AEM
Journals.ASM.org

The Secreted Esterase of *Propionibacterium freudenreichii* Has a Major Role in Cheese Lipolysis

María Claudia Abeijón Mukdsi,[a] Hélène Falentin,[b,c] Marie-Bernadette Maillard,[b,c] Victoria Chuat,[b,c] Roxana Beatriz Medina,[a] Sandrine Parayre,[b,c] Anne Thierry[b,c]

Centro de Referencia para Lactobacilos (CERELA-CONICET), Argentina, and Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina[a]; INRA, UMR1253, Science et Technologie du Lait et de l'Œuf, Rennes, France[b]; Agrocampus Ouest, UMR1253, Science et Technologie du Lait et de l'Œuf, Rennes, France[c]

Free fatty acids are important flavor compounds in cheese. *Propionibacterium freudenreichii* is the main agent of their release through lipolysis in Swiss cheese. Our aim was to identify the esterase(s) involved in lipolysis by *P. freudenreichii*. We targeted two previously identified esterases: one secreted esterase, PF#279, and one putative cell wall-anchored esterase, PF#774. To evaluate their role in lipolysis, we constructed overexpression and knockout mutants of *P. freudenreichii* CIRM-BIA1^T for each corresponding gene. The sequences of both genes were also compared in 21 wild-type strains. All strains were assessed for their lipolytic activity on milk fat. The lipolytic activity observed matched data previously reported in cheese, thus validating the relevance of the method used. The mutants overexpressing PF#279 or PF#774 released four times more fatty acids than the wild-type strain, demonstrating that both enzymes are lipolytic esterases. However, inactivation of the *pf279* gene induced a 75% reduction in the lipolytic activity compared to that of the wild-type strain, whereas inactivation of the *pf774* gene did not modify the phenotype. Two of the 21 wild-type strains tested did not display any detectable lipolytic activity. Interestingly, these two strains exhibited the same single-nucleotide deletion at the beginning of the *pf279* gene sequence, leading to a premature stop codon, whereas they harbored a *pf774* gene highly similar to that of the other strains. Taken together, these results clearly demonstrate that PF#279 is the main lipolytic esterase in *P. freudenreichii* and a key agent of Swiss cheese lipolysis.

Flavor is a basic criterion associated with food quality. Cheese flavor results from a complex mixture of flavor compounds, which include nonvolatile and volatile compounds. Most of them are produced during cheese manufacture and ripening and result from the activity of microbial enzymes on milk components, such as fat, proteins, and carbohydrates. Therefore, to control cheese flavor, it is important to identify the main actors involved in the formation of flavor compounds, so as to modulate their activity, depending on the flavor notes targeted (1). Free fatty acids are important flavor compounds in most cheeses, where they contribute to pungent, rancid, cheesy, and fruity notes (2). They result from the partial hydrolysis of the ester linkage between a fatty acid and the glycerol moiety of milk triacylglycerides by lipolytic esterases (2). The main sources of esterases in cheese are microorganisms. However, the indigenous milk lipoprotein lipase (LPL) is involved in the early lipolysis that occurs during the first steps of cheese manufacture, and some lipases present in traditional coagulant preparations can also play an important role in the lipolysis observed in varieties such as Italian cheeses (2).

Propionibacterium freudenreichii is used as a ripening culture in the manufacture of Swiss-type cheeses and some other semihard cheeses (3, 4) and is the main agent of Swiss cheese lipolysis. For example, 96% of the free fatty acids (FFA) released during the ripening of mini-Swiss-type cheeses resulted from the activity of *P. freudenreichii* CIRM-BIA1^T (5). The extent of lipolysis has been shown to vary significantly under the same cheesemaking conditions, depending on the strain used, with the amount of released FFA varying up to 3-fold (6–9). In particular, some strains exhibit very low lipolytic activity (8). Whether these diverse phenotypes result from differences in the number, sequence, or expression levels of esterase-encoding genes is unknown.

Twelve (putative) esterases were previously predicted from the genome sequence of *P. freudenreichii* CIRM-BIA1^T (10), all of which are expressed regardless of the presence or absence of milk fat in the culture medium (11). The majority of lipolysis takes place early during *P. freudenreichii* growth (5), at a stage at which the release of intracellular enzymes from lysed cells is very unlikely. Therefore, we hypothesized that lipolysis in Swiss cheese mainly results from the activity of surface-exposed or secreted lipolytic esterases, rather than intracellular enzymes. We thus targeted two putative esterases, PF#279 and PF#774, predicted to be secreted and cell wall anchored, respectively. Our previous work showed that esterase PF#279 is effectively secreted and active on milk triglycerides (11).

The aim of the present study was to determine the respective contributions of these two enzymes to lipolysis. We assessed the activity of the putative esterase PF#774 by overexpression in *P. freudenreichii* CIRM-BIA1^T. In the same strain, we also knocked out the genes encoding these two proteins, using recently developed genetic tools (12). In parallel, we screened a collection of previously sequenced *P. freudenreichii* strains for their lipolytic activity to determine whether variations in the sequences of the two targeted genes would explain the different phenotypes observed.

