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The X-prolyl dipeptidyl-peptidase PepX of *Streptococcus thermophilus* initially described as intracellular is also responsible for peptidase extracellular activity

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ABSTRACT

This study addresses the hypothesis that the extracellular cell-associated X-prolyl dipeptidyl-peptidase activity initially described in *Streptococcus thermophilus* could be attributable to the intracellular X-prolyl dipeptidyl-peptidase PepX. For this purpose, a PepX-negative mutant of *S. thermophilus* LMD-9 was constructed by interrupting the *pepX* gene and named LMD-9-Δ*pepX*. When cultivated, the *S. thermophilus* LMD-9 wild type strain grew more rapidly than its Δ*pepX* mutant counterpart. Thus, the growth rate of the LMD-9-Δ*pepX* strain was reduced by a factor of 1.5 and 1.6 in milk and LM17 medium (M17 medium supplemented with 2% lactose), respectively. The negative effect of the PepX inactivation on the hydrolysis of β-casomorphin-7 was also observed. Indeed, when incubated with this peptide, the LMD-9-Δ*pepX* mutant cells were unable to hydrolyze it, whereas this peptide was completely degraded by the *S. thermophilus* LMD-9 wild type cells. This hydrolysis was not due to leakage of intracellular PepX, as no peptide hydrolysis was highlighted in cell-free filtrate of wild type strain. Therefore, based on these results, it can be presumed that though lacking an export signal, the intracellular PepX might have accessed the β-casomorphin-7 externally, perhaps via its galactose-binding domain-like fold, this domain being known to help enzymes bind to several proteins and substrates. Therefore, the identification of novel distinctive features of the proteolytic system of *S. thermophilus* will further enhance its credibility as a starter in milk fermentation.

Key words: X-prolyl dipeptidyl peptidase, *Streptococcus thermophilus*, β-casomorphin-7

INTRODUCTION

In developed countries, demand for healthy foods has escalated due to increased consumer perception about the diet–health relationship (Mollet and Rowland, 2002). Among these healthy foods, fermented dairy products remain of interest because they can be consumed daily without influencing the balance of diet. Because of the numerous auxotrophies of lactic acid bacteria (**LAB**), proteolysis of milk proteins is an essential phenomenon of milk fermentation that determines their growth in milk (Savijoki et al., 2006). Control of caseinolysis in dairy products is not only an economical challenge but also a health challenge. Besides the growth of LAB, casein breakdown generates peptides, which either contribute to the physicochemical properties or enhance the health benefits of the finished dairy products. In the latter case, they are designated as bioactive peptides, which are known to be potentially able to modulate different body functions. A convenient and natural way to give desired health attributes to dairy products is the generation of bioactive peptides via casein hydrolysis, and one of the major actors in caseinolysis is the proteolytic system of LAB (Hafeez et al., 2014).

The LAB *Streptococcus thermophilus* is the second most important species of industrial dairy starter after *Lactococcus lactis* and is widely used in the manufacture of various dairy products such as yogurt and hard cooked cheeses (Hols et al., 2005), mainly for its fast acidification (Dandoy et al., 2011) and organoleptic properties. Moreover, some strains of *S. thermophilus* have the capacity to generate a variety of bioactive peptides from milk caseins owing to its proteolytic system (Miclo et al., 2012; Chang et al., 2014).

The proteolytic system of *S. thermophilus* is generally organized in 4 main parts. The first and principal actor of this system is the proteinase PrtS, which is usually anchored to the cell wall and belongs to the subtilisin-like serine protease family (Fernandez-Espla et al., 2000). However, it must be underlined that PrtS

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is neither always present nor associated with the cell wall of *S. thermophilus* strains; for example, strain CNRZ1066 lacks the *prt*S gene, whereas PrtS is wall anchored in strain LMD-9 or can also exist as a free form in strain 4F44 (Delorme et al., 2010; Chang et al., 2012). After their liberation from caseins by PrtS, peptides can be internalized by oligo- and dipeptide transport system (Garault et al., 2002; Jameh et al., 2016) or further hydrolyzed by extracellular peptidases (Hafeez et al., 2013, 2015). Once inside the cell, the peptides are exposed for further breakdown to a pool of intracellular exo- and endopeptidases that make the final constituent of this system (Savijoki et al., 2006).

About 15 different intracellular peptidase activities have been described in several *S. thermophilus* strains (Rul and Monnet, 1997). Only some of these enzymes [aminopeptidase PepN (Rul et al., 1994), cysteine aminopeptidase PepC (Chapot-Chartier et al., 1994), acidic residue-specific aminopeptidase PepA (Rul et al., 1995), metallo-aminopeptidase PepS (Fernandez-Espla and Rul, 1999), dipeptidase (Rabier and Desmazeaud, 1973), endopeptidase PepO (Chavagnat et al., 2000), and Xaa-Pro dipeptidyl-peptidase PepX (Tsakalidou et al., 1998)] have been characterized biochemically or genetically after either purification or gene cloning. PepX (EC 3.4.14.11) is an enzyme that catalyzes the removal of N-terminal dipeptides (Xaa-Pro) by hydrolyzing the peptide bond implying the carboxylic function of a proline residue and belongs to the MEROPS peptidase family S15 (http://www.ebi.ac.uk/interpro/entry/IPR008252).

In a previous work we reported the presence of extracellular cell-associated aminopeptidase, carboxypeptidase, peptidyl-dipeptidase, and X-prolyl dipeptidyl-peptidase activities in *S. thermophilus* LMD-9 and CNRZ1066 (Hafeez et al., 2013, 2015). Such activities did not result from activity of the known extracellular protease PrtS and HrtA, as inactivation in the strain LMD-9 of each of them or of both did not preclude the extracellular peptidase activities observed (Hafeez et al., 2013, 2015). Unfortunately, genes encoding these extracellular cell-associated peptidase activities have not yet been identified among the numerous genome sequences of *S. thermophilus* strains that are now available, especially those of strains LMD-9 and CNRZ 1066. In addition, genome sequence analysis of these strains failed to detect any other X-prolyl dipeptidyl-peptidase gene except PepX. Despite the fact that PepX does not possess any sorting signal according to SignalP or PredTAT prediction tools (Bagos et al., 2010; Petersen et al., 2011), we tested the possibility that this enzyme, initially described as an intracellular peptidase, was also responsible for the extracellular cell-associated X-prolyl dipeptidyl-peptidase activity we detected.

The strategy was to delete the *pepX* open reading frame in the *S. thermophilus* LMD-9 genome and replace it with the spectinomycin adenyl transferase gene, which provides resistance to the spectinomycin antibiotic. Growth of *S. thermophilus* LMD-9 and LMD-9-Δ*pepX* strains was characterized in 10% reconstituted skim milk and LM17 medium. Furthermore, the proteolysis experiments using the proline-rich peptide β-casomorphin-7, derived from bovine β-casein A1 protein variant (^{60}YPFPGPI^{66}; Brantl et al., 1979), were performed in the presence of intact cells of both strains in a nongrowth medium.

