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Identification of a Secreted Lipolytic Esterase in *Propionibacterium freudenreichii*, a Ripening Process Bacterium Involved in Emmental Cheese Lipolysis▽†

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Lipolysis plays an important role in the formation of cheese flavor. In Emmental cheese, the main part of lipolysis has been associated with the presence of *Propionibacterium freudenreichii*, a species used as a ripening culture. Our aim was to identify the most probable lipolytic esterase(s) involved in cheese lipolysis by *P. freudenreichii*. Since cheese lipolysis mainly occurs during *P. freudenreichii* growth, we hypothesized that *P. freudenreichii* possesses secreted lipolytic esterase(s). For 12 putative esterase genes previously identified from the genome of *P. freudenreichii* CIRM1, the level of expression was quantified by real-time reverse transcriptase (RT)-PCR, and the subcellular localization of esterases was predicted *in silico*. The esterase activity in extracellular and intracellular extracts of *P. freudenreichii* was characterized by zymography, and the extracellular esterases were identified by mass spectrometry. Finally, the best candidate was overexpressed in the same strain. All of the 12 genes encoding putative esterases were expressed. Esterase PF#279 was predicted to be secreted in the medium, PF#774 to be surface exposed, and the 10 remaining putative esterases to be intracellular. Zymography revealed that esterase activities in culture supernatant differed from the ones detected in intracellular extracts. PF#279 was identified as the sole esterase present in culture supernatant. Transformed *P. freudenreichii* CIRM1 clones overexpressing PF#279 showed 5 to 8 times more lipolytic activity on milk fat than the wild-type strain. Combining *in silico*, biochemical, and genetic approaches, we showed that PF#279 is the sole secreted esterase in *P. freudenreichii* and is active on milk fat. Therefore, it is likely a key component in cheese lipolysis by *P. freudenreichii*.

Lipolysis consists of the hydrolysis of fat by lipolytic esterases, commonly named lipases. It occurs at various degrees in all cheeses, results in the release of free fatty acids (FFA), and is essential for the formation of the correct flavor. The FFA released bring "pungent," "rancid," "cheese," and "fruity" flavor notes (1, 2). The enzymes involved in Emmental cheese lipolysis are milk lipoprotein lipase (LPL) and enzymes from cheese microorganisms (2, 3).

In Emmental cheese, the main part of lipolysis has been associated with the presence of *Propionibacterium freudenreichii*, a species used as a ripening culture. It has been shown that the intensity of lipolysis was four to five times greater in experimental Emmental-type cheeses inoculated with *P. freudenreichii* than in the control cheese without *P. freudenreichii* (26). The role of *P. freudenreichii* in lipolysis is thought to result from a direct involvement of lipolytic esterase(s) on cheese fat. However, the enzymes involved have not yet been identified. *P. freudenreichii* may also have an indirect role in cheese lipolysis by modifying the fat interface, thus facilitating the activity of lipolytic esterases from other sources. The main part of lipolysis takes place early during the growth of *P. freudenreichii* (6, 19). The release of intracellular enzymes from lysed cells at this early stage of growth is very unlikely, since lysis of *P. freudenreichii* in Emmental cheese has never been detected before the end of the ripening. Moreover, even *P. freudenreichii* strains with a high lytic activity *in vitro* showed only a weak and late lysis in Emmental-type cheese (27). From all of these observations, we hypothesized that *P. freudenreichii* possesses at least one secreted or surface-exposed lipolytic esterase able to hydrolyze milk fat.

Several esterases have been characterized in *P. freudenreichii* (9, 10, 15, 21, 24). Most of these studies concern the characterization of intracellular enzymes. The presence of cell-associated and secreted esterase activities was also reported in *P. freudenreichii* (9, 10). However, since the absence of cell lysis was not strictly demonstrated in the latter studies, the esterase activity observed in culture supernatant may have resulted from the release of intracellular enzyme(s) from lysed cells, as previously stressed by Collins et al. (2). Twelve putative esterases have been recently identified from the genome sequence of *P. freudenreichii* CIRM1^T (7).

In the present study, we aimed to identify the most probable esterase(s) involved in lipolysis in Emmental cheese, i.e., the

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description	Source or reference
Plasmids		
pPK705	*E. coli-P. freudenreichii* shuttle vector, 8.3 kb; Amp^r Hyg^r	16
pFB02lip	Derived from pPK705 vector, 9.8 kb; Amp^r Hyg^r; *pf279* under the control of the *Pbccp* promoter	This study
Strains		
E. coli DH5α	Cloning host strain	Gibco-BRL
CIRM1	*P. freudenreichii* subsp. *shermanii* type strain (wild type)	CRBIP[a]
CIRM1::pPK705	CIRM1 transformed with vector only	This study
CIRM1::pFB02lip	CIRM1 with *pf279* overexpressed from the *Pbccp* promoter	This study

[a] CRBIP, Collection of Institut Pasteur, Paris, France.

esterases that fulfill the following conditions: (i) to be expressed in *P. freudenreichii*, (ii) to be surface exposed or secreted, and (iii) to be active on milk fat. We developed the following strategy: (i) to confirm the lipolytic activity of *P. freudenreichii* CIRM1 in pure culture; (ii) to search for secreted esterases among a pool of putative esterases previously identified, by combining bioinformatics prediction, gene expression study, zymography, and identification of the esterases present in culture supernatant; and (iii) to confirm the lipolytic activity of the secreted esterase that we identified, by overexpression in *P. freudenreichii*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The origin of strains is given in Table 1. *P. freudenreichii* was grown at 30°C, either in yeast extract-lactate (YEL) medium (20) or in a previously described (8) chemically defined medium (CDM) containing sodium lactate as carbon source, seven amino acids (Glu, Asp, Cys, Arg, Lys, His, Arg and Tyr), minerals (K, Na, PO_4, Mg, Mn, Fe, Co, and Zn), and nine vitamins. The incubation was performed at 30°C, for 3 days or more, depending on the experiment. For growth of *P. freudenreichii* transformed clones, 250 μg/ml hygromycin B (Sigma) was added. *Escherichia coli* was grown in Luria-Bertani (LB) broth at 37°C with shaking at 200 rpm. For antibiotic selection, 100 μg/ml ampicillin was used.