Received 6 November 2013 **Accepted** 6 November 2013
Published ahead of print 15 November 2013
Address correspondence to Anne Thierry, anne.thierry@rennes.inra.fr.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03640-13.

doi:10.1128/AEM.03640-13

TABLE 1 Origins of the wild *Propionibacterium freudenreichii* strains used in this study[a]

Strain[b]	Environmental source (yr of isolation)
CIRM-BIA1	Unknown
CIRM-BIA9	Emmental cheese (1989)
CIRM-BIA118	Gruyère cheese (1973)
CIRM-BIA119	Gruyère cheese (1973)
CIRM-BIA121	Swiss cheese (1937)
CIRM-BIA122	Unknown (1992)
CIRM-BIA123	Morbier cheese (1992)
CIRM-BIA134	Unknown
CIRM-BIA135	Ewe raw milk (1994)
CIRM-BIA456	Raw milk (1992)
CIRM-BIA508	Gruyère cheese (1973)
CIRM-BIA512	Raw milk (1994)
CIRM-BIA513	Ras cheese (1995)
CIRM-BIA514	Hay (1994)
CIRM-BIA516	Yak cheese (2000)
CIRM-BIA527	Fribourg cheese (1992)
ITG P9	Actalia (origin not given)
ITG P18	Actalia (origin not given)
ITG P20	Actalia (origin not given)
ITG P23	Actalia (origin not given)
LSP108	Laboratoires Standa (origin not given)

[a] Strains were identified by PCR using species-specific primers according to Tilsala-Timisjarvi and Alatossava (19).
[b] CIRM-BIA, collection of the Centre International de Ressources Microbiennes—Bactéries d'Intérêt Alimentaire, INRA, Rennes, France; ITG, Actalia collection, Rennes, France; LSP, Laboratoires Standa, Caen, France. (Strain LSP108 is known for its weak lipolytic activity in Emmental cheese.)

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Twenty wild-type sequenced strains of *P. freudenreichii* were used (Table 1). In addition, strain LSP108 (from Laboratoires Standa, Caen, France), known for its very low lipolytic activity in Swiss cheese, was included in the present study.

The wild-type strain, CIRM-BIA1^T, and its genetically modified derivatives are listed in Table 2. All strains were grown at 30°C in yeast extract-lactate (YEL) broth (13) in glass tubes without agitation. In some cases, specified in the text, YEL was supplemented with chloramphenicol (10 μg ml^{-1}) or hygromycin B (750 μg ml^{-1} in YEL agar or 250 μg ml^{-1} in YEL broth).

Escherichia coli DH5α was used as a cloning vector and was obtained from Gibco-BRL Life Technologies (Saint Aubin, France). It was grown at 37°C with agitation (200 rpm) in Luria-Bertani medium (LB) containing ampicillin (100 μg ml^{-1}).

To determine the lipolytic activity of *P. freudenreichii*, cells were grown in a modified YEL medium containing 20 g/liter lactate and milk fat emulsified with a sodium caseinate solution (final fat concentration of 30% [wt/wt] in the broth medium), as previously described (11). Population levels of propionibacteria were monitored by measuring the optical density at 650 nm (OD_{650}) of a culture grown in the same medium without emulsion. Cultures were harvested at the end of log-phase growth. Triplicate (CIRM-BIA1^T mutants) or duplicate (wild-type strain) independent cultures were performed.

Construction of the genetically modified strains. Reagents were purchased from New England BioLabs (Ipswich, MA), and oligonucleotides were purchased from Sigma Genosys (Haverhill, Cambridgeshire, United Kingdom). Genomic DNA was extracted using the DNeasy tissue kit (Qiagen, Courtaboeuf, France) and automated sample preparation QIAcube (Qiagen), according to the manufacturer's instructions.

(i) Overexpression of esterase in *P. freudenreichii*. Transformed strains of *P. freudenreichii* CIRM-BIA1^T overexpressing esterase PF#279 or PF#774 were constructed. The esterase genes *pf279* (PFREUD_04340) and *pf774* (PFREUD_04240) were separately cloned into the pFB01 vector downstream of the P*tuf* promoter (Table 2). P*tuf* was chosen because the *tuf* gene was shown to be strongly expressed during the growth of this strain under the same conditions (14). To obtain this construct, *pf279* and *pf774* were amplified by PCR from *P. freudenreichii* CIRM1-BIA1 genomic DNA using primers containing restriction sites (see Table S1 in the supplemental material). High-fidelity PCR was performed to ensure accurate and reliable PCR amplification. PCR mixtures contained 1 mM MgSO4, 0.3 μM each primer, 300 μM each deoxynucleoside triphosphate (dNTP), and 1.43 U of Platinum *Pfx* high-fidelity polymerase in 1× *Pfx* amplification buffer (Invitrogen, Carlsbad, CA). PCR was performed in a thermocycler (Veriti, Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 94°C for 2 min, followed by 20 cycles of 94°C for 45 s and 60°C for 45 s, with a decrease by 0.5°C at each cycle, then 72°C for 90s, followed by 10 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 90 s, followed by a final step at 72°C for 5 min. Each of the *pf279* and *pf774* amplicons was purified with the QIAquick PCR purification kit (Qiagen) and double digested with restriction enzymes (XbaI-HindIII), and the digested fragments were then purified. The pFB01 plasmid was digested using the same restriction enzymes, and the digestion product was subjected to 1.5% agarose gel electrophoresis. Agarose pieces containing the digested plasmid were excised and purified using PCR cleanup gel electrophoresis (Qiagen). Fragments containing *pf279* and *pf774* were separately introduced into the digested plasmid by ligation performed according to the manufacturer's instructions (ligase from In-