MATERIALS AND METHODS

Peptide Synthesis and Bacterial Growth Conditions

The peptide β-casomorphin-7 used in this study was synthesized by Genosphere Biotechnologies (Paris, France). The *S. thermophilus* LMD-9 strain ATCC BAA-491, whose genome sequence is known (Makarova et al., 2006), came from the American Type Culture Collection (Manassas, VA). The *S. thermophilus* LMD-9 and *S. thermophilus* LMD-9-Δ*pepX* (this study) strains were conserved at −80°C in 10% sterile reconstituted skim milk. Both strains were cultivated at 42°C in either 10% reconstituted skim milk or LM17 (M17 medium supplemented with 2% lactose; Terzaghi and Sandine, 1975). *Escherichia coli* DH5-α was preserved at −80°C in Luria-Bertani broth containing 11.4% glycerol and cultured aerobically at 37°C in Luria-Bertani broth (Sambrook and Russell, 2001). When needed, spectinomycin was added to the culture medium at a concentration of 300 μg/mL to select the LMD-9-Δ*pepX* mutant strain.

DNA Extraction and PCR Amplifications

For DNA extraction, *E. coli* DH5-α and the 2 *S. thermophilus* strains used in this work were grown in Luria-Bertani broth and LM17 medium, respectively, as described by Hafeez et al. (2013). Plasmid pSET4s (Takamatsu et al., 2001) possessing the spectinomycin adenyltransferase gene *specR* was purified from *E. coli* DH5-α using a GeneJET Plasmid Miniprep Kit (Fermentas, Villebon-sur-Yvette, France) according to supplier recommendations, and genomic DNA of *S. thermophilus* was extracted according to Fischer et al. (1997). Plasmid pSET4s and genomic DNA of *S. thermophilus* LMD-9 were used as template DNA for amplification

of the *specR* gene and of upper (**Up**) and down (**Dn**)-flanked regions of the *pepX* gene, respectively (Figure 1). All PCR were conducted in a Mastercycler® pro thermal cycler (Eppendorf, Hamburg, Germany). Primers (Table 1) were designed using Primer3Plus software (Untergasser et al., 2007) and were purchased from Eurogentec (Serain, Belgium). To create overlap regions between flanked regions of the *pepX* and *specR* genes, a short nucleotide sequence homologous to the Up- and Dn-flanked regions of PepX was added to each of the primers used to amplify the specR fragment (Figure 1). The PCR amplifications and agarose gel electrophoresis were performed as described previously (Hafeez et al., 2013). Overlapping PCR reactions were performed as previously described (Lecomte et al., 2014). The PCR-amplified fragments were purified using a high pure PCR product purification kit (Roche Applied Science, Meylan, France), and sequence analyses of both DNA strands of PCR fragments were conducted by Beckman Coulter Genomics (Essex, UK).

S. thermophilus LMD-9-Δ*pepX* Mutant Construction and Transformation

To construct the *S. thermophilus* LMD-9-Δ*pepX* mutant strain, the *pepX* gene [locus tag: STER_1633/STER_RS08015; National Center for Biotechnology Information (Bethesda, MD) reference sequence: NC_008532.1] of *S. thermophilus* LMD-9 was replaced by the *specR* gene using overlap extension PCR (Figure 1). First, an 862-bp upstream sequence (starting just before the start codon and comprising the promoter and ribosome binding site of the *pepX* gene) and an 825-bp downstream sequence (including 71 nucleotides before the stop codon and terminator of the *pepX* gene) were amplified from *S. thermophilus* LMD-9 genomic DNA using UpPepX_F/UpPepX_R and PepXDn_F/PepXDn_R primer couples, respectively (Table 1). The *specR* gene corresponding to a 1,200-bp sequence was amplified from pSET4s plasmid DNA using the UpPepX-Spec_F/Spec-PepXDn_R primer pair (Figure

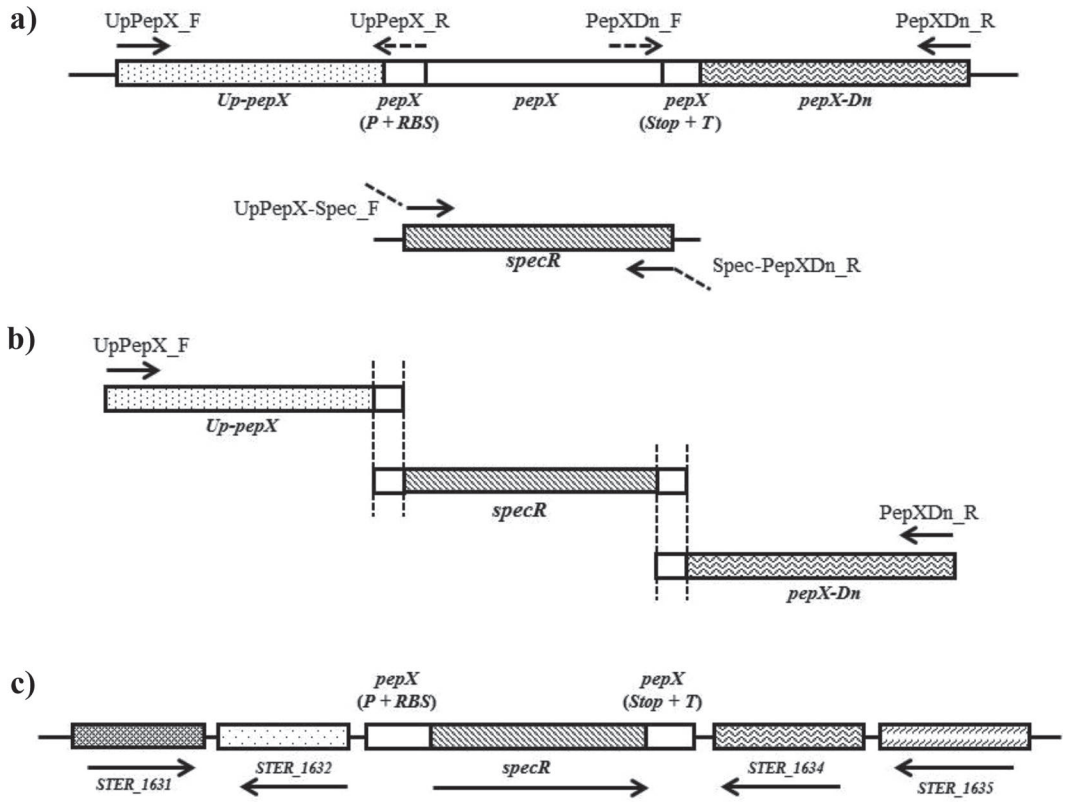

Figure 1. Schematic representation of different steps of overlap extension PCR to replace the *pepX* gene by the *specR* gene and resultant mutant locus. (a) Amplification of the PepX upper flanked region (UpPepX), resistance to antibiotic spectinomycin (specR), and PepX down flanked region (PepXDn) fragments using appropriate primer pairs. (b) The overlap extension PCR using the outermost primer pair and the 3 amplicons. (c) The resulting chromosomal locus carrying the *specR* gene obtained after transformation with the final overlap extension PCR amplicon. Dotted lines indicate the homologous/complementary regions. P, RBS, stop, and T correspond to promoter, ribosome binding site, stop codon, and transcription terminator of *pepX* gene, respectively.

1a). The amplicon specR contains extremities homologous to the UpPepX and PepXDn amplicons (Figure 1b). Then, the 3 amplicons (UpPepX, specR, and PepXDn) were mixed in equimolar concentrations and attached together using external primers UpPepX_F and PepXDn_R (Figure 1b). Finally, linear DNA fragment obtained by overlap extension PCR was introduced in *S. thermophilus* LMD-9 competent cells by natural transformation according to Hafeez et al. (2013). The validity of construction was checked by sequencing the different PCR fragments obtained using specific primers (Table 1).