To determine the lipolytic activity of *P. freudenreichii*, cells were grown in culture medium (CDM or YEL) containing milk fat emulsified with a sodium caseinate solution, as previously described (6). Briefly, an emulsion was prepared from commercial sodium caseinate and anhydrous milk fat, by emulsifying 1,300 × *g* of a preheated 4% (wt/wt) solution of sodium caseinate with 900 g of anhydrous milk fat using a blender to obtain a 45% (wt/wt) fat emulsion. The emulsion was then homogenized at 600 bars in a high-pressure homogenizer (Stansted Fluid Power, Ltd., United Kingdom). The final emulsion was sterilized by autoclaving at 115°C for 20 min and stored at room temperature. It was added to culture media to obtain a fat concentration of 30%. The medium containing milk fat emulsion was stable several weeks.

Population levels of propionibacteria were monitored by measuring the optical density at 650 nm (OD_{650}) and by plate counting on YEL-agar plates (agar, 10 g/liter). The absence of culture contamination was checked on PCA agar plates (Biokar Diagnostics, Beauvais, France) incubated under an air atmosphere for 1 to 3 days at 30°C.

Determination of free fatty acids. Individual free fatty acids (FFA) were determined in duplicate according to the method of De Jong (5). Briefly, lipids were extracted from 1 g culture medium using ether-heptane (50:50 [vol/vol]) at acidic pH in the presence of anhydrous sodium sulfate. FFA were then separated from fat using an aminopropyl solid-phase extraction column and quantified by gas chromatography on a BP21 semicapillary column (25 m by 0.53 mm by 0.5-μm film thickness; SGE, Ringwood, Victoria, Australia), as previously described (4).

***In silico* search of exportation signal.** Several computational tools were used for predicting the subcellular localization of proteins since the accuracy of methods varies in terms of both sensitivity and specificity. Tools based on algorithm diversity were selected and organized in "sequence features boxes." The "lipoprotein prediction box" contains results from DOLOP, LIPO, LipoP, LipPred, LIPO, PRED-LIPO, and SpepLIP (see Table S1 in the supplemental material), used with their default settings. Protein secretion by the Tat export pathway was evaluated by TatP and Tat-find, both tools that focus twin-arginine motif and the presence of consecutive transmembrane helix (see Table S2 in the supplemental material). Sec-secreted proteins were predicted using 13 specialized tools (see Table S3 in the supplemental material) that identify the N-terminal signal peptide. The "transmembrane segment boxes" were assessed using 18 membrane (α-helices and β-barrel) topology predictors as well as one cell wall-anchored protein predictor (CW_pred; see Table S4 in the supplemental material).

Gene expression. Gene expression was followed by reverse transcriptase quantitative PCR (RT-qPCR). Cells (~10^9) were pelleted by centrifugation (8,000 × *g* for 10 min at 20°C). The sequences of the primers used are given in Table 2.

RNA extraction and total RNA and transcript quantification were performed as previously described (H. Falentin, F. Postollec, S. Parayre, N. Henaff, P. Le Bivic, R. Richoux, A. Thierry, and D. Sohier, unpublished data). Briefly, RNA was extracted in triplicate from pelleted cells using a heavy phaseLock gel microtube (Eppendorf, Hamburg, Germany) and RNeasy minikit (Qiagen,

TABLE 2. Oligonucleotides used in this study

Primer name	Oligonucleotide sequence (5′→3′)
RT-qPCR of esterase genes	
pf169 FWD	ACCCGTCGCACATTCAGTATCG
pf169 REV	GGCACGCTGGTGTCCTCATC
pf279 FWD	GCTGGGTTCCGACCACACG
pf279 REV	CGATGAGCGGTACTGCGACTG
pf379 FWD	GACTGGGTGGCGGGGATC
pf379 REV	CGGTCTGCTCATAGATGTACTTCG
pf667 FWD	GCGTCGGGTGAGGAGTTCG
pf667 REV	AGGCTCATCGGCTTGTAGTAGTTG
pf774 FWD	AACCGCTCCTATGGACTGTC
pf774 REV	GGTGCCGTTGAACATCGC
pf962 FWD	TGGCGAGGGCGAATGGAAC
pf962 REV	GGTGAGCACGGGCGTATCG
pf1509 FWD	TGGGATCACGGCGTCAGG
pf1509 REV	GCAGCACGAAGCCACGAATG
pf1637 FWD	CCAGCAACTACGGACGAACTC
pf1637 REV	ATGGCGACCGACGAAGACG
pf1655 FWD	TGAGCATCTCCACGACCAC
pf1655 REV	ATCGGCGTCCTTGTCCTTG
pf1861 FWD	GGATTCCATACCGCTTTCATCG
pf1861 REV	AATTTCGGATCGTCCCAGTAGG
pf2042 FWD	CGCCATCACGCCCGACAC
pf2042 REV	GCCGCCCAACAGGTAGACG
pf3004 FWD	TCGGGATCGTTGGAGAACATTG
pf3004 REV	GCTTCGGCTGGGCAGAGG
Cloning experiments	
pf279-XbaI-FWD	TATGCTCTAGAATGCGACGTCGCAC
pf279-HindIII-REV	TATGCAAGCTTTCACCGGGATTCCC
Pbccp-BamHI-FWD	TATGCGGATCCTGGCGCCATCC TCCC
Pbccp-XbaI-REV	TATGCTCTAGAATCGGTGTCGC GCCA

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Hilden, Germany). Nucleic acids were quantified with a NanoDrop spectrophotometer. RNAs were retrotranscribed in cDNAs, and cDNAs were then amplified by qPCR. The mRNA copy number of each gene was determined using Opticon Monitor freeware (Bio-Rad Laboratories) and expressed as the number of targeted RNA copies contained in 100 ng of total extracted RNA.