TABLE 2 Genetically modified strains and plasmids used in this study

Strain or plasmid	Description	Origin or reference
Strains		
E. coli DH5α	Cloning host strain	Gibco-BRL
P. freudenreichii		
CB1^{Ta}	*P. freudenreichii* subsp. *shermanii* type strain (wild type)	CIRM-BIA
CB1::pFB01:*pf279*	CB1 with *pf279* overexpressed downstream of P*tuf* promoter	This study
CB1-KO-*pf279*	CB1 with inactivated *pf279*	This study
CB1-KO-*pf279*-C	CB1 with inactivated *pf279* and complemented with *pf279*	This study
CB1::pFB01:*pf774*	CB1 with *pf774* overexpressed downstream of the P*tuf* promoter	This study
CB1-KO-*pf774*	CB1 with inactivated *pf774*	This study
Plasmids		
pPK705	*E. coli-P. freudenreichii* shuttle vector, 8.3 kb; Amp^r Hyg^r	20
pFB01	Derived from pPK705 vector, 8.5 kb; Amp^r Hyg^r; P*tuf* promoter	14
pFB01:*pf279*	Derived from pFB01 vector, 9.0 kb; Amp^r Hyg^r; *pf279* under control of P*tuf* promoter	This study
pFB01:*pf774*	Derived from pFB01 vector, 9.0 kb; Amp^r Hyg^r; *pf774* under control of P*tuf* promoter	This study
pUC:*pf279*:CmR	Derived from pUC18 vector, 4.7 kb, Cm^r; truncated fragment of *pf279*	This study
pUC:*pf774*:CmR	Derived from pUC18 vector, 4.7 kb, Cm^r; truncated fragment of *pf774*	This study

[a] CB1^T = CIRM-BIA1^T.

vitrogen, Carlsbad, CA). The resulting product was used to transform *E. coli* DH5α competent cells by thermal shock (15). Cells were regenerated by incubation in LB broth (90 min, 37°C, 200 rpm), and transformants were selected on LB agar-ampicillin. Recombinant plasmids were extracted with Nucleospin Multi-8-Plasmid kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's instructions and used to transform *P. freudenreichii* CIRM-BIA1^T competent cells as previously described (16), with the following modifications. *P. freudenreichii* was grown in YEL medium supplemented with 0.5 M sucrose and 1% (wt/wt) glycine and incubated to an OD_{650} of 0.4 to 0.7. After electrotransfection (2.5 kV, 200 Ω, and 25 μF), cells were regenerated by incubation for 16 h at 30°C, and *P. freudenreichii* clones harboring the inserted vector were selected on YEL agar containing 250 μg/ml of hygromycin B and incubated for 4 days at 30°C under anaerobiosis (Anaerocult A; Merck, Darmstadt, Germany).

(ii) Insertional inactivation of *pf279* and *pf774* genes (KO mutants). The insertional inactivation of both genes was conducted separately as previously described (12). Briefly, a suicide vector was constructed by inserting a chloramphenicol resistance gene into a pUC18 plasmid. In the resulting plasmid, internal fragments of 515 bp of the *pf279* open reading frame and 516 bp of the *pf774* open reading frame (accession no. AM944371 and AM944376) (10) of strain CIRM-BIA1^T were separately cloned, resulting in the vectors pUC:*pf279*:CmR and pUC:*pf774*:CmR, respectively. To make these constructs, *pf279* and *pf774* were amplified by PCR from *P. freudenreichii* CIRM-BIA1^T genomic DNA using primers containing restriction sites (see Table S1 in the supplemental material).

PCR mixtures contained 2 mM $MgCl_2$, 1 μM each primer, 200 μM each dNTP, and 2.5 U of *Taq* polymerase (Fermentas International, Inc., Burlington, Ontario, Canada) in 1× amplification buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl). PCR was performed as previously described (14). PCR products were purified, and each amplicon and the pUC18:CmR plasmid were double digested (XbaI-BamHI) and purified again as described above. Fragments containing *pf279* and *pf774* were separately ligated to the digested plasmid, and each ligation product was used to transform *E. coli* DH5α competent cells. Transformants were selected on LB agar-ampicillin, and recombinant plasmids were isolated. *P. freudenreichii* CIRM-BIA1^T was transformed with each inactivation vector, and *ad hoc* transformants were selected on YEL agar containing chloramphenicol.