Growth of S. thermophilus LMD-9 and S. thermophilus LMD-9-ΔpepX

Growth of *S. thermophilus* LMD-9 wild type and Δ*pepX* mutant strains was monitored in 10% reconstituted skim milk and LM17 broth. Before growth in each medium, strains were inoculated at 1% in 10% reconstituted skim milk and grown overnight at 42°C to provide preculture. For studies in milk medium, optical density (**OD**) of the preculture, diluted 10× with EDTA solution 0.2%, pH 12 (a clarifying agent), was measured at 480 nm as previously described (Garault et al., 2000; Galia et al., 2016). Then, the preculture was diluted in 40 mL of 10% reconstituted skim milk to obtain an $OD_{480\ nm}$ of 0.01, and aliquots of 1.3 mL were incubated at 42°C. Afterward, $OD_{480\ nm}$ was measured after the same dilution using EDTA every 30 min until the bacterial culture reached the stationary growth phase. Measurements were made in duplicate. In the case of the Δ*pepX* mutant strain, each medium and clarifying solution was supplemented with spectinomycin at 300 μg/mL.

To monitor growth in LM17 broth, preculture of each strain was diluted to an $OD_{600\ nm}$ of 0.01 in LM17 broth and cultured as in reconstituted skim milk. Then, the $OD_{600\ nm}$ was measured after 1 h and then every 30 min until the bacterial culture reached the stationary growth phase. Growth rates of both strains were calculated from the maximum slope of a logarithmic representation of growth curves assessed by OD measurements at 480 and 600 nm of bacterial cultures grown in reconstituted skim milk and LM17, respectively.

Hydrolysis of Peptide β-Casomorphin-7

The ability of *S. thermophilus* LMD-9 and LMD-9-Δ*pepX* mutant strains to hydrolyze β-casomorphin-7 was evaluated in phosphate-acetate buffer (**PAB**; 12.5 m*M*, pH 6.5). For this purpose, both strains were grown in LM17 medium up to exponential phase ($OD_{600\ nm}$ of 1.00 ± 0.05), and cells were harvested by centrifugation at 3,100 × *g* for 10 min at 20°C. After twice washing with PAB, the cell pellet was resuspended in the same buffer to obtain an initial $OD_{600\ nm}$ of 1.00 ± 0.05. Then, the β-casomorphin-7 was added to the cell suspensions to a final concentration of 20 nmol/mL and incubated at 42°C under shaking at 200 rpm. In

Table 1. Primers used for the construction of the *Streptococcus thermophilus* LMD-9-Δ*pepX* mutant and the sequence verification of the construction obtained

Primer	Sequence 5′–3′	Annealing temperature (°C)	Purpose
UpPepX_F[1]	GCAAGAGATGGTGAAGAAGAC	54.0	Amplify upper-flanked region of *pepX* gene
UpPepX_R	ATTCAAAAACCTCAAAAATAATACG		
UpPepX-Spec_F	*CGTATTATTTTTGAGGTTTTTGAAT*TGACTCCCCGTCGTGTAGATAACTAG	58.0	Amplify *specR* gene
Spec-PepXDn_R	*GCTCAAAGTCTGTGGTATAGAGAAC*CGCTACGATAACGCCTGTTT		
PepXDn_F	GTTCTCTATACCACAGACTTTGAGC	54.0	Amplify down-flanked region of *pepX* gene
PepXDn_R[1]	AACTGGTGTTTATCGTTTGC		
PepXSpec1_F	AAAGGTAGAGAGCTTTAAACAAG	54.1	Sequencing
PepXSpec1_R	GGATACCTTATTGCACAACTTC		
PepXSpec2_F	GCACGATTACGATTTTCTGG	57.9	Sequencing
PepXSpec2_R	AAATATCTCTTGCCAGTCACG		
PepXSpec3_F	CTCCCCGTCGTGTAGATAAC	56.7	Sequencing
PepXSpec3_R	TTCCTTCTGAGGAATGTATCC		
PepXSpec4_F	TGGTACCGTGGAATCATCCT	57.9	Sequencing
PepXSpec4_R	AGTGTGCTCAAAGTCTGTGG		
PepXSpec5_F	TTTGGGAAATATTCATTCTAATTG	55.4	Sequencing
PepXSpec5_R	TGGTAACTTTGTAGGCGTTG		
PepXSpec6_F	GACAAAACAATGGCCGTAAG	58.8	Sequencing
PepXSpec6_R	TTATGACAAGCGGACAAGTG		

[1]Primers were used for overlap extension PCR with annealing temperature of 50°C. UpPepX-Spec_F and Spec-PepXDn_R italic sequences are homologous to the reverse complement of UpPepX_R and PepXDn_F, respectively. F = forward; R = reverse.

parallel, β-casomorphin-7 was incubated alone in PAB under the same conditions as a control of the peptide stability. Each sample (500 μL) aliquoted at 0, 1, 2, 4, 6, and 22 h of incubation was mixed with 15 μL of trifluoroacetic acid to stop enzyme activity, the cells were removed by filtration through a 0.45-μm filter (Phenomenex, Le Pecq, France), and the hydrolysis products were stored at −20°C until reversed-phase HPLC (**RP-HPLC**) analysis.

Stability of Extracellular Cell-Associated PepX Activity and Detection of Bacterial Cell Lysis

The stability of extracellular cell-associated X-prolyl dipeptidyl-peptidase activity in PAB (12.5 m*M*, pH 6.5) was explored as well as hydrolysis of β-casomorphin-7 by intracellular peptidases, particularly PepX, that may be released in the incubation medium as a result of spontaneous cell lysis. For this, *S. thermophilus* LMD-9 wild type and LMD-9-Δ*pepX* mutant strains were grown in LM17 medium to an $OD_{600\ nm}$ of 1.00 ± 0.05 (see "Growth of *S. thermophilus* LMD-9 and *S. thermophilus* LMD-9-Δ*pepX*"). The cells recovered by centrifugation were washed twice with PAB and resuspended in it to obtain the initial OD. Both strains were then incubated at 42°C under shaking at 200 rpm. After 22 h of incubation, the cell pellet and supernatant were separated by centrifugation at 3,100 × *g* for 10 min at 20°C. The supernatant was filtered through a 0.22-μm filter (Merck Millipore Corp., Billerica, MA) and named cell-free filtrate (**CFF**). The cell pellet and CFF were incubated with β-casomorphin-7 (20 nmol/mL), and samples drawn at 0 and 6 h of incubation were treated and analyzed as described above.

RP-HPLC Analysis of Hydrolysis Products

The RP-HPLC analysis of hydrolysis products from β-casomorphin-7 was conducted as described previously by Hafeez et al. (2013) with some modifications. Aliquots of hydrolysis products (70 μL) recovered at different time intervals were injected into a LiChrospher 100 RP-18 column (150 × 2 mm, 5-μm particle size, 10-nm porosity; Merck Millipore Corp.) connected to an Alliance 2690 HPLC unit (Waters, Milford, MA). Samples were eluted during 60 min by applying a gradient of 0.5% min^{-1} acetonitrile containing 0.1% trifluoroacetic acid in water at a flow rate of 0.25 mL/min. The column temperature was maintained at 30°C. Detection of eluted samples was monitored using a photodiode array detector 996 (Waters) between 200 and 300 nm. The percentage of residual peptide quantity was calculated by comparing the peak area at 215 nm obtained at the chosen time of hydrolysis with that of the initial area of the peptide at 0 min of hydrolysis.

