The fast milk acidifying phenotype of *Streptococcus thermophilus* can be acquired by natural transformation of the genomic island encoding the cell-envelope proteinase PrtS

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From 10th Symposium on Lactic Acid Bacterium
Egmond aan Zee, the Netherlands. 28 August - 1 September 2011

**Abstract**

**Background:** In industrial fermentation processes, the rate of milk acidification by *Streptococcus thermophilus* is of major technological importance. The cell-envelope proteinase PrtS was previously shown to be a key determinant of the milk acidification activity in this species. The PrtS enzyme is tightly anchored to the cell wall via a mechanism involving the typical sortase A (SrtA) and initiates the breakdown of milk casein into small oligopeptides. The presence or absence of PrtS divides the *S. thermophilus* strains into two phenotypic groups i.e. the slow and the fast acidifying strains. The aim of this study was to improve the milk acidification rate of slow *S. thermophilus* strains, and hence optimise the fermentation process of dairy products.

**Results:** In the present work, we developed for the first time a strategy based on natural transformation to confer the rapid acidification phenotype to slow acidifying starter strains of *S. thermophilus*. First, we established by gene disruption that (i) *prtS*, encoding the cell-envelope proteinase, is a key factor responsible for rapid milk acidification in fast acidifying strains, and that (ii) *srtA*, encoding sortase A, is not absolutely required to express the PrtS activity. Second, a 15-kb PCR product encompassing the *prtS* genomic island was transfered by natural transformation using the competence-inducing peptide in three distinct *prtS*-defective genetic backgrounds having or not a truncated sortase A gene. We showed that in all cases the milk acidification rate of transformants was significantly increased, reaching a level similar to that of wild-type fast acidifying strains. Furthermore, it appeared that the *prtS*-encoded activity does not depend on the *prtS* copy number or on its chromosomal integration locus.

**Conclusion:** We have successfully used natural competence to transfer the *prtS* locus encoding the cell-envelope proteinase in three slow acidifying strains of *S. thermophilus*, allowing their conversion into fast acidifying derivatives. The efficient protocol developed in this article will provide the dairy industry with novel and optimised *S. thermophilus* starter strains.
Introduction

Lactic acid bacteria (LAB) are widely used as starter cultures in the manufacture of dairy products due to their efficient utilisation of milk constituents, principally lactose and caseins. Their capacity to produce lactic acid as the main metabolic end-product of lactose fermentation is of major economic importance, since acidification inhibits the growth of spoilage organisms. Previous studies highlighted a link between the presence of an efficient casein proteolytic system and fast growth and acidification rate of LAB in milk [1]. Casein breakdown is initiated by the cell-envelope proteinases (CEPs) and the resulting oligopeptides are then transported into the cell where they are further hydrolysed by a set of various intracellular peptidases [2,3]. Similar to many surface proteins such as adhesins, CEPs generally contain a C-terminal LPXTG motif and are anchored to the cell wall via a mechanism involving the typical sortase A (SrtA) in Gram-positive bacteria [3,7]. Transpeptidation of LPXTG proteins by the membrane-bound SrtA involves two steps: (i) a cleavage reaction inside the LPXTG motif, and (ii) covalent attachment of the processed and exported N-terminal form to the pentapeptide chain of one peptidoglycan repetition unit [8,9].