(iii) Complementation of KO mutants with plasmid harboring *pf279*. To perform the genetic complementation of the CB1-KO-*pf279* strain, pFB01:*pf279* overexpression plasmids were multiplied in *E. coli* DH5α cells, extracted, and used to transform the CB1-KO-*pf279* knockout strain, as described above. Transformants were selected on YEL agar containing hygromycin B.

Comparison of the sequences of genes encoding esterases. The sequences of both *pf279* and *pf774* genes were extracted from the complete genomes of the 20 sequenced strains of *P. freudenreichii* by using the genome annotation system AGMIAL (17). In addition, the sequence of the *pf279* gene was determined in the commercial strain LSP108. The sequence was also confirmed in the nonlipolytic strain CIRM-BIA514, in which a specific mutation was observed in the *pf279* sequence. For this, *pf279* was amplified by high-fidelity PCR from the genomic DNA of the CIRM-BIA514 and LSP108 strains using primers *pf279*-XbaI-F and *pf279*-HindIII-R (see Table S1 in the supplemental material). The PCR mixture (50 μl) contained 1.5 mM $MgCl_2$, 200 μM dNTP, 0.5 μM each primer, ~50 ng DNA template, 3% (vol/vol) dimethyl sulfoxide (DMSO), and 1 U high-fidelity Phusion DNA polymerase (New England BioLabs) in 1× Phusion GC buffer. A 2-step thermocycling protocol was used: initial denaturation at 98°C for 1 min, followed by 30 cycles of 98°C for 15 s and 72°C for 30 s, then 72°C for 10 min, and then a final step at 12°C for 4 min. Amplification products were purified as described above and sequenced (GATC Biotech AG, Mulhouse, France). The sequences of the *pf279* and *pf774* genes were analyzed using Vector NTI software (Invitrogen) and aligned at the gene and protein levels using ClustalW 2.1.

TABLE 3 EMBL accession numbers of the sequences of *Propionibacterium freudenreichii* lipolytic esterases PF#279 and PF#774

Accession no.	Gene	Strain
HG426277	*pf279*	CIRM-BIA9
HG426278	*pf279*	CIRM-BIA118
HG426279	*pf279*	CIRM-BIA119
HG426280	*pf279*	CIRM-BIA121
HG426281	*pf279*	CIRM-BIA122
HG426282	*pf279*	CIRM-BIA123
HG426283	*pf279*	ITG P18
HG426284	*pf279*	ITG P20
HG426285	*pf279*	LSP108
HG426286	*pf279*	CIRM-BIA134
HG426287	*pf279*	CIRM-BIA135
HG426288	*pf279*	ITG P9
HG426289	*pf279*	ITG P23
HG426290	*pf279*	CIRM-BIA456
HG426291	*pf279*	CIRM-BIA508
HG426292	*pf279*	CIRM-BIA512
HG426293	*pf279*	CIRM-BIA513
HG426294	*pf279*	CIRM-BIA514
HG426295	*pf279*	CIRM-BIA516
HG426296	*pf279*	CIRM-BIA527
HG426297	*pf774*	CIRM-BIA9
HG426298	*pf774*	CIRM-BIA118
HG426299	*pf774*	CIRM-BIA119
HG426300	*pf774*	CIRM-BIA121
HG426301	*pf774*	CIRM-BIA122
HG426302	*pf774*	CIRM-BIA123
HG426303	*pf774*	ITG P18
HG426304	*pf774*	ITG P20
HG426305	*pf774*	CIRM-BIA134
HG426306	*pf774*	CIRM-BIA135
HG426307	*pf774*	ITG P9
HG426308	*pf774*	ITG P23
HG426309	*pf774*	CIRM-BIA456
HG426310	*pf774*	CIRM-BIA508
HG426311	*pf774*	CIRM-BIA512
HG426312	*pf774*	CIRM-BIA513
HG426313	*pf774*	CIRM-BIA514
HG426314	*pf774*	CIRM-BIA516
HG426315	*pf774*	CIRM-BIA527

Determination of FFA. Individual FFA were analyzed using gas chromatography (GC) according to the method of De Jong and Badings (18). Briefly, lipids were extracted from 1 g of culture medium using ether-heptane (50:50 [vol/vol]) at acidic pH in the presence of anhydrous sodium sulfate. FFA were isolated from total lipids using an aminopropyl solid-phase column and quantified using gas chromatography on a BP21 column (SGE, Ringwood, Victoria, Australia) (25 m by 0.53 mm by 0.5-μm film thickness) under the following conditions: on-column injection at 65°C; carrier gas, hydrogen, 31 kPa; temperature program, heating rate of 10°C/min from 65°C up to 240°C, maintained for 10 min; flame-ionization detector operated at 240°C.