Electrophoresis and zymography of intracellular extracts and culture supernatants. Intracellular extracts were obtained after bead beating (20 min at 30 Hz) of 300 μl of cell suspension in deionized water (standardized to an OD_{650} of 20) in the presence of 200 mg of 0.1-mm zirconia-silica beads (BioSpec Products, Inc., Bartlesville, OK) in 1.5 ml Eppendorf's Safelock. Culture supernatants from CDM and YEL medium were 100-fold and 20-fold concentrated, respectively, by centrifugation at 6,000 × *g*, using ultrafiltration of cells with a Vivaspin 20 centrifugal concentrator with a polyethersulfone (PES) 10,000-molecular-weight-cutoff membrane (Sartorius AG, Goettingen, Germany).

Intracellular and supernatant protein extracts were separated by native PAGE to reveal esterase activity by zymography and by SDS-PAGE before protein identification. Native PAGE (i.e., a gel electrophoresis based on SDS-PAGE, but without SDS or any reducing agent) was conducted in 10% polyacrylamide gels on a Hoefer SE 300 miniVE unit (Hoefer, San Francisco, CA). To preserve esterase activity, protein separation was performed at 120 V/40 mA maximum for two gels, and the tank was immerged in ice-cold water. Esterase activity was assayed by zymography according to Dupuis et al. (9), with modifications. Briefly, native gels were rinsed in distilled water. Gels were then incubated overnight at 30°C in 0.1% fast red TR-salt (4-chloro-2-methylbenzenediazonium salt; Serva, Heidelberg, Germany) in 0.1 M sodium phosphate buffer (pH 7.0) containing 2% of either a 1% (wt/vol) 1-naphthyl acetate or 1-naphthyl propionate solution or a 1% (vol/vol) 1-naphthyl butyrate solution in acetone. Naphthyl esters were purchased at Sigma-Aldrich (St. Louis, MO). Gels were then rinsed in 0.1 M sodium phosphate buffer (pH 7.0), and activities were visualized as brown bands. SDS-PAGE was conducted in 12% polyacrylamide gels on the Protean II system (Bio-Rad Laboratories), according to Laemmli (18). The molecular weight was determined using a low-molecular-weight marker (LMW marker kit; GE Healthcare UK, Ltd., Buckinghamshire, United Kingdom). To identify the esterases responsible for the bands of activity visualized by zymography, activity bands were treated as follows. Two native gels were performed with pure culture supernatant, at two growth phases. Activity bands were quickly revealed on 2 wells of each gel, and the 8 corresponding unrevealed bands were cut and boiled in Laemmli sample buffer. The blend obtained was loaded in a double Protean II well, sealed by molten agar, and separated by SDS-PAGE.

Protein identification. After separation by SDS-PAGE, proteins of the supernatant were identified by mass spectrometry (MS) after an in-gel trypsin digestion adapted from Shevchenko (23). Briefly, gel pieces were excised from the gel, washed with acetonitrile and NH_4HCO_3 solution, and then dried under vacuum in a SpeedVac concentrator (SVC100H-200; Savant, Thermo Fisher Scientific, Waltham, MA). In-gel trypsin digestion was performed overnight at 37°C and stopped with spectrophotometric-grade trifluoroacetic acid (TFA) (Sigma-Aldrich). The supernatants containing peptides were then vacuum dried in a SpeedVac concentrator and stored at −20°C until mass spectrometry analysis.

Mass spectrometry (MS) experiments were performed using an on-line liquid chromatography tandem mass spectrometry (MS/MS) setup using a LC Packings nano-liquid chromatography (nano-LC) system (Dionex) fitted to a QSTAR XL (MDS SCIEX, Ontario, Canada) equipped with a nano-electrospray ion source (ESI) (Proxeon Biosystems A/S, Odense, Denmark). The instrument was calibrated by multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein, β-CN(193–209). Samples were first concentrated on a PepMap 100 reverse-phase column (C_{18}, 5 μm, 300-μm inner diameter (i.d.) by 5-mm length) (LC-Packings, Dionex, Amsterdam, The Netherlands). Peptides were separated on a reverse-phase PepMap column (C_{18}, 3 μm, 75 μm i.d. by 150 mm length) (LC-Packings) at room temperature, using solvent A (2% [vol/vol] acetonitrile, 0.08% [vol/vol] formic acid, and 0.01% [vol/vol] TFA in deionized water) and solvent B (95% [vol/vol] acetonitrile, 0.08% [vol/vol] formic acid, and 0.01% [vol/vol] TFA in deionized water). A linear gradient from 10 to 50% of solvent B in 70 min was applied for the elution at a flow rate of 0.2 μl/min. Eluted peptides were directly electrosprayed into the mass spectrometer operated in positive mode. A full continuous MS scan was carried out followed by three data-dependent MS/MS scans. Spectra were collected in the selected mass range 450 to 2,000 *m/z* for MS and 60 to 2,000 *m/z* for MS/MS spectra. The three most intense ions from the MS scan were selected individually for collision-induced dissociation (1+ to 4+ charged ions were considered for the MS/MS analysis). The mass spectrometer was operated in data-dependent mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software.

The proteins present in samples were identified from MS and MS/MS data by using MASCOT v.2.2 software (22) for search into two concatenated databases: (i) a homemade (unpublished) database containing all the predicted proteins of *P. freudenreichii* from the CIRM1 genome and (ii) the Swiss-Prot database (http://www.expasy.ch/sprot/). Search parameters were set as follows. A trypsin enzyme cleavage was used, the peptide mass tolerance was set to 0.2 Da for both MS and MS/MS spectra, and finally two variable modifications (oxidation of methionine and deamidation of asparagines and glutamine residues) were selected. For each protein identified, it was necessary to obtain a minimum of two peptides with MASCOT score corresponding to a *P* value below 0.05 for validation with a high degree of confidence.