RESULTS

Characterization of S. thermophilus LMD-9 and LMD-9-ΔpepX Mutant Growth in Milk and LM17

The growth behavior of *S. thermophilus* LMD-9 wild type and *S. thermophilus* LMD-9-Δ*pepX* strains was evaluated in reconstituted skim milk (medium poor in free AA and peptides but rich in caseins) and LM17 (medium rich in AA and peptides). After overnight preculture in 10% reconstituted skim milk, both strains were inoculated in each medium and OD was measured either at 480 nm for reconstituted milk or at 600 nm for LM17 broth.

The *S. thermophilus* LMD-9 wild type strain grew faster in milk than the Δ*pepX* mutant and reached the exponential growth phase after 3 h of incubation, whereas the lag phase was prolonged for the Δ*pepX* mutant strain, and it took almost 4 h to attain exponential growth phase. After 6 h of incubation, the wild type strain was already in the stationary phase, whereas the Δ*pepX* mutant strain was still in the exponential growth phase. The difference in growth rates (determined from the maximum slope of a logarithmic representation of growth curves assessed by OD_{480nm} measurements) of both strains was obvious in the exponential growth phase (Figure 2). During this phase, growth rate of the wild type strain was higher by a factor of 1.5 compared with the Δ*pepX* mutant counterpart. Growth rate of the Δ*pepX* mutant strain was not influenced by the presence or absence of spectinomycin in the growth medium (Figure 2; Table 2).

After 4 h of incubation in LM17 medium, both *S. thermophilus* LMD-9 and LMD-9-Δ*pepX* mutant strains were in their exponential growth phase (Figure 2) as determined by the OD measurements at 600 nm. However, a difference in growth rate was also observed in the exponential growth phase: the growth rate of the wild type strain was 0.95, which corresponded to about 1.6-fold higher growth rate compared with the Δ*pepX* mutant strain (Table 2). As before, the presence of spectinomycin had no significant effect on the mutant growth because the mutant showed more or less similar growth pattern in the absence of this antibiotic. Thus, the results obtained for both media suggest an important role of the peptidase PepX during growth because the growth rate of the wild type strain in the 2 media—milk (a medium poor in AA and peptides) and LM17 (a medium rich in AA and peptides) is higher than that of the mutant.

Table 2. Growth rates (GR; h^{-1}) and generation times (GT; h) of *Streptococcus thermophilus* LMD-9 wild type and LMD-9-Δ*pepX* mutant strains during their growth in milk and LM17 media

	Milk		LM17	
Strain	GR	GT	GR	GT
LMD-9	0.42	0.71	0.95	0.33
LMD-9-Δ*pepX* + spectinomycin	0.27	1.1	0.56	0.51
LMD-9-Δ*pepX*	0.27	1.1	0.58	0.54

Hydrolysis of β-Casomorhin-7 by PepX

The *S. thermophilus* LMD-9 wild type strain has the capacity to hydrolyze proline residue-rich bioactive peptide β-casomorphin-7 through its cell-associated X-prolyl dipeptidyl-peptidase activity. Indeed, we previously demonstrated by liquid chromatography/electrospray ionization MS that when incubated with cells of *S. thermophilus* LMD-9, β-casomorphin-7 was hydrolyzed into the shorter peptides FPGI, GPI, FP, and YP (Hafeez et al., 2013). Therefore, to investigate whether the deficiency of intracellular PepX has an influence on this type of activity, the β-casomorphin-7 was incubated at 42°C in PAB (25 m*M*, pH 6.5) for 22 h with the nonproliferating cells of *S. thermophilus* LMD9 wild type and LMD-9-Δ*pepX* mutant strain, which were beforehand grown to the exponential growth phase in LM17 medium. The breakdown pattern of the peptide was then compared between the 2 strains. The RP-HPLC chromatograms of β-casomorphin-7 showed that no hydrolysis of the peptide occurred after 4 h of contact with cells of the *S. thermophilus* LMD-9-Δ*pepX* mutant strain (Figure 3b), and the peptide degradation was almost insignificant even after 22 h of incubation as determined by calculating the residual peptide quantity (Table 3). On the other hand, a significant portion of the peptide was already hydrolyzed (84% loss of the initial peptide quantity) after 4 h of exposure to the cells of *S. thermophilus* LMD-9 wild type strain (Figure 3c). Moreover, the residual peptide quantity calculation exposed that the peptide was fully degraded by the wild type strain after 22 h of contact because no peptide traces were observed at the end of the incubation period (Table 3). Total hydrolysis of β-casomorphin-7 by *S. thermophilus* LMD-9 was an expected result, as the wild type strain was shown to possess an extracellular X-prolyl dipeptidyl-peptidase activity. Moreover, this hydrolysis cannot be correlated with the instability of the peptide because it remained intact during the 22-h incubation period under the same conditions without bacterial cells (data not shown). Conversely, the inactivation of the *pepX* gene led to an absence of hydrolysis of β-casomorphin-7 even after 22 h of incubation. This shows that the peptidase PepX, initially described as intracellular, was responsible for the extracellular X-prolyl dipeptidyl-peptidase activity previously described in the wild type LMD-9 strain.

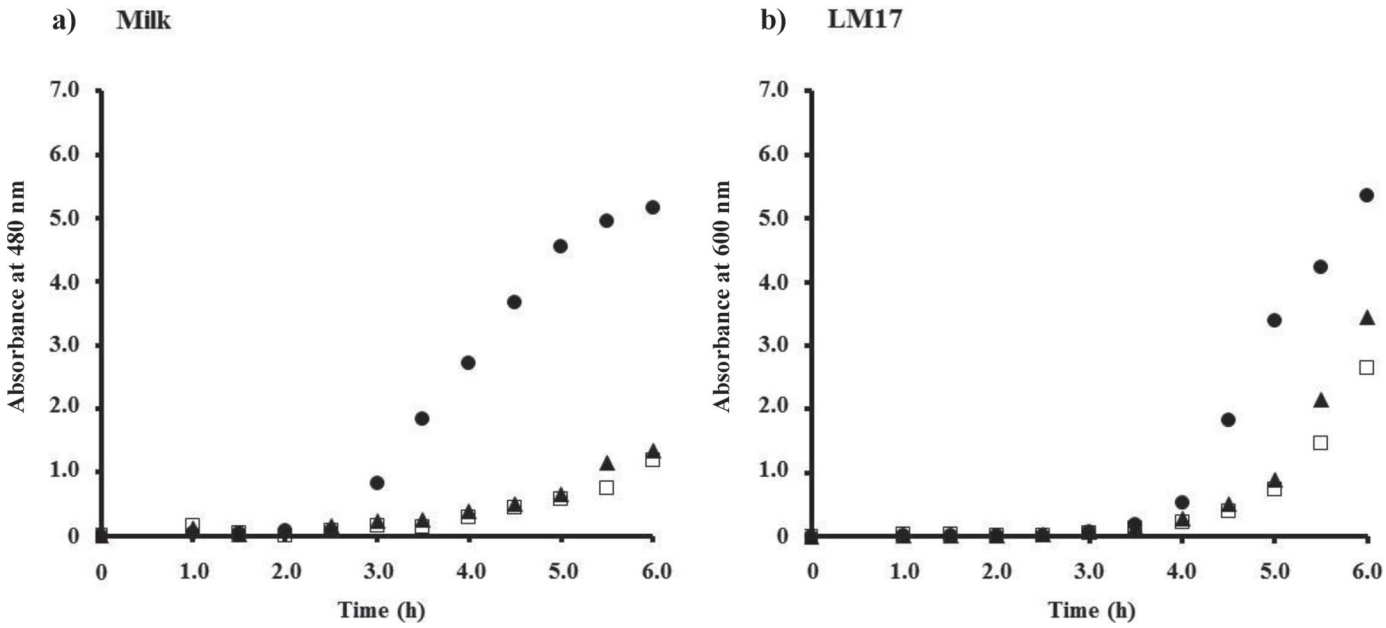

Figure 2. Growth curves of *Streptococcus thermophilus* LMD-9 wild type (filled circles) and *S. thermophilus* LMD-9-Δ*pepX* in the presence (filled triangles) and absence (empty squares) of spectinomycin in (a) milk and (b) LM17 medium. Values correspond to the mean of 2 assays.