Statistical analyses. One-way analyses of variance were performed using R (http://www.R-project.org) to determine the effect of the strains on the concentrations of FFA and on their net production. Differences between the treatment means were compared at the 5% level of significance by using the Fisher's least significance difference (LSD) test.

Nucleotide sequence accession numbers. The sequences of the *pf279* and *pf774* genes have been deposited in the EMBL data library, and the accession numbers are given in Table 3.

TABLE 4 Concentrations of FFA at the end of growth of *Propionibacterium freudenreichii* CIRM-BIA1^T and five genetically modified strains derived from this strain in a medium containing a milk fat emulsion[a]

FFA	FFA concn (μg/g) in[b]:						
	Control[c]	CB1^T	CB1::pFB01:*pf279*	CB1-KO-*pf279*	CB1-KO-*pf279*-C	CB1::pFB01:*pf774*	CB1-KO-*pf774*
$C_{4:0}$	120.7 D	222.0 B	250.7 A	176.3 C	234.3 AB	252.3 A	229.3 B
$C_{6:0}$	11.7 C	37.0 B	59.0 A	16.0 C	56.0 A	56.7 A	40.7 B
$C_{8:0}$	8.7 E	26.7 C	49.7 A	14.7 D	42.0 B	46.3 AB	29.7 C
$C_{10:0}$	20.3 D	47.7 C	104.7 A	30.7 D	90.3 B	97.3 AB	54.0 C
$C_{12:0}$	24.7 D	57.3 C	150.3 A	36.3 D	127.0 B	133.7 B	66.7 C
$C_{14:0}$	81.7 D	201.3 C	643.0 A	122.3 D	520.0 B	526.7 B	224.7 C
$C_{15:0}$	10.0 E	18.0 CD	52.3 A	12.7 DE	44.3 B	44.0 B	19.0 C
$C_{16:0}$	250.3 E	478.7 CD	1,591.3 A	289.3 DE	1,353.0 B	1,362.7 B	562.3 C
$C_{16:1}$	23.7 C	47.7 B	107.7 A	32.7 C	101.3 A	105.3 A	54.3 B
$C_{18:0}$	108.0 D	143.7 CD	446.7 A	106.7 D	379.7 B	387.3 B	175.7 C
$C_{18:1}$	247.7 C	469.7 B	1,151.0 A	276.7 C	1,065.7 A	1,147.3 A	559.3 B
$C_{18:2}$	15.7 C	29.7 B	72.3 A	17.7 C	68.0 A	70.0 A	32.0 B
$C_{18:3}$	5.7 C	7.7 BC	17.0 A	6.7 BC	17.0 A	16.3 A	8.3 B
Total	946.3 D	1,810.0 C	4,744.0 A	1,164.0 D	4,144.0 AB	4,290.7 B	2,076.0 C

[a] See Table 2 for strain nomenclature.
[b] Means within a row followed by a common letter do not significantly differ according to the LSD test ($P > 0.05$).
[c] Noninoculated control medium.

RESULTS AND DISCUSSION

This study aimed to determine the role of two putative esterases in lipolysis by *P. freudenreichii*, combining targeted mutation experiments and the exploration of the natural phenotypic and genomic biodiversity in 21 wild-type strains.

Phenotype of mutants of *P. freudenreichii* CIRM-BIA1^T either overexpressing or knocked out for the two targeted genes. All wild-type and mutant strains displayed similar growth and propionic fermentation rates. At harvest time (from 64 to 74 h of incubation), the OD_{650} value was 3.3 ± 0.4 (i.e., about 3.10^9 CFU/g).

The concentrations of the 13 main FFA observed for the wild-type strain and the five genetically modified strains in the presence of a milk fat emulsion are detailed in Table 4, and the profile of the released FFA is illustrated in Fig. 1. The concentration of total FFA in the noninoculated control medium was 946.3 ± 118.6 μg/g, and it did not significantly change during incubation (data not shown). In cultures of the wild-type strain, the concentration of FFA was about 2-fold higher than that in the control medium (Table 4).

The two strains overexpressing either *pf279* or *pf774* and the complemented *pf279* knockout strain exhibited similarly high lipolytic activity levels (Table 4). They released 4-fold larger FFA amounts, on average, than the wild-type strain, with marked differences for some FFA (Fig. 1). The amounts of medium- and long-chain FFA released, in particular saturated FFA, were 3.8- to 4.8-fold higher than those of the wild-type strain, whereas short-chain FFA release was less affected (1.4-fold). Consequently, the profile of released FFA by these strains differed from that of the wild-type strain, with 42.9% saturated long-chain FFA and only 6.1% short-chain FFA, versus 30.6% and 16.7%, respectively, in the wild-type strain. The results of overexpression of *pf279* are in agreement with our previous results that showed that a mutant overexpressing *pf279* under the control of another promoter released 5 to 8 times more FFA from milk fat than the wild-type strain (11). The results of overexpression of the *pf774* gene, previously annotated as encoding a putative cell-bound esterase (11), showed that PF#774 (i) is effectively an esterase and (ii) is active on milk triglycerides. *P. freudenreichii* CIRM-BIA1^T thus possesses a second lipolytic esterase active on milk fat, PF#774, which exhibits an apparent specificity for milk fat that did not significantly differ from that of PF#279.