Overexpression of esterase in *P. freudenreichii*. Reagents were purchased from New England Biolabs (Ipswich, MA), and oligonucleotides were purchased from Sigma Genosys (Haverhill, Cambridgeshire, United Kingdom). A transformed strain of *P. freudenreichii* CIRM1 overexpressing esterase PF#279 was constructed as follows. The esterase gene *pf279* (PFREUD_04340) was cloned into pPK705 vector downstream the *Pbccp* promoter (Table 1), promoting the biotin-dependent carboxytransferase Bccp (EC 2.1.3.1). This promoter was chosen because Bccp was found highly expressed in previous proteomics studies (14). To perform this construction, *pf279* and *Pbccp* were amplified by PCR from *P. freudenreichii* CIRM1 genomic DNA using primers containing restriction sites (described in Table 2). For *Pbccp* amplification, PCR mixtures contained 2 mM $MgCl_2$, 1 μM each primer, 400 μM each deoxynucleoside triphosphate, and 1.25 U of *Taq* polymerase (Fermentas International, Inc., Burlington, Ontario, Canada) in 1× buffer (20 mM Tris HCl [pH 8.4], 50 mM KCl). 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 65°C for 45 s, with a decrease by 0.5°C at each cycle; and 72°C for 45 s, followed by 10 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s; and then a final step at 72°C for 5 min.

High-fidelity PCR was required for cloning *pf279*, because no reliable PCR product was obtained with classical PCR. Platinum *Taq* high-fidelity polymerase (Invitrogen, Carlsbad, CA) was used and PCR was performed under the same conditions as described above. The PCR mixture was then purified on QIAquick PCR purification kit (Qiagen) and incubated at 72°C for 4 min in 1× buffer, containing 100 μM ATP, 2 mM $MgCl_2$, and 2 U *Taq* polymerase (Fermentas) to perform adenylation.

Each of the *pf279* and *Pbccp* amplicons was cloned into a pGEM-T Easy vector (Promega Corporation, Madison, WI) according to the manufacturer's instructions. pGEM-T plasmids were multiplied in *E. coli* DH5α and extracted with Nucleospin Multi-8-Plasmid kit (Macherey-Nagel, Hoerdt, France) according to the manufacturer's instructions. pGEM-T:*pf279* and pGEM-T:*Pbccp* were double digested by BamHI-HindIII and by XbaI-HindIII, respectively. Digestion products were subjected to 1.5% agarose gel electrophoresis. Agarose pieces containing *pf279* and *Pbccp* were excised, and fragments were purified using PCR cleanup gel electrophoresis (Qiagen) according to the manufacturer's instructions. *Pbccp* was introduced into a double-digested (XbaI-HindIII) pPK705 plasmid. Ligation was performed according to the manufacturer's instructions (Ligase, Invitrogen). The product was used to transform *E. coli* DH5α competent cells according to Hanahan et al. (13), and transformants were selected on ampicillin. Plasmids were extracted, double digested with BamHI-HindIII, and ligated to fragments containing *pf279*. The resulting ligation products were then used to transform CIRM1 competent cells as previously described (12), with the following modifications. To prepare electrocompetent cells, *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 (at 2.5 kV, 200 Ω, and 25 μF), cells were regenerated by incubation for 3 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.

RESULTS

Ability of *P. freudenreichii* to hydrolyze milk fat in growth medium. The lipolytic activity of *P. freudenreichii* CIRM1 was assessed in CDM containing or not containing an emulsion of milk fat. *P. freudenreichii* grew similarly in the presence or in the absence of milk fat emulsion (Fig. 1). Regarding lipolysis, a significant amount of FFA was released throughout the incubation time and predominantly during the growth (before stage t1 in Fig. 1). FFA were not released in uninoculated

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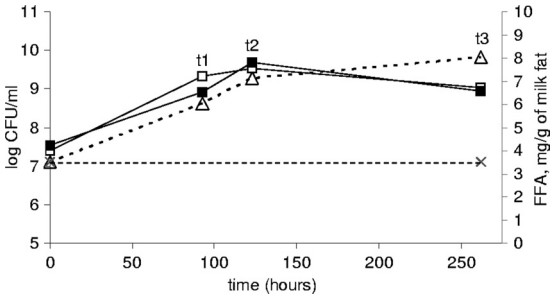

FIG. 1. Growth and lipolytic activity of *P. freudenreichii* in a chemically defined medium (CDM) containing an emulsion of milk fat. Shown are the CFU of *P. freudenreichii* grown in CDM in the absence (black diamonds) or presence (white squares) of milk fat emulsion. X's on dotted lines and black squares represent concentrations of free fatty acids (FFA) in the culture media seeded or not seeded, respectively, with strain CIRM1.

control CDM containing milk fat emulsion incubated under the same conditions, demonstrating the absence of indigenous lipolytic activity in the medium. The absence of contaminating bacteria was also checked in all cultures on PCA plates.

Prediction of esterase subcellular localization. Twelve putative esterases were previously identified from the genome of *P. freudenreichii* CIRM1 (7). In the present study, *ab initio* methods were used to predict the subcellular localization of these 12 putative esterases.

The results of the prediction of cell localization are summarized in Fig. 2. The six tools of the "lipoprotein box" agree to predict that none of the 12 esterases have signal peptide or cleavage site specific of lipoproteins. The two tools dedicated to the twin-arginine translocation (Tat) secretion pathway are in agreement to conclude that one of the 12 putative esterases previously identified, PF#279, is a Tat-dependent secreted protein with a cleavage site predicted between positions 53 and 54 (ASA-CG) for TatP and positions 23 and 24 (AQA-AP) for TatFind. However, the Tat-targeting (ST)RRXøøK (ø is a hydrophobic residue) consensus pattern was not detected. A motif with three arginine residues (RRRTTI) is proposed by TatFind, but the TT residues are hydrophilic, which is unusual for a Tat motif. The N-terminal signal peptide of PF#279 was confirmed by all 13 methods used to predict Sec signal peptides with two positions proposed for the cleavage site, between positions 23 and 24 (AQA-AP) or 28 and 29 (ASA-DG). As a conclusion, PF#279 was undoubtedly predicted to be released into the extracellular medium, but the system (Tat or Sec) is still uncertain. PF#774, a second putative esterase previously identified, was also predicted to be translocated via the Sec translocon (but not Tat) with a signal peptidase I (SPase I)-cleaved signal peptide detected by all tools between positions 22 and 23 [VAS-TI] or 33 and 34 [AHA-DD]. However, PF#774 also contains an additional C-terminal α-helix from positions 346 to 369 and is probably not released in the extracellular medium but rather anchored to the cell wall (LPXTG anchorage predicted by CW-Pred). The other 10 putative esterases were predicted to be cytoplasmic (Fig. 2).

