1·6)	3·3 (2·4)	0·4 (0·2)	8·1 (8·4)	11·5 (9·2)	6·7 (6·2)	3·5 (2·1)	x	–	–
C16:0	1·0 (0·4)	2·3 (1·4)	2·1 (0·8)	1·6 (1·6)	0·5 (0·2)	0·8 (1·2)	0·9 (0·5)	0·9 (0·5)	x	–	–
C17:0	3·6 (1·5)	3·6 (2·3)	7·8 (1·1)	1·7 (0·6)	10·5 (4·9)	12·5 (5·8)	12·0 (6·3)	8·4 (1·5)	xxx	–	–
C18:0	0·6 (0·6)	0·7 (0·0)	1·1 (0·7)	0·8 (1·0)	0·3 (0·0)	0·5 (0·7)	0·7 (0·5)	0·8 (0·5)	–	–	–
C19:0	0·0 (0·1)	0·1 (0·1)	0·2 (0·0)	ND	0·4 (0·1)	0·4 (0·5)	0·6 (0·2)	0·5 (0·1)	xx	–	–
Total	6·9 (1·7)	8·7 (5·5)	15·0 (1·5)	4·7 (3·3)	20·9 (14·2)	27·8 (19·5)	21·7 (13·6)	14·7 (4·5)	xx	–	–
Concentrations of short BCFAs in the medium (mg l^{-1})§											
Anteiso-C5	7·2 (0·7)	3·6	4·0 (1·5)	16·5 (4·8)	1·8 (0·1)	1·4	1·1 (0·6)	2·3 (0·6)	xxx	xx	x
Iso-C5	ND	ND	0·6 (0·5)	ND	ND	0·1	0·3 (0·1)	ND	–	–	–
% of anteiso-BC in odd-numbered BCFAs											
Membrane FAs	94·8 (3·4)	95·9 (0·4)	84·5 (7·3)	99·1 (0·4)	96·1 (0·5)	83·8 (10·4)	76·2 (7·4)	99·8 (0·1)	–	xxx	–
Short BCFAs	98·1 (2·6)	97·7 (2·6)	87·8 (6·7)	100 (0·0)	100 (0·0)	82·4 (14·5)	79·2 (1·6)	100 (0·0)	x	xxx	–

*BCFAs, branched-chain fatty acids; BCAAs, branched-chain fatty acids; ND, not detected.

†cdmB, basal chemically defined medium; cdmVLI, medium containing valine, leucine and isoleucine; cdmVL, medium containing valine and leucine; cdmI, medium containing isoleucine.

‡Results of the anova of fatty acid proportions as function of strain (str), medium (med) and the interaction (str × med). –, not significantly different; x, $P < 0{\cdot}05$; xx, $P < 0{\cdot}01$; xxx, $P < 0{\cdot}001$.

§Values are means and standard deviations (in parenthesis) of two to three replicate experiments.

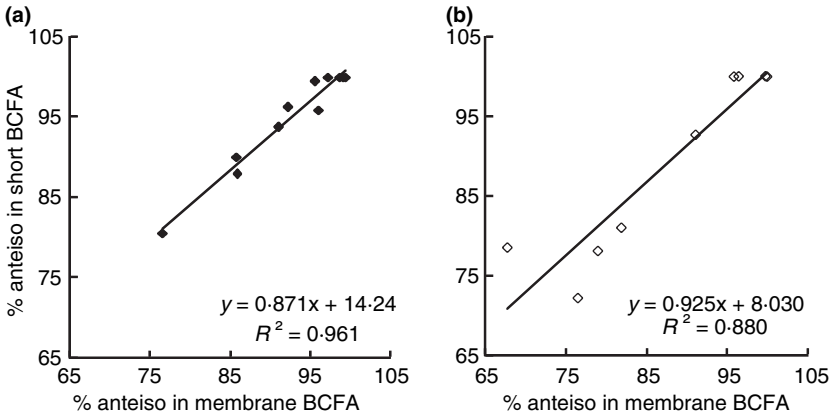

Figure 4 Relationship between the proportions of the anteiso-branched forms in short odd-numbered branched-chain fatty acids (BCFAs) and odd-numbered membrane BCFAs in *P. freudenreichii* TL4 (a) and ITGP17 (b) grown in four chemically defined media containing different combinations of branched-chain amino acids.

odd- and even-numbered iso-BCFAs were the greatest in cells grown in cdmVL, which contained valine and leucine as sole BC precursors (20·3 ± 4·2%).