The two strains knocked out for the *pf279* and *pf774* esterase genes (CB1-KO-*pf279* and CB1-KO-*pf774*) exhibited very different phenotypes (Fig. 1). Inactivation of the *pf279* gene induced a dramatic decrease in the lipolytic activity compared to that of the wild-type strain, with a particularly sharp decrease (−84%) in the amount of long-chain FFA released (Fig. 1). Only two FFA, butanoic and octanoic acids, were found in significantly larger

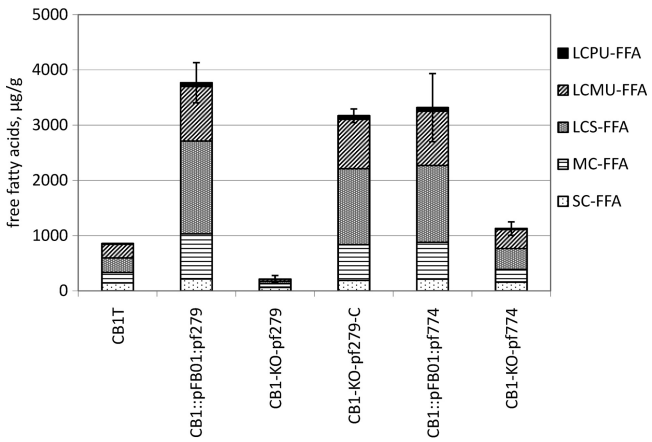

FIG 1 Net production of free fatty acids released from a milk fat emulsion in cultures of *Propionibacterium freudenreichii* CIRM-BIA1^T and five genetically modified strains derived from this strain. See Table 2 for strain nomenclature. FFA are expressed as μg total FFA per g culture. SC-FFA, short-chain free fatty acids ($C_{4:0}$ to $C_{8:0}$); MC-FFA, medium-chain free fatty acids ($C_{10:0}$ to $C_{15:0}$); LCS-FFA, long-chain saturated free fatty acids (sum of $C_{16:0}$ and $C_{18:0}$); LCMU-FFA, long-chain monounsaturated free fatty acids (sum of $C_{16:1}$ and $C_{18:1}$); LCPU-FFA, long-chain polyunsaturated free fatty acids (sum of $C_{18:2}$ and $C_{18:3}$). Values are means and standard deviations of triplicate experiments.

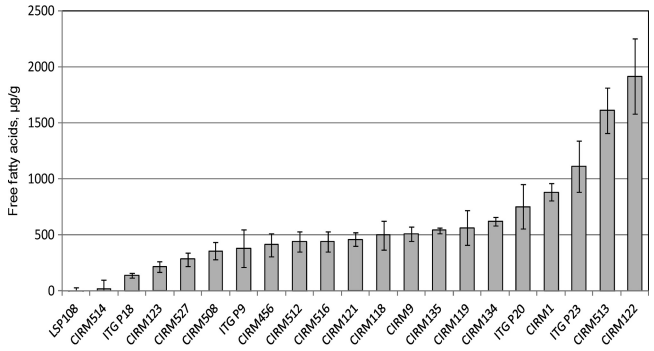

FIG 2 Net production of free fatty acids released from a milk fat emulsion in cultures of wild *Propionibacterium freudenreichii* strains. Values are means and standard deviations of duplicate experiments.

amounts in cultures of CB1-KO-*pf279* compared to the noninoculated control medium (Table 4). Inactivation of the *pf774* gene, in contrast, did not induce any significant change in the lipolytic activity and the profiles of the released FFA compared to the wild-type strain (Table 4 and Fig. 1). These results suggest that either PF#774 was not highly expressed in the wild-type strain under our experimental conditions, or the activity of this esterase is low compared to that of PF#279.

The results of these targeted mutation experiments clearly demonstrate that PF#279 is responsible for the majority of the FFA released from an emulsion of milk fat during *P. freudenreichii* growth.

Exploration of phenotypic and genomic biodiversity within a collection of wild-type *P. freudenreichii* strains. The lipolytic activity of 21 wild-type strains, including some strains previously characterized for their lipolytic activity in Swiss cheese, was investigated in a medium containing an emulsion of milk fat (Fig. 2).

At harvest time (66 to 71 h of incubation), the value of OD_{650} was 4.4 ± 0.3. Nineteen strains out of 21 released FFA during their growth in the presence of milk fat, with a net production of FFA ranging from 137 to 1,915 μg/g culture, whereas two strains (LSP108 and CIRM-BIA514) did not display any detectable lipolytic activity under our experimental conditions (production lower than 50 μg/g).