Streptococcus thermophilus is a thermophilic LAB which is considered as the second most important industrial dairy starter culture after Lactococcus lactis [10]. The CEP from S. thermophilus, PrtS, is a LPXTG-containing serine proteinase of the subtilisin family, similar to the cell envelope protease from other LAB [7,11,12]. In monoculture, PrtS is essential for rapid growth of S. thermophilus in milk and therefore confers a competitive advantage compared to PrtS-deficient strains [1]. However despite its relevant role for growth in milk, only 21 strains among the 135 strains of the INRA historical collection displayed a high level of proteinase activity, indicating that this characteristic is not common in this species [13]. Since S. thermophilus is generally found in mixed cultures, growth of PrtS-deficient strains relies on the use of oligopeptides released by other LAB such as L. delbrueckii subsp. bulgaricus, Lactobacillus helveticus or L. lactis [1]. However, during the last 10 years, this important metabolic trait for milk adaptation was drastically selected by the dairy industry, resulting in a strong increase of PrtS+ strains in industrial dairy products [14,15]. These probably result from the attractive technological implications associated with PrtS+ strains, such as optimal development in milk containing high protein content, rapid milk acidification, and acceleration of cheese ripening. Recently, Delorme and co-workers showed that in S. thermophilus, prtS is located in a 15-kb genomic island that was probably acquired by horizontal gene transfer [13]. Indeed, this region which consists of three open reading frames (ORF) present upstream of prtS is flanked by tandem repeats of IS elements (Fig. 1).
Besides the fast acidifying phenotype, some *S. thermophilus* strains harbour other industrially relevant phenotypes such as texturing properties, bacteriocins production, and phage resistance. However, these features are rarely found together in a single starter strain. Our objective was to develop a strategy to transfer the rapid acidification phenotype in different genetic backgrounds. In a first step, we defined the minimum genetic information associated with this phenotype by investigating the contribution of *prtS* and Sortase A. In a second step, we took advantage of competence to transfer *prtS* from the fast-acidifying strain DGCC7710 into three different slow strains. The *PrtS*-positive transformants were characterised at the genetic level by PCR mapping and at the phenotypic level by performing proteinase plate assays and monitoring milk acidification.

**Materials and methods**

**Bacterial strains and growth conditions**

The *S. thermophilus* strains used in this study are listed in Table 1. Strains were grown at 37°C or at 40°C. For natural transformation experiments, M17 broth (Difco Laboratories Inc., Detroit, MI) and reconstituted CDM [16] were used. These media were supplemented with 1% (wt/vol) of lactose (M17L or CDML, respectively). Two different skimmed milks were used to assess the proteinase phenotype of strains: 10% (vol/vol) UHT milk (Candida GrandLait, Candia, France) and reconstituted 9% (wt/vol) milk (Humana Milchunion, Germany). Solid agar plates were typically prepared by adding 2% (w/v) agar to the medium. When necessary, 5 µg ml⁻¹ chloramphenicol was added to the media. Inoculated solid plates, solely, were incubated in anaerobic conditions (BBL GasPak Systems, Becton Dickinson, Franklin lakes, NJ).

**Proteinase plate assay**

The PrtS proteinase phenotype of *S. thermophilus* strains was determined on bacterial colonies grown on Fast Slow Difference Agar (FSDA) medium after 48 to 72 h of incubation at 37°C, as previously reported [17]. This medium contains 1.5% (wt/v) agar, 1.9% (wt/v) Sodium glycerophosphate (Prolabo, Merck, West Chester, PA), 10% (vol/vol) UHT skimmed milk (Candida GrandLait, Candia, France) and 0.001% (wt/v) of the bromocresol purple (BCP) indicator (Prolabo, Merck, West Chester, PA). On FSDA plates, bacteria possessing a proteinase activity appear as yellow, big, opaque colonies surrounded by a yellow area (fast acidifying phenotype), whereas proteinase-negative colonies appear small, flat, and translucent (slow acidifying phenotype).

**Determination of the kinetics parameters of acidification**

The acidification activity of *S. thermophilus* strains was evaluated by calculating the maximum acidification rate and time necessary to reach pH 5.2. Practically, reconstituted 10% (wt/vol) UHT skimmed milk (autoclaved during 10 minutes, 110°C) was inoculated with 2% (vol/vol) of a 5ml-culture of *S. thermophilus* grown at 37°C during 24h in M17L broth. After 24h of incubation at 37°C, the cultures were inoculated at 1% (vol/vol) in 150ml skimmed milk i.e. either 10% (vol/vol) UHT milk (Candida GrandLait) or 9% reconstituted (wt/vol) milk (Humana Milchunion, Germany). The cultures were then incubated in a water bath at 40°C and the pH (pH electrode Mettler 405 DPAS SC, Toledo, Spain) was monitored during 16 hours using the CINAC system (Alliance Instruments, France) as previously described [18,19]. The pH was measured every second and values obtained during 5 minutes were averaged.