Table 3. Quantity (% ± SEM) of intact β-casomorphin-7 recovered at different time intervals after incubation with nonproliferating cells of *Streptococcus thermophilus* LMD-9 wild type and LMD-9-Δ*pepX* mutant

	β-Casomorphin-7 remaining	
Incubation time (h)	*S. thermophilus* LMD-9	*S. thermophilus* LMD-9-Δ*pepX*
1	83 ± 5	100
2	50 ± 2	100
4	16 ± 1	100
6	3.0 ± 0.1	97 ± 2
22	0	90.5 ± 2

Stability of Cell-Associated PepX Activity and Control of Integrity of Bacterial Cells

The *S. thermophilus* strains possess a variety of intracellular peptidases that could be released in incubation medium as a result of spontaneous cell lysis. To check the integrity of bacterial cells as well as the stability of cell-associated PepX activity, *S. thermophilus* LMD-9 wild type and LMD-9-Δ*pepX* mutant strain were grown in LM17 medium up to an $OD_{600\ nm}$ of 1.00 ± 0.05 and then incubated for 22 h in PAB. The potential hydrolysis of β-casomorphin-7 was investigated in the obtained cell pellet and CFF. No significant peptide hydrolysis in CFF of both strains was observed even after 6 h of incubation, whereas the peptide was significantly hydrolyzed by the cell pellet of the wild type strain, contrary to the mutant strain (Figure 4). This thus confirmed an undetectable cell lysis and release of intracellular peptidases in the incubation medium. Similarly, hydrolysis of the peptide by the cell pellet of the wild type strain confirmed the stability of the cell-associated PepX activity.

DISCUSSION

Besides the cell wall protease PrtS and peptide transport system, the proteolytic system of *S. thermophilus* comprises a variety of intracellular and extracellular cell wall-associated peptidases (Savijoki et al., 2006; Hafeez et al., 2014). Peptidases play an essential role in *S. thermophilus* growth in milk because they release AA from casein-derived peptides, previously generated by the hydrolysis of caseins by PrtS. The Xaa-Pro dipeptidyl-peptidase PepX of *S. thermophilus* is postulated to be important for the hydrolysis of proline-rich peptides because bovine milk caseins are a group of proline-rich proteins, particularly β-casein and κ-casein containing 16.7 and 11.8% proline residues, respectively (Casey and Meyer, 1985; Smid et al., 1991; El-Agamy, 2017). Four proline-specific peptidases were reported in *S. thermophilus*: PepX, aminopeptidase P, a prolinase, and a prolidase. PepX displayed the highest activity of all the aminopeptidases found in this bacterium species (Rul and Monnet, 1997). The hydrolysis of different substrates and peptides containing proline residue(s) by the intracellular peptidase PepX has been widely described in different *S. thermophilus* strains (Meyer

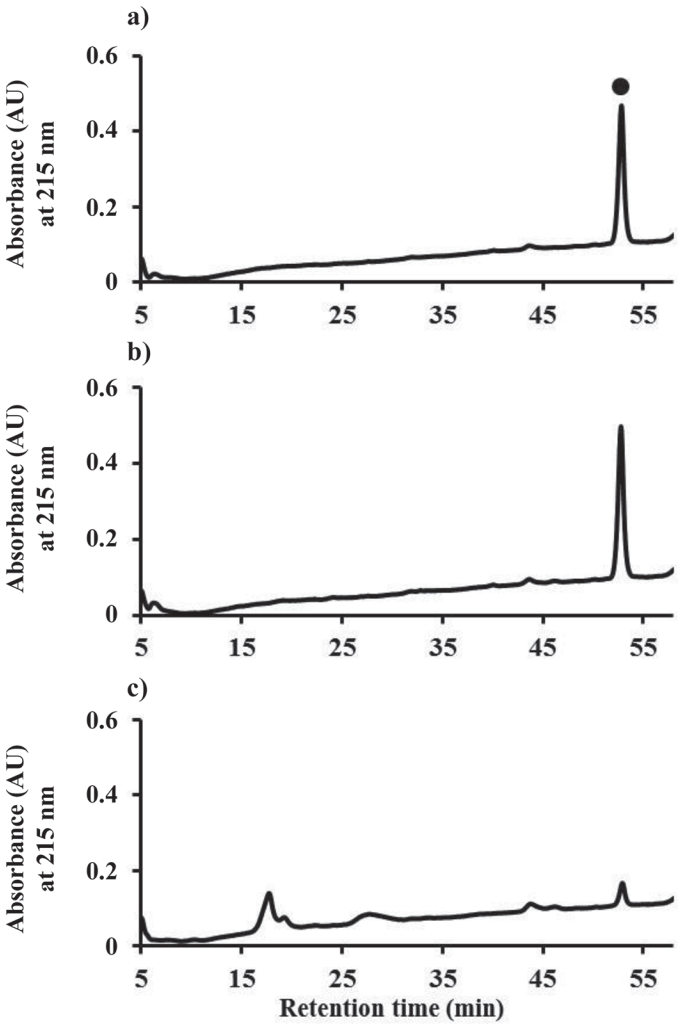

Figure 3. Reversed-phase HPLC chromatograms of β-casomorphin-7 (a, control) after 4 h of incubation with cells of (b) *Streptococcus thermophilus* LMD-9-Δ*pepX* mutant and (c) *S. thermophilus* LMD-9 wild type strain in phosphate-acetate buffer (12.5 m*M*, pH 6.5). Peak corresponding to β-casomorphin-7 is represented by filled circle.