Interestingly, the lipolytic activities of *P. freudenreichii* were similar under the *in vitro* conditions used and in cheese. Specifically, ITGP18 was previously shown to exhibit low lipolytic activity, ITGP9 exhibited medium activity, and ITGP23 exhibited high activity in cheese, with net increases in FFA concentrations of 0.2, 2.1, and 3 to 4 mg/g cheese, respectively, compared to the control cheeses manufactured without propionibacteria (6, 8). Under the *in vitro* conditions of the present study, these three strains released 0.137, 0.379, and 1.109 mg FFA/g culture medium, respectively. Moreover, the commercial strain LSP108, known for its very low lipolytic activity in Swiss cheese, did not display any detectable activity *in vitro*. These results demonstrate the relevance of the conditions used in the present study to assess *in vitro* the lipolytic activity of *P. freudenreichii* in cheese.

The results of sequence alignments of the *pf279* and *pf774* genes showed that both genes are highly conserved in *P. freudenreichii* (data not shown). The predicted esterase PF#774 is a 412-residue protein in all but two strains (CIRM-BIA516 and CIRM-BIA122, for which the predicted proteins have 399 amino acid residues). In most strains (16 out of 21), the predicted sequence of the esterase PF#774 is identical. The size of the *pf279* gene is 1,332 bp (for 14 strains) or 1,329 bp (for 5 strains). In the latter case, *pf279* presents the same deletion of 3 consecutive bp near the 3′ end of the gene (codon TCG [Ala] or GCG [Ser] at position 1308) (results not shown). This deletion leads to a protein that is shorter by one residue (Ala or Ser) at the C-terminal end than the 443-residue protein predicted in the majority of strains.

For two strains (CIRM-BIA514 and LSP108), however, the *pf279* gene shared the same single-nucleotide deletion near the 5′ end of the *pf279* gene, at position 107 (see Fig. S1 in the supplemental material). This mutation results in a frameshift and the introduction of a TGA stop codon at position 190. Therefore, the translation product would consist of a 63-residue peptide with a 35-residue N-terminal sequence homologous to that of the other strains, followed by a 28-residue sequence without homology. Interestingly, these strains are the two identified as nonlipolytic (Fig. 2). This result strongly supports the conclusion that the secreted esterase PF#279 is the main agent of milk fat lipolysis in *P. freudenreichii*.

Significance for cheese ripening. Taken together, the results of the present study clearly demonstrate that the secreted esterase PF#279 is the main agent of lipolysis in cheese by *P. freudenreichii*. The identification of this key target gene offers new screening opportunities to identify nonlipolytic strains. We also observed great variation in lipolytic activities within the pool of lipolytic strains, which should now be further investigated. The variation could result from several factors or combinations of factors, including differences in the expression levels of the *pf279* gene and variations in the efficiency of secretion of PF#279. Moreover, even if the sequences of esterase genes appeared to be highly conserved within the species, we cannot exclude slight changes in sequence that could affect the secondary structure of the esterase and consequently its affinity for the substrate.

The esterases responsible for lipolysis have not been identified in most cheese-related bacteria, although the FFA released from lipolysis participate in cheese flavor in most cheeses. Lactic acid bacteria possess only intracellularly located esterases (2). Their activity on cheese fat is thus made possible only after their release in the cheese matrix and is generally very weak. The present results highlight why the contribution of *P. freudenreichii* to lipolysis is decisive in the internally ripened (semi-) hard cheese varieties that contain propionibacteria, such as Emmental and Maasdam cheeses. The contribution of *P. freudenreichii* esterases is greater than that of LPL, whose activity can be observed during the earliest steps of cheese manufacture, and far greater than that of lactic acid bacteria.

ACKNOWLEDGMENTS

M. C. Abeijón Mukdsi benefited from a grant from ECOS-MINCyT (Action no. A08B02) and a postdoctoral Bernardo Houssay grant. The sequencing was supported by INRA AIP Bioressource PropioDive 2009.

Valentin Loux and Julien Buratti created databases and implemented the AGMIAL tool. We are grateful to Alyson Yee for English language corrections. We thank Pascal Pachot, Stat-Plan, for support concerning statistical analyses. We also thank Y. Murooka, Osaka University, for providing the pPK705 vector used in this study.

REFERENCES

1. **Urbach G.** 1997. The flavour of milk and dairy products. II. Cheese: contribution of volatile compounds. Int. J. Dairy Technol. **50:**79–89.