**Table 1 Characteristics of *S. thermophilus* strains used in this work**

| Strain    | Phenotype | Genotype          | Transformation rate |
|-----------|-----------|-------------------|---------------------|
|           | F  | T  | prtS | strA | (+ 1mM ComS17-24)[20] |
| LMG18311  | F+ | T+ | -   | Trunc.| 1.3 x 10⁻¹                  |
| CNRZ1066  | F+ | T+ | -   | Trunc.|                          |
| LMD-9     | F+ | T+ | +   |      | 5.4 x 10⁻³                  |
| DGCC7790  | F+ | T+ | -   |      | 1.0 x 10⁻²                  |
| DGCC7710  | F+ | T+ | +   |      |                          |
| DGCC7853  | F+ | T+ | -   |      |                          |
| DGCC7879  | F+ | T+ | +   |      |                          |
| DGCC715   | F+ | T+ | -   |      |                          |
| DGCC7773  | F+ | T+ | -   | Trunc.|                          |
| DGCC7785  | F+ | T+ | -   |      |                          |
| DGCC7796  | F+ | T+ | +   |      |                          |
| DGCC7854  | F+ | T+ | +   |      |                          |
| DGCC7891/ND03 | F+ | T+ | +   | +   | 1.4 x 10⁻⁴                  |
| DGCC7666  | F+ | T+ | -   | Trunc.| 4.2 x 10⁻²                  |
| DGCC7694  | F+ | T+ | +   |      |                          |
| DGCC7809  | F+ | T+ | +   |      |                          |
| DGCC7909  | F+ | T+ | -   | Trunc.|                          |
| DGCC7984  | F+ | T+ | +   |      |                          |
| DGCC47    | F+ | T+ | -   | Trunc.|                          |
| DGCC7766  | F+ | T+ | -   |      |                          |
| DGCC855   | F+ | NT | +   |      |                          |
| DGCC2057  | F+ | T+ | -   |      |                          |
| DGCC2058  | F+ | T+ | -   |      |                          |
| DGCC9791  | F+ | NT | +   |      |                          |
| DGCC7856  | F+ | T+ | -   |      |                          |
| DGCC8014  | F+ | T+ | +   |      |                          |

All strains are from Danisco collection, excepted strains LMD-9 (ATCC collection), CNRZ1066 (CNRZ collection), ND03[29] and LMG18311 (LMG collection)

For phenotype, F+ and F- respectively refer to fast and slow acidifying strains when grown in pasteurized milk, T+ and T- respectively refer to the presence and absence of texturing properties when grown in pasteurized milk, and NT for not tested.

For genotype, + indicates that the gene is detected and entire, – indicates that *prtS* is no detected, Trunc. indicates a truncated version of *strA*.
maximum acidification rate \( (V_m) \), defined as the maximum slope of the pH curve \((\text{d}pH/\text{d}t)\), was calculated using the CINAC v2.07 software and expressed as pH units/minute.

Natural transformation experiments
Transformation experiments were performed as previously described [20,21]. Briefly, an overnight culture of \( S. \) \( \text{thermophilus} \) grown in M17L at 37°C was washed twice (5,000 x g, 9 min, room temperature) in one volume of CDML and resuspended in one volume of CDML. The washed culture was then 30-fold diluted in CDML and DNA was added to small volumes (300 μl). The amount of DNA used was either 25 ng (purified overlapping PCR products) or 10 μg (PCR product encompassing the \( \text{prtS} \) genomic island). After 1h30 of incubation at 37°C, 1 μM of peptide ComS17-24 (purity >95%; supplied by Peptide 2.0 (Chantilly, VA)) was added to the culture. After 5 hours, samples (100 μl of serial dilutions in M17 broth) containing DNA, or not (negative control), were spread on selective plates and incubated anaerobically at 37°C. These plates contained M17L broth supplemented with 5 μg/ml chloramphenicol (transformation of \( \text{lox66-} \text{P32-} \text{cat-} \text{lox71} \)-containing PCR products) or FSDA medium (transformation of the \( \text{prtS} \) genomic island). In the latter case, 10⁶ cells were plated on FSDA dishes. After 72 hours of incubation, colonies displaying a fast acidifying phenotype (see above) i.e. colonies emerging on the lawn of slow acidifying colonies were recovered and isolated on FSDA plates. The presence of \( \text{prtS} \) in fast acidifying colonies was then validated by PCR extension.