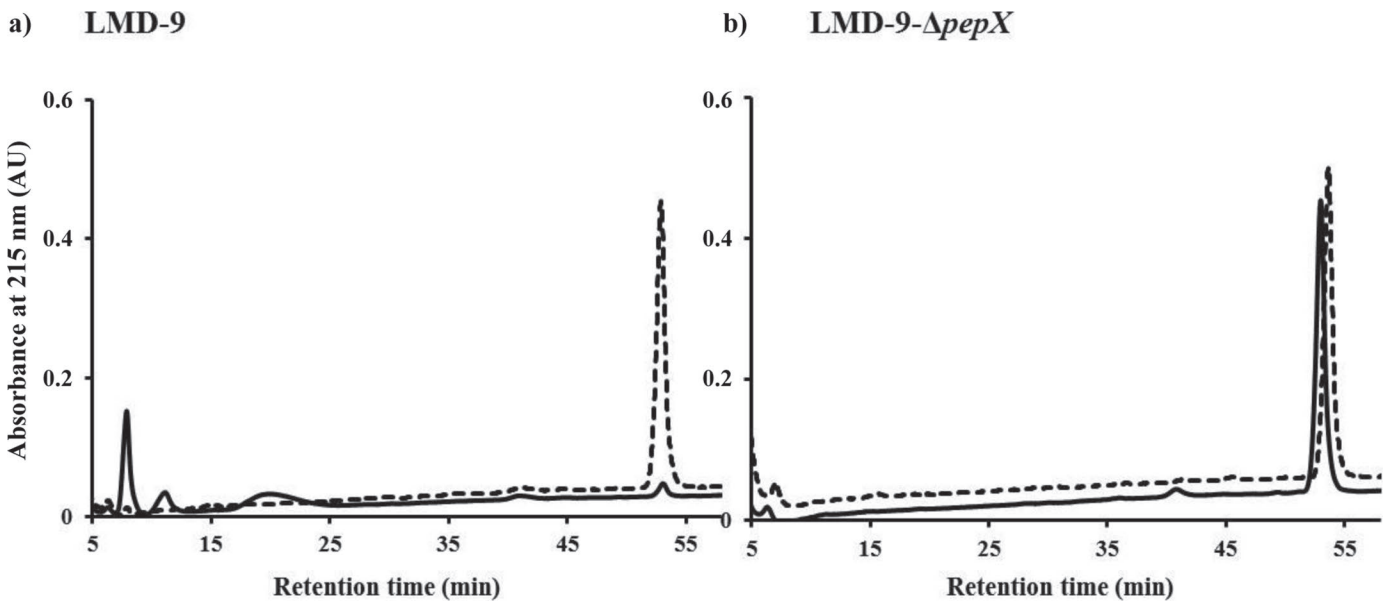

Figure 4. Comparison of reversed-phase HPLC chromatograms obtained after 6 h of hydrolysis of the β-casomorphin-7 by cell pellet (solid line) and cell-free filtrate (CFF; dashed line) of (a) *Streptococcus thermophilus* LMD-9 wild type and (b) *S. thermophilus* LMD-9-Δ*pepX* mutant strain. For clarity, the superimposed chromatogram of β-casomorphin-7 obtained from CFF of LMD-9-Δ*pepX* mutant strain was shifted to the right. After 22 h of incubation of both strains in phosphate-acetate buffer (12.5 m*M*, pH 6.5), cell suspensions were centrifuged to obtain the cell pellet and the CFF. Both the cell pellet and the CFF were further incubated with the β-casomorphin-7 for a period of 6 h.

and Jordi, 1987; Tsakalidou et al., 1998), but to our knowledge the implication of intracellular PepX in extracellular cell-associated X-prolyl dipeptidyl-peptidase activity has never been reported.

In this work, we showed that the inactivation of the *pepX* gene had a negative effect on the growth of the *S. thermophilus* LMD-9-*ΔpepX* mutant in the 2 media tested (i.e., milk and LM17). The overall growth of the *ΔpepX* mutant appeared to be more markedly decreased in milk than in the LM17 medium (Figure 2). Nevertheless, growth rates of the *ΔpepX* mutant strain were decreased almost equally in both media. This decrease cannot be attributed to the presence of the *specR* gene used in the construction of the mutant (Figure 1) because the presence or absence of spectinomycin had not influenced the growth of the Δ*pepX* mutant regardless of the culture medium. As reported in *Lactobacillus helveticus* (Vesanto et al., 1995), the transcription of the *pepX* gene is a typical feature of the exponential phase. Therefore, if it is true, it is not surprising that the inactivation of PepX leads to a decreased growth of the corresponding mutant because during the exponential phase protein synthesis is active, and so the bacterium needs AA. However, several conflicting results about the implication of PepX or other peptidases in the growth of LAB in milk and other complex growth media have to be cited here. Some of them support our findings, whereas others oppose them. It has been stated that the PepX-negative mutant of *Lactobacillus helveticus* CNRZ32 grew in milk at a decreased growth rate but not in de Man, Rogosa and Sharpe medium (Yüksel and Steele, 1996). Likewise, Mierau et al. (1996) reported a decrease in growth rate for a single or multiple peptidase-deficient mutants of *L. lactis* compared with the wild type strain when grown in milk, but no difference in growth rates of wild type and mutants was observed in GM17. In another study conducted on the PepX-deficient mutant of *L. lactis*, the authors on one hand observed that PepX is not essential for growth in milk because the PepX mutant grows normally in milk but on the other hand proposed that alteration in expression of the *pepX* gene may lead to dramatic changes in the peptide composition of finished fermented milk products (Mayo et al., 1993). Cow milk is a medium poor in free AA and peptides and rich in caseins, which are proteins with a high content of proline residues (about 17% for β-casein; 1 of the 2 major caseins of bovine milk according to UniProtKB P02666). As it was shown that PepX displayed the highest activity of all the aminopeptidases of *S. thermophilus* (Rul and Monnet, 1997), its inactivation was expected to affect the growth of the Δ*pepX* mutant in milk. Conversely, LM17 is a medium rich in AA and peptides that are provided by meat, soy, yeast extracts, and casein hydrolysates. The effect of inactivation of PepX on the growth of the mutant that we observed

in this medium could result from the incapacity of the Δ*pepX* mutant to hydrolyze peptides rich in proline residues brought by casein hydrolysates.

In this work, we brought genetic proof of the implication of PepX in the extracellular degradation of β-casomorphin-7 that did not result from cellular lysis. Indeed, we showed that the *S. thermophilus* LMD-9-Δ*pepX* mutant strain was unable to hydrolyze the β-casomorphin-7 in a nonproliferating growth medium. In contrast, β-casomorphin-7 was fully hydrolyzed in the same conditions by the cells of the wild type strain, and this observation is consistent with the previous studies in which hydrolysis of proline-rich peptides by the extracellular cell-associated X-prolyl dipeptidyl-peptidase of *S. thermophilus* strains has been established (Hafeez et al., 2013, 2015). The spontaneous lysis of cells and eventual release of intracellular peptidases in the incubation medium leading to degradation of the peptide can be ruled out because no peptide hydrolysis was observed after 6 h of incubation in CFF of the wild type strain. Kiefer-Partsch et al. (1989) also observed hydrolysis of β-casomorphin-7 by the PepX of *L. lactis*. Further, in a recent work, extracellular peptidase activity was postulated in *L. lactis*. Indeed, analysis of the extracellular peptidome of this bacterium showed peptides mainly originating from surface proteins and having been generated by PrtP, HtrA, or an unknown extracellular peptidase (Guillot et al., 2016).

How the intracellular PepX reaches the cell membrane or the wall to become extracellular remains unknown, especially as shaving experiments conducted on the strain LMD-9 did not allow its detection (Lecomte et al., 2014). In bacteria, most of the exported proteins display a Sec-signal peptide that target them to the Sec secretion pathway. Other Sec-independent secretion pathways have been described, such as the twin-arginine translocation secretion pathway that was distinguished by the presence of a twin-arginine consensus motif in the signal peptide (Berks, 1996; Berks et al., 2000). Nevertheless, some bacterial proteins have been found to be secreted without any apparent signal peptide due to a nonclassical secretion (Bendtsen et al., 2005). Concerning *S. thermophilus*, if the protease PrtS displays a Sec-signal peptide, the sequence analysis of PepX did not reveal any N-terminal signal sequence that could account for translocation from the cytoplasm but highlighted the presence of a galactose-binding domain-like fold (IPR008979). Several studies revealed that the galactose-binding domain-like fold confers the ability to proteins and enzymes to bind to specific ligands such as cell-surface-attached carbohydrate substrates (Gaskell et al., 1995; Boraston et al., 2002; Newstead et al., 2005), phosphatidylserine-rich procoagulant membranes (Macedo-Ribeiro et al., 1999), single-stranded DNA (Marintchev et al., 1999), and several proteins (Kitami et al., 2011). Further, it has been proposed that the C-terminal moiety of PepX of *L. lactis* could act as a tropic factor toward the cell membrane (Tan et al., 1992; Rigolet et al., 2002). Finally, the secretion of proteins by a nonclassical manner could be controlled by the AA composition, the secondary structure, and the presence of disordered regions (Bendtsen et al., 2004, 2005). Concerning PepX, SecretomeP software (http://www.cbs.dtu.dk/services/SecretomeP) gives a probability of nonclassical secretion of 0.449, very close to the threshold set at 0.5. Thus, this type of secretion cannot be excluded in the case of PepX. Therefore, for all these reasons, we propose that the intracellular PepX might (1) be located in the plasmic membrane with its catalytic domain orientated toward peptidoglycan or (2) cross the cell membrane due to its galactose-binding domain-like fold or by a nonclassical manner and then is buried inside the peptidoglycan. In both hypotheses, β-casomorphin-7 or small peptides would be hydrolyzed after their penetration into the peptidoglycan.