2. **Collins YF, McSweeney PLH, Wilkinson MG.** 2003. Lipolysis and free fatty acid catabolism in cheese: a review of current knowledge. Int. Dairy J. **13:**841–866. http://dx.doi.org/10.1016/S0958-6946(03)00109-2.
3. **Thierry A, Falentin H, Deutsch SM, Jan G.** 2011. Bacteria, beneficial: *Propionibacterium* spp., p 403–411. *In* Fuquay JW, Fox PF, McSweeney P (ed), Encyclopedia of dairy science. Elsevier, London, United Kingdom.
4. **Thierry A, Maillard MB, Bonnarme P, Roussel E.** 2005. The addition of *Propionibacterium freudenreichii* to Raclette cheese induces biochemical changes and enhances flavour development. J. Agric. Food Chem. **53:** 4157–4165. http://dx.doi.org/10.1021/jf0481195.
5. **Dherbécourt J, Bourlieu C, Maillard MB, Aubert-Frogerais L, Richoux R, Thierry A.** 2010. Time course and specificity of lipolysis in Swiss cheese. J. Agric. Food Chem. **58:**11732–11739. http://dx.doi.org/10.1021/jf102572z.
6. **Thierry A, Maillard MB, Richoux R, Kerjean JR, Lortal S.** 2005. *Propionibacterium freudenreichii* strains quantitatively affect production of volatile compounds in Swiss cheese. Lait **85:**57–74. http://dx.doi.org/10.1051/lait:2004036.
7. **Fröhlich-Wyder MT, Bachmann HP.** 2004. Cheeses with propionic acid fermentation, p 141–156. *In* Fox PF, McSweeney PLH, Cogan TM, Guinee TP (ed), Cheese. Chemistry, physics and microbiology. Elsevier, London, United Kingdom.
8. **Chamba JF, Perréard E.** 2002. Contribution of propionibacteria to lipolysis of Emmental cheese. Lait **82:**33–44. http://dx.doi.org/10.1051/lait:2001003.
9. **Bachmann HP.** 1998. Lipolysis in cheese: not too much, not too little. Agrarforschung **5:**293–295.
10. **Dherbécourt J, Falentin H, Canaan S, Thierry A.** 2008. A genomic search approach to identify esterases in *Propionibacterium freudenreichii* involved in the formation of flavour in Emmental cheese. Microb. Cell Fact. **7:**16. http://dx.doi.org/10.1186/1475-2859-7-16.
11. **Dherbécourt J, Falentin H, Jardin J, Maillard MB, Baglinière F, Barloy-Hubler F, Thierry A.** 2010. Identification of a secreted lipolytic esterase in *Propionibacterium freudenreichii*, a ripening process bacterium involved in Emmental cheese lipolysis. Appl. Environ. Microbiol. **76:**1181–1188. http://dx.doi.org/10.1128/AEM.02453-09.
12. **Deutsch SM, Le Bivic P, Herve C, Madec MN, LaPointe G, Jan G, Le Loir Y, Falentin H.** 2010. Correlation of the capsular phenotype in *Propionibacterium freudenreichii* with the level of expression of *gtf*, a unique polysaccharide synthase-encoding gene. Appl. Environ. Microbiol. **76:** 2740–2746. http://dx.doi.org/10.1128/AEM.02591-09.
13. **Malik AC, Reinbold GW, Vedamuthu ER.** 1968. An evaluation of the taxonomy of *Propionibacterium*. Can. J. Microbiol. **14:**1185–1191. http://dx.doi.org/10.1139/m68-199.
14. **Deutsch SM, Parayre S, Bouchoux A, Guyomarc'h F, Dewulf J, Dols-Lafargue M, Baglinière F, Cousin FJ, Falentin H, Jan G, Foligné B.** 2012. Contribution of surface β-glucan polysaccharide to physicochemical and immunomodulatory properties of *Propionibacterium freudenreichii*. Appl. Environ. Microbiol. **78:**1765–1775. http://dx.doi.org/10.1128/AEM.07027-11.
15. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580. http://dx.doi.org/10.1016/S0022-2836(83)80284-8.
16. **Gautier M, Rouault A, Sommer P, Briandet R, Cassin R.** 1995. Bacteriophages infecting dairy propionibacteria. Lait **75:**427–434. http://dx.doi.org/10.1051/lait:19954-532.
17. **Bryson K, Loux V, Bossy R, Nicolas P, Chaillou S, van de Guchte M, Penaud S, Maguin E, Hoebeke M, Bessieres P, Gibrat JF.** 2006. AGMIAL: implementing an annotation strategy for prokaryote genomes as a distributed system. Nucleic Acids Res. **34:**3533–3545. http://dx.doi.org/10.1093/nar/gkl471.
18. **De Jong C, Badings HT.** 1990. Determination of free fatty acids in milk and cheese. Procedures for extraction clean up and capillary gas chromatographic analysis. J. High Resolut. Chromatogr. **13:**94–98.
19. **Tilsala-Timisjarvi A, Alatossava T.** 2001. Characterization of the 16S-23S and 23S-5S rRNA intergenic spacer regions of dairy propionibacteria and their identification with species-specific primers by PCR. Int. J. Food Microbiol. **68:**45–52. http://dx.doi.org/10.1016/S0168-1605(01)00462-7.
20. **Kiatpapan P, Hashimoto Y, Nakamura H, Piao YZ, Ono H, Yamashita M, Murooka Y.** 2000. Characterization of pRGO1, a plasmid from *Propionibacterium acidipropionici*, and its use for development of a host-vector system in propionibacteria. Appl. Environ. Microbiol. **66:**4688–4695. http://dx.doi.org/10.1128/AEM.66.11.4688-4695.2000.