DNA techniques
For general molecular biology techniques, we followed the instructions given by Sambrook et al. [22]. Preparation of \( S. \) \( \text{thermophilus} \) chromosomal DNA was performed as described previously [23]. The primers used in this study were purchased from Eurogentec (Seraing, Belgium) and are listed in Table S1 of the Additional file 1. PCR reactions were performed with Phusion high-fidelity DNA polymerase (Finnzymes Espoo, Finland) or LA Taq™ Polymerase (Takara bio, Otsu, Japan) in a GeneAmp PCR system 2400 (Applied Biosystems, Foster City, CA).

Construction of \( \text{prtS} \) and \( \text{srtA} \) deletion mutants
Mutant strains \((\Delta \text{srtA::} \text{lox66-} \text{P32-} \text{cat-} \text{lox72} \) and \( \Delta \text{prtS::} \text{lox66-} \text{P32-} \text{cat-} \text{lox72} \) of LMD-9 and DGCC7710 were constructed by replacing the sequence between the start and stop codons of \( \text{srtA} \) and \( \text{prtS} \) with the chloramphenicol resistance cassette \( \text{lox66-} \text{P32-} \text{cat-} \text{lox71} \) according to the procedure described by Fontaine et al. [20]. The primers used to construct these strains are listed in Table S1 of the Additional file 1.

Results and discussion
Fast acidifying strains contain the \( \text{prtS} \) genomic island and a full-length \( \text{srtA} \) gene
The strategy chosen to optimise the acidification rate of \( S. \) \( \text{thermophilus} \) strains consists of transferring the minimal genetic requirements associated with the fast acidifying phenotype. Based on previous knowledge on the functional role of \( \text{PrtS} \) and Sortase A in the acidification rate [1,11,12] and activity of LPXTG-surface proteins [24-26], respectively, we focused our attention on the co-occurrence of \( \text{srtA} \) and \( \text{prtS} \) in fast strains. For this purpose, we analysed a sample of fast and slow acidifying strains of \( S. \) \( \text{thermophilus} \). Their phenotype was determined by a FSDA plate assay (Data not shown and [20]). They were selected by industrial manufacturers for their relevant phenotypes related to milk fermentation [20].

The presence of \( \text{prtS} \) was firstly analysed by PCR using primers specific to the \( \text{prtS} \) ORF or flanking genes (Additional file 1, Table S1). These primers were designed based on the sequence of the \( \text{prtS} \) genomic island in strain LMD-9. Amplification products of the expected size were obtained for 11 out of the 26 strains tested (22 strains plus the 4 sequenced strains LMD-9, LMG18311, CNRZ1066 and ND03) (Data not shown). As expected, the presence or absence of \( \text{prtS} \) was in perfect agreement with the fast or slow acidifying phenotypes of strains on FSDA medium, respectively (Table 1). In addition, the systematic finding of a PCR amplification product with primers targeting the two genes flanking \( \text{prtS} \) strongly suggests that its genetic context is similar to that described by Delorme and co-workers (Additional file 1, Fig. S1) [13]. Next, the 11 \( \text{prtS} \) ORFs were sequenced and the corresponding proteins were aligned with known sequences from strains LMD-9, CNRZ385 and JIM8232 (Additional file 1, Fig. S2). \( \text{PrtS} \) sequences share a high level of conservation at the protein level (between 96% and 100% of identity in pairwise alignments), which suggests a recent acquisition as hypothesised by Delorme et al. [13]. However, the phylogenetic tree deduced from the multiple alignment of \( \text{PrtS} \) sequences shows that they form three distinct groups (Additional file 1, Fig. S1). Each cluster is similarly represented, which suggests that at least three different sub-populations have emerged since the initial acquisition of the \( \text{prtS} \) locus.