To conclude, this study provides the first evidence of the potential implication of intracellular PepX in the hydrolysis of proline-rich peptides extracellularly and that a single PepX might be responsible for the concurrent intra- and extracellular enzyme activity. Identification of novel distinctive features of a proteolytic system of *S. thermophilus* will further enhance its credibility as a starter in milk fermentation.

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REFERENCES

Bagos, P. G., E. P. Nikolaou, T. D. Liakopoulos, and K. D. Tsirigos. 2010. Combined prediction of Tat and Sec signal peptides with hidden Markov models. Bioinformatics 26:2811–2817.

Bendtsen, J. D., L. J. Jensen, N. Blom, G. Von Heijne, and S. Brunak. 2004. Feature-based prediction of non-classical and leaderless protein secretion. Protein Eng. Des. Sel. 17:349–356.

Bendtsen, J. D., L. Kiemer, A. Fausboll, and S. Brunak. 2005. Non-classical protein secretion in bacteria. BMC Microbiol. 5:58.

Berks, B. C., F. Sargent, and T. Palmer. 2000. The Tat protein export pathway. Mol. Microbiol. 35:260–274.

Berks, B. C. 1996. A common export pathway for proteins binding complex redox cofactors? Mol. Microbiol. 22:393–404.

Boraston, A. B., D. Nurizzo, V. Notenboom, V. Ducros, D. R. Rose, D. G. Kilburn, and G. J. Davies. 2002. Differential oligosaccharide recognition by evolutionarily-related β-1,4 and β-1,3 glucan-binding modules. J. Mol. Biol. 319:1143–1156.

Brantl, V., H. Teschemacher, A. Henschen, and F. Lottspeich. 1979. Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein peptone. Hoppe Seylers Z. Physiol. Chem. 360:1211–1216.

Casey, M. G., and J. Meyer. 1985. Presence of X-prolyl-dipeptidyl-peptidases in lactic acid bacteria. J. Dairy Sci. 68:3212–3215.
Chang, O. K., C. Perrin, W. Galia, F. Saulnier, L. Miclo, E. Roux, A. Driou, G. Humbert, and A. Dary. 2012. Release of the cell-envelope protease PrtS in the growth medium of *Streptococcus thermophilus* 4F44. Int. Dairy J. 23:91–98.
Chang, O. K., E. Roux, A. A. Awussi, L. Miclo, J. Jardin, N. Jameh, A. Dary, G. Humbert, and C. Perrin. 2014. Use of a free form of the *Streptococcus thermophilus* cell envelope protease PrtS as a tool to produce bioactive peptides. Int. Dairy J. 38:104–115.
Chapot-Chartier, M. P., F. Rul, M. Nardi, and J. C. Gripon. 1994. Gene cloning and characterization of PepC, a cysteine aminopeptidase from *Streptococcus thermophilus*, with sequence similarity to the eucaryotic bleomycin hydrolase. Eur. J. Biochem. 224:497–506.
Chavagnat, F., J. Meyer, and M. G. Casey. 2000. Purification, characterisation, cloning and sequencing of the gene encoding oligopeptidase PepO from *Streptococcus thermophilus* A. FEMS Microbiol. Lett. 191:79–85.
Dandoy, D., C. Fremaux, M. Henry de Frahan, P. Horvath, P. Boyaval, P. Hols, and L. Fontaine. 2011. The fast milk acidifying phenotype of *Streptococcus thermophilus* can be acquired by natural transformation of the genomic island encoding the cell-envelope proteinase PrtS. Microb. Cell Fact. 10(Suppl. 1):S21.
Delorme, C., C. Bartholini, A. Bolotine, S. D. Ehrlich, and P. Renault. 2010. Emergence of a cell wall protease in the *Streptococcus thermophilus* population. Appl. Environ. Microbiol. 76:451–460.
El-Agamy, E. S. I. 2017. Camel milk. Pages 409–480 in Handbook of Milk of Non-Bovine Mammals. 2nd ed. Y. W. Park, G. F. W. Haenlein, and W. L. Wendorff, ed. Wiley Blackwell, Hoboken, NJ.
Fernandez-Espla, M. D., P. Garault, V. Monnet, and F. Rul. 2000. *Streptococcus thermophilus* cell wall-anchored proteinase: Release, purification, and biochemical and genetic characterization. Appl. Environ. Microbiol. 66:4772–4778.
Fernandez-Espla, M. D., and F. Rul. 1999. PepS from *Streptococcus thermophilus*. A new member of the aminopeptidase T family of thermophilic bacteria. Eur. J. Biochem. 263:502–510.
Fischer, G., B. Decaris, and P. Leblond. 1997. Occurrence of deletions, associated with genetic instability in *Streptomyces ambofaciens*, is independent of the linearity of the chromosomal DNA. J. Bacteriol. 179:4553–4558.
Galia, W., N. Jameh, C. Perrin, M. Genay, and A. Dary-Mourot. 2016. Acquisition of PrtS in *Streptococcus thermophilus* is not enough in certain strains to achieve rapid milk acidification. Dairy Sci. Technol. 96:623–636.
Garault, P., D. Le Bars, C. Besset, and V. Monnet. 2002. Three oligopeptide-binding proteins are involved in the oligopeptide transport of *Streptococcus thermophilus*. J. Biol. Chem. 277:32–39.
Garault, P., C. Letort, V. Juillard, and V. Monnet. 2000. Branched-chain amino acid biosynthesis is essential for optimal growth of *Streptococcus thermophilus* in milk. Appl. Environ. Microbiol. 66:5128–5133.
Gaskell, A., S. Crennell, and G. Taylor. 1995. The three domains of a bacterial sialidase: A β-propeller, an immunoglobulin module and a galactose-binding jelly-roll. Structure 3:1197–1205.
Guillot, A., M. Boulay, E. Chambellon, C. Gitton, V. Monnet, and V. Juillard. 2016. Mass spectrometry analysis of the extracellular peptidome of *Lactococcus lactis*: Lines of evidence for the coexistence of extracellular protein hydrolysis and intracellular peptide excretion. J. Proteome Res. 15:3214–3224.
Hafeez, Z., C. Cakir-Kiefer, J. M. Girardet, J. Jardin, C. Perrin, A. Dary, and L. Miclo. 2013. Hydrolysis of milk-derived bioactive peptides by cell-associated extracellular peptidases of *Streptococcus thermophilus*. Appl. Microbiol. Biotechnol. 97:9787–9799.
Hafeez, Z., C. Cakir-Kiefer, J. M. Girardet, X. Lecomte, C. Paris, W. Galia, A. Dary, and L. Miclo. 2015. New insights in the proteolytic system of *Streptococcus thermophilus*: Use of isracidin to characterize cell-associated extracellular peptidase activities. J. Agric. Food Chem. 63:7522–7531.
Hafeez, Z., C. Cakir-Kiefer, E. Roux, C. Perrin, L. Miclo, and A. Dary-Mourot. 2014. Strategies of producing bioactive peptides from milk proteins to functionalize fermented milk products. Food Res. Int. 63:71–80.
Hols, P., F. Hancy, L. Fontaine, B. Grossiord, D. Prozzi, N. Leblond-Bourget, B. Decaris, A. Bolotin, C. Delorme, S. D. Ehrlich, E. Guédon, V. Monnet, P. Renault, and M. Kleerebezem. 2005. New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. FEMS Microbiol. Rev. 29:435–463.
Jameh, N., W. Galia, A. A. Awussi, E. Roux, M. Genay, C. Perrin, and A. Dary. 2016. Characterization of a new peptide transport system in *Streptococcus thermophilus*. Food Res. Int. 86:34–45.
Kiefer-Partsch, B., W. Bockelmann, A. Geis, and M. Teuber. 1989. Purification of an X-prolyl-dipeptidyl aminopeptidase from the cell wall proteolytic system of *Lactococcus lactis* ssp. *cremoris*. Appl. Microbiol. Biotechnol. 31:75–78.
Kitami, M., T. Kadotani, K. Nakanishi, S. Atsumi, S. Higurashi, T. Ishizaka, A. Watanabe, and R. Sato. 2011. *Bacillus thuringiensis* Cry toxins bound specifically to various proteins via domain III, which had a galactose-binding domain-like fold. Biosci. Biotechnol. Biochem. 75:305–312.
Lecomte, X., V. Gagnaire, V. Briard-Bion, J. Jardin, S. Lortal, A. Dary, and M. Genay. 2014. The naturally competent strain *Streptococcus thermophilus* LMD-9 as a new tool to anchor heterologous proteins on the cell surface. Microb. Cell Fact. 13:82.
Macedo-Ribeiro, S., W. Bode, R. Huber, M. A. Quinn-Allen, S. W. Kim, T. L. Ortel, G. P. Bourenkov, H. D. Bartunik, M. T. Stubbs, W. H. Kane, and P. Fuentes-Prior. 1999. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. Nature 402:434–439.
Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Díaz-Muñiz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills. 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. USA 103:15611–15616.
Marintchev, A., M. A. Mullen, M. W. Maciejewski, B. Pan, M. R. Gryk, and G. P. Mullen. 1999. Solution structure of the single-strand break repair protein XRCC1 N-terminal domain. Nat. Struct. Biol. 6:884–893.
Mayo, B., J. Kok, W. Bockelmann, A. Haandrikman, K. J. Leenhouts, and G. Venema. 1993. Effect of X-prolyl dipeptidyl aminopeptidase deficiency on *Lactococcus lactis*. Appl. Environ. Microbiol. 59:2049–2055.
Meyer, J., and R. Jordi. 1987. Purification and characterization of X-prolyl-dipeptidyl aminopeptidase from *Lactobacillus lactis* and from *Streptococcus thermophilus*. J. Dairy Sci. 70:738–745.
Miclo, L., E. Roux, M. Genay, E. Brusseaux-Lorson, C. Poirson, N. Jameh, C. Perrin, and A. Dary. 2012. Variability of hydrolysis of β-, αs1- and αs2-caseins by 10 strains of *Streptococcus thermophilus* and resulting bioactive peptides. J. Agric. Food Chem. 60:554–565.
Mierau, I., E. R. S. Kunji, K. J. Leenhouts, M. A. Hellendoorn, A. J. Haandrikman, B. Poolman, W. N. Konings, G. Venema, and J. Kok. 1996. Multiple-peptidase mutants of *Lactococcus lactis* are severely impaired in their ability to grow in milk. J. Bacteriol. 178:2794–2803.
Mollet, B., and I. Rowland. 2002. Functional foods: At the frontier between food and pharma. Curr. Opin. Biotechnol. 13:483–485.
Newstead, S. L., J. N. Watson, A. J. Bennet, and G. Taylor. 2005. Galactose recognition by the carbohydrate-binding module of a bacterial sialidase. Acta Crystallogr. D Biol. Crystallogr. 61:1483–1491.
Petersen, T. N., S. Brunak, G. von Heijne, and H. Nielsen. 2011. SignalP 4.0: Discriminating signal peptides from transmembrane regions. Nat. Methods 8:785–786.