The concentrations of anteiso-C5 released in the medium were significantly influenced by the strain, the medium, and the interaction of both factors (Table 2). TL4 released concentrations of short BCFAs about two- to sevenfold greater than ITGP17, and produced significantly higher concentrations in cdmI (16·5 ± 4·5 mg l^{-1}), compared with the concentrations produced in the three other media (3·6–7·2 mg l^{-1}, Table 2). As observed in YEL, short BCFAs essentially consisted of anteiso-C5. The proportions of anteiso-C5 : iso-C5 varied from 79 : 21 and 100 : 0 depending on the medium.

Anteiso-BCFAs constituted the main portion of BCFAs, in both membrane BCFAs and short extracellular BCFAs, and their proportions varied similarly as function of the BCAAs present in the medium (Table 2). We further investigated the relationships between the branching patterns in short BCFAs and in membrane BCFAs, by calculating the proportions of the anteiso forms in odd-numbered BCFAs, for all individual values of replicate cultures obtained in each growth medium, for each strain (Fig. 4). Both strains showed significant ($P < 0·001$) correlations between the proportions of anteiso forms in short extracellular BCFAs and the proportions of anteiso forms in membrane BCFAs (Fig. 4).

Discussion

Previous studies reported that the concentrations of 'isovaleric acid' released by *P. freudenreichii* vary from one strain to another by factors of approximately two- to threefold in culture media, and up to sixfold in Swiss cheese (Thierry *et al.* 2002, 2004b). In the present study, the results of the screening performed under conditions that simulated Swiss cheese conditions show that a minority of strains (approx. 10%) exhibited a high capacity to release short BCFAs (Fig. 2). In particular, the strain TL4 produced BCFAs at concentrations about 3·5 fold greater than the average concentrations of the other strains tested, and up to nine times more than the less productive strains. We also showed that anteiso-C5 predominates in short BCFAs (approx. 72–100%), regardless of which growth medium was used. Interestingly, *P. freudenreichii* still produced short extracellular BCFAs when no exogenous BCAAs were provided (in cdmB, Table 2). This result highlights the probable physiological role of short BCFA formation.

To investigate whether the production of short BCFAs is related to the biosynthesis of membrane BCFAs in *P. freudenreichii*, we first selected, from the screening results, five strains showing distinct abilities to produce short BCFAs, and determined their membrane FA composition. The membrane FA compositions were compared for cells harvested in early stationary phase, because preliminary studies showed that FA compositions significantly differed during growth (Dherbécourt *et al.*, unpublished results). BCFAs, in particular anteiso-branched FAs, were the main FAs in the five tested strains of *P. freudenreichii* (Fig. 3). In addition to BCFAs, significant proportions (approx. 13%) of odd-numbered straight-chain FAs (C15:0 and C17:0) were detected in four of the five *P. freudenreichii* strains tested (Table 2). The precursor for the biosynthesis of C15:0 and C17:0 in propionibacteria could be propionyl-CoA, which is produced via the Wood Werkman cycle involved in the formation of propionic acid (Wood 1981). The presence of BCFAs in propionibacteria lipids had been early reported (Moss *et al.* 1969; Hofherr *et al.* 1983), but at smaller proportions: 44–75% of BCFAs *vs* 85–96% in the present study, and 24–61% of anteiso-C15:0 *vs* 66–72% in the present study. These differences can be due to the marked improvements of the resolution and the accuracy of peak identification of GC analyses since the 1970s

(Moss *et al.* 1980). Membrane BCFAs are present in many bacteria (Kaneda 1991). They consist of BCFAs derived either from the three BCAAs (valine, leucine, and isoleucine), as in *Bacillus subtilis* (Willecke and Pardee 1971), or from leucine and isoleucine only, as in *S. xylosus* (Beck *et al.* 2004) and propionibacteria (Moss *et al.* 1969; Hofherr *et al.* 1983; this study). In contrast, odd-numbered straight-chain FAs (C15:0 and C17:0) do not appear to be common in bacteria. They are however found in some clostridia, such as *C. sporogenes* and *C. bifermentens* (Elsden *et al.* 1980).