Expression of putative esterase genes. All of the 12 putative esterase genes previously identified (7) were expressed both in the presence and in the absence of milk fat in culture medium (see Table S5 in the supplemental material). Regardless of the targeted gene, the stage of cell harvest, and the presence of milk fat emulsion in the culture medium, the number of

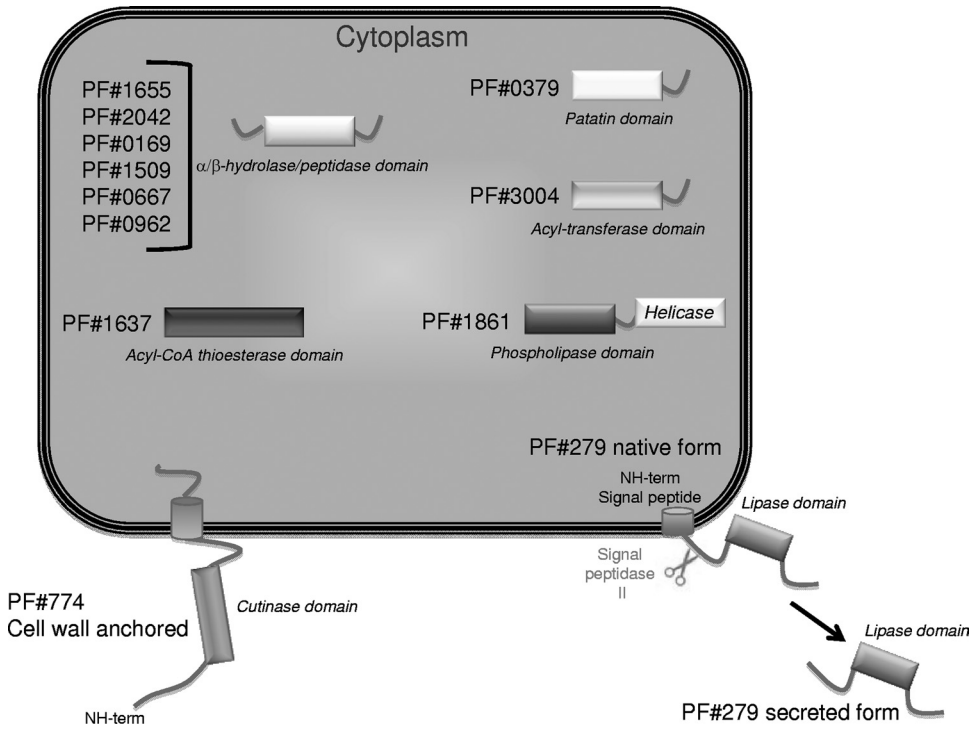

FIG. 2. Schematic representation of the subcellular localization of 12 putative esterases in *Propionibacterium freudenreichii*, predicted from a combination of computational tools (see Materials and Methods). CoA, coenzyme A.

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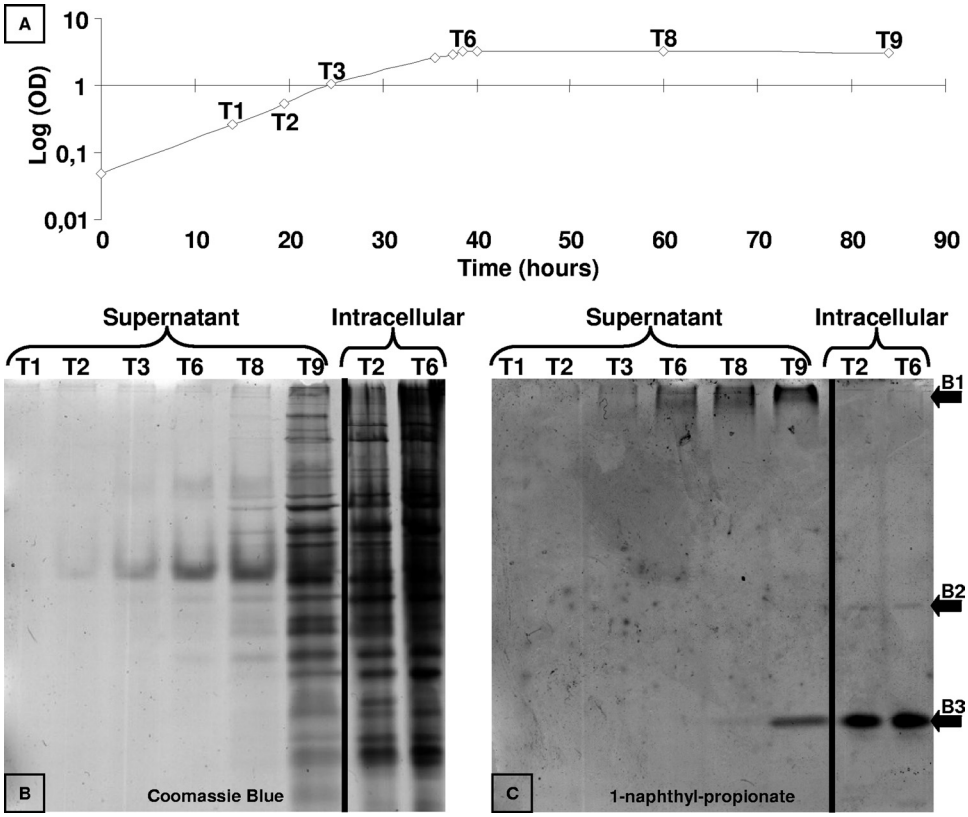