Rabier, D., and M. J. Desmazeaud. 1973. Inventaire des différentes activités peptidasiques intracellulaires de *Streptococcus thermophilus*: Purification et propriétés d'une dipeptide-hydrolase et d'une aminopeptidase. Biochimie 55:389–404.

Rigolet, P., I. Mechin, M. M. Delage, and J. F. Chich. 2002. The structural basis for catalysis and specificity of the X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis.* Structure 10:1383–1394.

Rul, F., J. C. Gripon, and V. Monnet. 1995. St-PepA, a *Streptococcus thermophilus* aminopeptidase with high specificity for acidic residues. Microbiology 141:2281–2287.

Rul, F., and V. Monnet. 1997. Presence of additional peptidases in *Streptococcus thermophilus* CNRZ 302 compared to *Lactococcus lactis.* J. Appl. Microbiol. 82:695–704.

Rul, F., V. Monnet, and J. C. Gripon. 1994. Purification and characterization of a general aminopeptidase (St-PepN) from *Streptococcus salivarius* ssp. *thermophilus* CNRZ 302. J. Dairy Sci. 77:2880–2889.

Sambrook, J., and D. W. Russell. 2001. Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Savijoki, K., H. Ingmer, and P. Varmanen. 2006. Proteolytic systems of lactic acid bacteria. Appl. Microbiol. Biotechnol. 71:394–406.

Smid, E. J., B. Poolman, and W. N. Konings. 1991. Casein utilization by lactococci. Appl. Environ. Microbiol. 57:2447–2452.

Takamatsu, D., M. Osaki, and T. Sekizaki. 2001. Thermosensitive suicide vectors for gene replacement in *Streptococcus suis.* Plasmid 46:140–148.

Tan, P. S. T., M. P. Chapot-Chartier, K. M. Pos, M. Rousseau, C. Y. Boquien, J. C. Gripon, and W. N. Konings. 1992. Localisation of peptidases in lactococci. Appl. Environ. Microbiol. 58:285–290.

Terzaghi, B. E., and W. E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807–813.

Tsakalidou, E., R. Anastasiou, K. Papadimitriou, E. Manolopoulou, and G. Kalantzopoulos. 1998. Purification and characterisation of an intracellular X-prolyl-dipeptidyl aminopeptidase from *Streptococcus thermophilus* ACA-DC 4. J. Biotechnol. 59:203–211.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. M. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35:W71–W74.

Vesanto, E., K. Savijoki, T. Rantanen, J. L. Steele, and A. Palva. 1995. An X-prolyl dipeptidyl aminopeptidase (*pepX*) gene from *Lactobacillus helveticus.* Microbiology 141:3067–3075.

Yüksel, G. U., and J. L. Steele. 1996. DNA sequence analysis, expression, distribution, and physiological role of the Xaa-prolyldipeptidyl aminopeptidase gene from *Lactobacillus helveticus* CNRZ32. Appl. Microbiol. Biotechnol. 44:766–773.