The links between the biosynthesis of short BCFAs and membrane BCFAs were investigated in two strains, TL4 and ITGP17, which differed for their membrane branching patterns and the concentrations of short BCFAs they released. This study shows for the first time that the branching pattern of short BCFAs exactly matched the branching pattern of membrane BCFAs in both strains (Fig. 4), whatever the BCAAs present in the medium. At least part of short and membrane BCFAs are synthesized in *P. freudenreichii* from the available exogenous BCAAs, as shown by the experiments performed using labelled leucine and different combinations of BCAAs (Table 2). However, whatever the branching pattern of the exogenous BCAAs provided, anteiso-BCFAs constituted the main part of BCFAs. For example, in the presence of only iso-BCAAs (cdm-VL), *P. freudenreichii* still synthesized 75–85% of anteiso-BCFAs. This result shows that short BCFAs do not result from a simple conversion of the exogenous BCAAs, but derive from the pool of BC molecules synthesized *de novo* in *P. freudenreichii* to be used in membrane BCFAs synthesis.

Many bacteria species that contain BCFAs in membrane lipids do produce short BCFAs, as reported in *B. subtilis* (Kaneda 1977), *Listeria monocytogenes* (Daneshvar *et al.* 1989), *Brevibacterium linens* (Ganesan *et al.* 2004), and some staphylococci (Beck *et al.* 2004; Beck 2005). In clostridia, of 23 species tested, only the 16 species that contained BCFAs in membrane lipids had the ability to convert BCAAs to short BCFAs (Elsden *et al.* 1980). This was expected because membrane and short BCFAs are synthesized from methyl-branched-chain C4 and C5 precursors derived from carbon skeletons of the three BCAAs valine, isoleucine and leucine or their derivatives (Fig. 1). However, the existence of an exact match between the branching patterns of short and membrane BCFAs has not been reported, to our knowledge, in other species. In *S. carnosus*, for example, cells grown in a complex medium contained approx. 66%, 14% and 5% of odd-numbered anteiso-BCFAs, odd-numbered iso-BCFAs and even-numbered iso-BCFAs, respectively, while the proportions of the corresponding short BCFAs were 23%, 61% and 16% (anteiso-C5, iso-C5 and iso-C4, respectively) (Beck 2005). This result suggests that membrane and short BCFAs could derive, at least partly, from different origins in *S. carnosus*.

The exact match between the branching patterns of short and membrane BCFAs in *P. freudenreichii* let us think that the formation of short BCFAs may result from a 'leak' in the pathway of biosynthesis of membrane BCFAs (Fig. 1). To support this hypothesis, we estimated the molar parts of BC intermediates either used for membrane lipid synthesis, or converted to short BCFAs, respectively. 81 μmol equivalent of anteiso-C15 were produced per litre culture, whereas, in the same time, 21–48 μmol (and up to 175 in strain TL4) of anteiso-C5 were produced per litre of supernatant. Hence, the latter values are too high to consider the formation of short BCFAs only as a 'leak' in the pathway of biosynthesis of membrane BCFAs, in particular in the high-producing strain TL4. The formation of short BCFAs could be a way for the cells to eliminate excess BC precursors, and may constitute a reserve of precursor re-entering in the cell when the concentrations of intracellular BC precursors decrease.

In conclusion, this study confirms the hypothesis that the formation of short BCFAs by *P. freudenreichii* is closely related to the biosynthesis of membrane BCFAs. Short BCFAs are constitutively produced in *P. freudenreichii*, they consist mainly of anteiso-C5, and the levels produced depend more on the strain than on the presence of BCAAs in the growth medium. The branching pattern of BCFAs exactly matches that of the intracellular pool of BC precursors for membrane BCFA biosynthesis, whatever BCAA composition of the medium. One strain was distinguished by both its particular FA membrane composition and its capacity to release high concentrations of anteiso-C5. Short BCFAs may be released in the medium during growth to eliminate excess BC intermediates and constitute a reserve of BC precursors, but could also fulfil other physiological roles, in particularly in stationary phase.

Acknowledgement

The authors wish to acknowledge Pascal Pachot for advice on statistical analyses.

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