FIG. 3. Intracellular and secreted esterases of *P. freudenreichii* at different times of growth in YEL medium. (A) Growth of *P. freudenreichii* CIRM1[T]; (B and C) native PAGE gels of 20-fold-concentrated supernatant and intracellular fractions, either Coomassie blue stained (B) or stained for esterase activity (zymogram) using 1-naphthyl propionate as a substrate (C). The arrows indicate the 3 bands of activity representing supernatant esterase activity (B1) and intracellular esterase activities (B2 and B3).

mRNA copies ranged from 10^5 to 10^8 copies per 100 ng of extracted RNA.

Evidence of secreted esterases in *P. freudenreichii*. The esterase activity in YEL culture supernatants was visualized by zymography and compared with the esterase activity in intracellular extracts at six stages of culture (Fig. 3A). YEL was chosen as culture medium in this experiment because *P. freudenreichii* reached a higher number of cells and then a potentially higher yield of proteins than in CDM. An example of zymogram showing the esterase bands active on 1-naphthyl propionate and the corresponding native Coomassie blue-stained gel is presented in Fig. 3B and C. Three activity bands, named B1, B2, and B3, were distinguished according to their relative migration distance in zymography (see the example in Fig. 3). Table 3 summarizes the results of zymogram assays for all the extracts tested, with each of the three naphthyl esters used as a substrate to test esterase activity.

During the exponential growth phase (times T1 to T6), only one band of activity (B1 in Fig. 3C) was detected in culture supernatant, whereas two distinct activity bands, B2 and B3, were detected in intracellular extracts (Fig. 3C). Band B1, on one hand, and B2 and B3, on the other hand, differed by their substrate specificity (Table 3). Hence, band B1 was clearly visualized using either 1-naphthyl propionate or 1-naphthyl butyrate as a substrate, but was almost undetectable using 1-naphthyl acetate. On the contrary, bands B2 and B3 showed activity on 1-naphthyl acetate and 1-naphthyl propionate, but not on 1-naphthyl butyrate (Table 3).

During the late stationary phase (T8 to T9), the profile of intracellular esterase activity did not change (data not shown). In contrast, the profile of esterase activity of culture supernatants showed two additional activity bands, corresponding to bands B2 and B3 (Fig. 3C). These two bands were hardly detected at time T8 (20 h after the end of growth) and were clearly visible in late stationary phase (45 h after the end of growth, time T9) after a 20% OD_{650} decrease. Concomitantly, numerous proteins, forming an electrophoretic pattern similar to the one of intracellular proteins, appeared in culture supernatant, as revealed in the Coomassie blue-stained gel (Fig. 3B). These changes in culture supernatants very likely result from the release of intracellular proteins from lysed cells during the stationary phase.

To summarize, zymography results showed that an esterase activity band, B1, was detected in the culture supernatant and that this activity had distinct biochemical properties and specificity compared to the two activity bands detected in intracellular extracts.

Identification of esterase(s) in culture supernatant. Three samples of culture supernatant harvested during the exponential growth phase were analyzed by SDS-PAGE, trypsinolysis of proteins, and nano-LC-ESI-MS/MS analysis of the peptides obtained. One sample was a CDM supernatant (time t2 in Fig.

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TABLE 3. Intensity and substrate specificity of esterase activity bands from *P. freudenreichii* CIRM1 extracts harvested at different stages of culture revealed by zymography on native PAGE gels

Substrate and esterase activity band[a]	Relative intensity at time shown[b]							
	Supernatant						Intracellular	
	T1	T2	T3	T6	T8	T9	T2	T6
1-Naphthyl acetate								
B1	−	−	−	+/−	+/−	+/−	−	−
B2	−	−	−	−	+/−	+	++	++
B3	−	−	−	−	+/−	+	++	++
1-Naphthyl propionate								
B1	+/−	+/−	+	++	++	+++	−	−
B2	−	−	−	−	−	+/−	+	+
B3	−	−	−	−	+	++	+++	+++
1-Naphthyl butyrate								
B1	+/−	+/−	+	++	++	+++	−	−
B2	−	−	−	−	−	−	−	−
B3	−	−	−	−	−	−	−	−

[a] Bands B1 to B3 are referred to according to Fig. 3.
[b] Stages T1 to T3 (exponential growth), T6 (end of growth), and T8 and T9 (stationary phase) are shown in Fig. 3.

1), and two samples were YEL supernatant (times T3 and T6 in Fig. 3). The corresponding SDS-PAGE gels are shown in Fig. 4A and B. Figure 4 also shows the pieces cut from these gels to be analyzed (38 gel pieces cut from lane cdm-t2 and 6 gel pieces cut from lane yel-T6 in Fig. 4A and B).

Only 1 of the 12 putative esterases, PF#279, was identified in the 44 gel pieces analyzed. PF#279 was detected in gel pieces C17 to C19, within a range of experimental molecular masses from 47 to 56 kDa (lane yel-T6 in Fig. 4A). PF#279 was also identified in gel pieces S1 to S4, in the same range of molecular masses (44 to 58 kDa) (Fig. 4B). The experimental molecular mass fit the molecular mass deduced from PF#279 sequence (46.4 kDa with the signal peptide, 43.6 to 44.0 kDa for the mature protein, regarding the predicted cleavage sites).

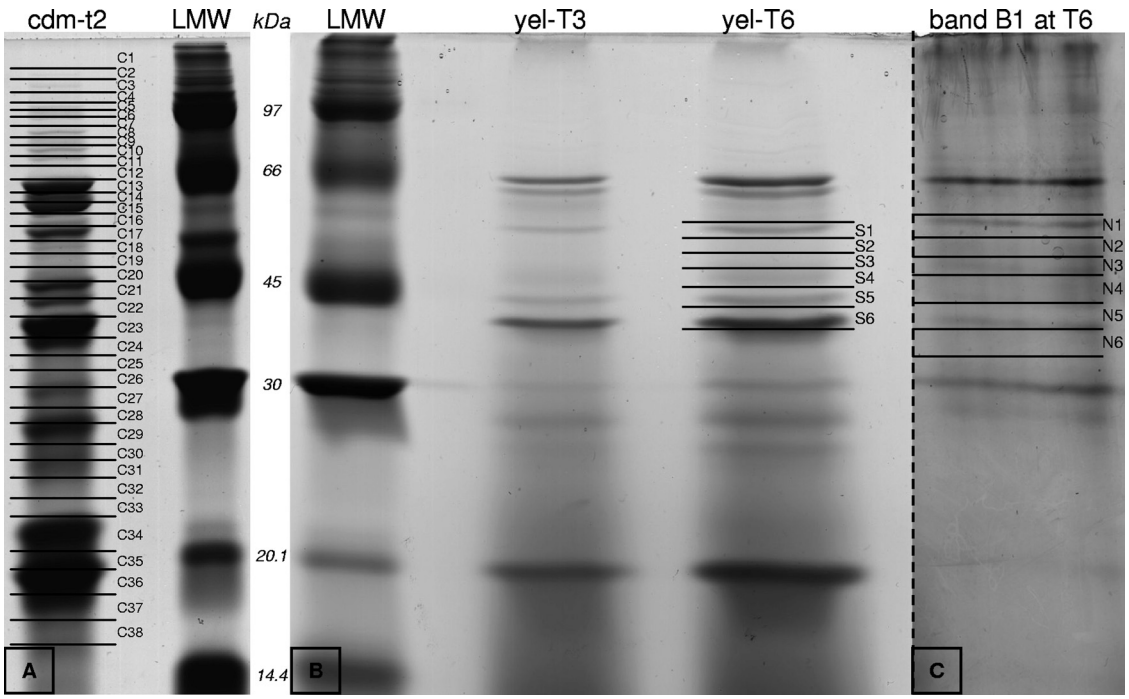

FIG. 4. SDS-PAGE of culture supernatant proteins of *P. freudenreichii* CIRM1^T grown in CDM (A) or YEL medium (B and C). (A) Coomassie blue-stained proteins of 100-fold-concentrated CDM supernatant (time t2 in Fig. 1); (B) Coomassie blue-stained proteins of 20-fold-concentrated YEL supernatant at two stages of growth (times T3 and T6 in Fig. 2); (C) silver-stained proteins from 8 unstained pooled gel bands of B1 (corresponding to the extracellular activity) cut from zymogram at time T6 (see Fig. 2) and reseparated by SDS-PAGE. Panels B and C are part of the same gel. LMW, low-molecular-weight marker (GE HealthCare). Bands C1 to C38, S1 to S6, and N1 to N6 show the bands cut from gels for protein identification by mass spectrometry.

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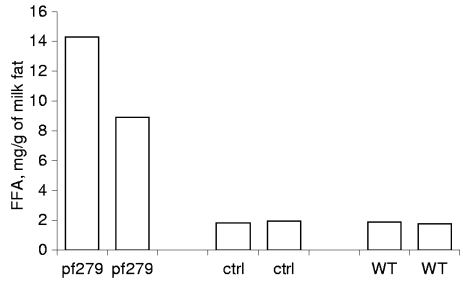

FIG. 5. Production (final minus initial concentrations) of free fatty acids (FFA) by transformed *P. freudenreichii* CIRM1 overexpressing the *pf279* gene under the control of the *Pbccp* promoter, after 60 h of growth in YEL containing a milk fat emulsion. ctrl, control strains (transformed strains harboring the empty pPK705 vector); WT, wild-type strain CIRM1. Values of FFA in the inoculated medium incubated under the same conditions were subtracted from values determined in *P. freudenreichii* cultures. Results are shown for two clones of each construct.

Neither PF#279 nor another esterase was detected in the early exponential growth (from lane yel-T3), likely because the relative amount of PF#279 was insufficient at this stage of culture to be detected by this approach. However, by using 2D electrophoresis followed by mass spectrometry analysis, PF#279 was also identified as the sole of the 12 putative esterases in the same sample (lane yel-T3; result not shown).

Identification of the esterase(s) active in culture supernatant. To identify the esterase(s) responsible for the activity band B1 detected in culture supernatant by zymography, 8 gel bands corresponding to uncolored B1 bands at time T6 (Fig. 3A) were pooled. The proteins contained in these bands were remigrated in SDS-PAGE. The proteins present in gel pieces N1 to N6 in the resulting gel (Fig. 4C) were identified after trypsinolysis and nano-LC-ESI-MS/MS analysis of the peptides obtained. Out of the 12 putative esterases previously identified in *P. freudenreichii* genome, only PF#279 was identified. It was detected only in gel piece N2, i.e., at molecular masses ranging from 50 to 54 kDa. This result shows that the esterase PF#279 was the esterase responsible for the activity detected in band B1.

Overexpression of esterase PF#279 in *P. freudenreichii*. Two cultures of wild-type strain CIRM1, two transformed clones harboring the pPK705 vector with the *pf279* gene under the control of the *Pbccp* strong promoter, and two transformed clones harboring only the pPK705 vector used as controls were incubated in YEL medium in the presence or the absence of milk fat emulsion. Generation times were calculated from OD_{650} data in YEL medium without emulsion. They ranged from 5.5 h to 5.9 h for all of the clones. Maximal populations of 6×10^9 CFU/ml were reached at ~60 h of incubation for all cultures. The production of FFA was assessed after 60 h of incubation in all cultures and in a control uninoculated medium incubated under the same conditions. The control clones (transformed CIRM1 with empty pPK705 vector) and the wild-type strain showed the same lipolytic activity, with a production level (final minus initial concentrations) of 553 ± 22 μg FFA/g medium. The production of FFA was 5- to 8-fold greater in the two transformed clones overexpressing *pf279* than in the control strains (Fig. 5).

DISCUSSION

P. freudenreichii has a major role in lipolysis of Emmental cheese and is used as a ripening culture in the manufacture of Emmental and other Swiss-type cheeses (11, 25, 26). The present study aimed to identify the most probable esterase(s) of *P. freudenreichii* involved in cheese lipolysis.

We first demonstrated the lipolytic activity of *P. freudenreichii* CIRM1. To date, most results showing the role of *P. freudenreichii* in fat hydrolysis were observed in Swiss-type cheeses, in the presence of a complex bacterial ecosystem. Under cheese conditions, the esterases of lactic acid bacteria and LPL of milk could also contribute to the formation of FFA. By growing *P. freudenreichii* CIRM1 in pure culture in the presence of an emulsion of milk fat, we showed for the first time that *P. freudenreichii* possesses enzymes able to hydrolyze milk fat. The amount of FFA produced was ~8 mg/g fat: i.e., an amount of the same order of magnitude as that observed in Emmental cheese (8 to 15 mg/g fat). The kinetics of lipolysis in culture medium occurred simultaneously with growth (Fig. 1), as reported in Emmental cheese for the same strain and other *P. freudenreichii* strains (19, 26). This result reinforces the hypothesis that at least one secreted or surface-exposed esterase is involved in the lipolysis of Emmental cheese by *P. freudenreichii*.

To identify secreted or surface-exposed esterases in *P. freudenreichii*, we combined *in silico* and biochemical approaches. We first investigated *in silico* the subcellular localization of a pool of 12 potential esterases previously predicted from the *P. freudenreichii* genome (7), by combining different computational tools. Many tools are available for predicting protein locations, each performing well with certain data sets. Here, an iterative strategy was used by integrating heterogeneous localization predictors of lipoprotein-, Tat-, or Sec-dependent signal peptides and transmembrane domains. We showed that one esterase, PF#279, was predicted to be secreted in the medium, and one putative esterase, PF#774, was predicted to be anchored in the plasma membrane, in agreement with previous suggestions made using SignalP as a unique tool (7). In contrast, unclear results were previously found for another esterase, PF#1509, which was clearly predicted as intracellular in the present study. PF#279 and PF#774 were expressed under all of the tested conditions and thus are the two best candidates to be involved in the lipolysis of milk fat observed during *P. freudenreichii* growth. The esterase activity of both these proteins was previously assessed by overexpression in *E. coli* and zymography (7). Only recombinant PF#279 was confirmed as an esterase, PF#774 being expressed as inclusion bodies in *E. coli*.

We then characterized the secreted esterase(s) of *P. freudenreichii* by zymography. Previous reports mentioned the existence of extracellular esterase activity in *P. freudenreichii* (10), but the esterase activity observed in culture supernatant may have, in the cited report, at least partly resulted from the activity of intracellular enzyme(s) released in culture supernatant. The results obtained in the present study showed that (i) one esterase activity band, B1, was detected in culture supernatants early during the growth phase (Fig. 3); (ii) band B1 was not detected in intracellular extracts regardless the stage of culture (Fig. 3); and (iii) band B1 presented biochemical prop-

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erties and substrate specificity distinct from the ones of the two activity bands detected in intracellular extracts, B2 and B3 (Table 3). When the incubation of *P. freudenreichii* was prolonged during the stationary phase, several events indicated that *P. freudenreichii* lysed: (i) OD_{650} decreased, (ii) many proteins appeared in the culture supernatants, and (iii) additional bands of esterase activity similar to those detected in the intracellular extracts were detected in zymograms from supernatants. Taken all together, these results show that *P. freudenreichii* CIRM1 secretes an extracellular esterase with distinct properties compared to intracellular esterases. The specificity of substrate of this extracellular esterase versus naphthyl esters was similar to the specificity previously reported in culture supernatant of *P. freudenreichii* subsp. *freudenreichii* CIP103026 (activity on 1-naphthyl propionate and 1-naphthyl butyrate, but not on 1-naphthyl acetate) (10).

The extracellular esterase detected by zymography was identified as PF#279 by mass spectrometry. PF#279 was also the sole esterase identified in *P. freudenreichii* CIRM1 culture supernatants of the two media used, under our experimental conditions, in agreement with the results of *in silico* prediction.

Finally, to investigate the activity of PF#279 on milk fat, and thus its potential role in cheese lipolysis, we overexpressed the *pf279* gene in *P. freudenreichii* CIRM1 under the control of a strong promoter. As no inactivation tool was available for *P. freudenreichii*, we chose to overexpress *pf279* using the expression vector pPK705 previously developed in *P. freudenreichii* (17). Transformants overexpressing *pf279* released 5 to 8 times more FFA from milk fat than the control strains. This result demonstrates that PF#279 is effectively active on milk fat and then can be referred to as a lipolytic esterase (also called lipase). It also shows that the intensity of lipolysis depends on the strength of *pf279* promoter, which could explain part of the strain dependency of lipolysis observed in *P. freudenreichii*.

The possible role in Emmental cheese lipolysis of PF#774, a putative esterase predicted to be anchored in the plasma membrane, cannot be discarded and should be clarified. Inactivation tools would be useful to evaluate the respective contribution of PF#279 and PF#774 in cheese lipolysis.

In conclusion, we have demonstrated for the first time the existence of a secreted lipolytic esterase in *P. freudenreichii* active on milk fat, PF#279. PF#279 is a Tat- or Sec-secreted esterase. It has a predicted molecular mass of 46.4 kDa, possesses an atypical motif, TXSXG, instead of the classical GXSXG motif of esterases, and shares only weak homology with the proteins of the NCBI nr database, as previously highlighted (7). Due to its activity on milk fat during *P. freudenreichii* growth, PF#279 is likely a key component in Emmental cheese lipolysis by *P. freudenreichii*.

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