A conserved LPNTG sorting motif was identified in the C-terminus part of all \( \text{PrtS} \) proteins (Additional file 1, Fig. S2), supporting our hypothesis of a putative role for Sortase A in \( \text{PrtS} \) activity of fast isolates.
Consequently, the srtA ORFs were PCR-amplified from the 22 strains, sequenced and compared to srtA from strains LMD-9 (ster_1255), LMG18311 (stu1277-stu1278), CNRZ1066 (str1277-str1278) and ND03 (STND1228). The SrtA proteins are more divergent than PrtS proteins since they share between 93% and 100% identity (deduced from pairwise alignments) (Additional file 1, Figs. S3 and S4). We found that srtA from 7 out of 26 strains contains the same nonsense mutation that shortens the ORF size (378 bp instead of 758 bp) (Additional file 1, Fig. S4). The Y123STOP mutation probably abolishes SrtA activity in those strains. Interestingly, all PrtS- isolates encode a full-length srtA ORF. Their SrtA protein also contains the conserved catalytic residues (H147, C212 and R220) and essential for sortase activity [8,9]. The co-occurrence of a full-length srtA and prtS in fast acidifying isolates strongly suggests that these two components are both required for an optimal fast acidifying phenotype in S. thermophilus.

PrtS is essential for fast milk acidification while SrtA is not required  
The individual contribution of SrtA and PrtS proteinase on milk acidification was investigated by replacing the corresponding ORFs in fast strain LMD-9 and DGCC7710 by a chloramphenicol resistance marker. The physiological effects in milk-based medium were investigated by plating the parental and mutant strains on FSDA medium and by measuring their acidification rates in UHT skimmed milk. As expected, both ΔprtS::lox66-P32-cat-lox71 derivative strains displayed the typical phenotype of slow acidifying strains on FSDA medium. Surprisingly, no striking morphological difference was observed between colonies of WT and ΔsrtA::lox66-P32-cat-lox71 derivatives (Data not shown). These results were confirmed by monitoring the pH and measuring the acidification kinetics of strains growing in two skimmed milks: UHT and reconstituted milk. Similar acidification rates were obtained for wild-type and SrtA-defective strains, while prtS-negative mutants were severely impaired in their acidification capacities (Data shown for DGCC7710 and its derivatives in Fig. 2). Altogether, our results show that prtS is the predominant genetic determinant required for the fast acidification phenotype of S. thermophilus in milk-based culturing conditions.

Isolation of fast acidifying PrtS+ transformants deriving from three slow strains  
The transfer of prtS from fast to slow strains was achieved using the natural competence protocol that was recently developed [20]. The model strains selected for the proof of concept were DGCC7710 as donor, and LMG18311, DGCC7666 and DGCC7891 as receivers. These latter strains were chosen for different reasons: (i) they encode, or not, a full-length srtA ORF (Table 1), (ii) they display different transformation rates [20], (iii) they display different industrially-relevant properties [20], and (iv) their genome has been sequenced (draft genome for DGCC7666, unpublished data; and DGCC7891 is closely related to the recently sequenced strain ND03) [29]. A 15,283-kb PCR product encompassing the whole prtS island (between ster_0839 and ster_0850) (Fig.1) from strain DGCC7710 was used as donor DNA. The entire region was sequenced and shares 99% identity with the prtS region of LMD-9. The IS elements (IS3th1 and IS1167) flanking the prtS locus would mediate double homologous recombination events between the PCR-amplified DNA fragment and the chromosome of receiver strains. Indeed, these elements are widespread among S. thermophilus genomes (Table 2; Additional file 1, Fig. S3). The natural competence experiments were performed in the presence of 10µg of the purified PCR fragment and transformants displaying a fast acidifying phenotype were recovered on FSDA plates. After three successive rounds of isolation on FSDA plates, the stable acquisitions of the prtS genomic island in fast acidifying clones were confirmed by PCR mapping. The integration site(s) of the prtS island were then determined by PCR on 3 to 5 clones per strain using a primer specific to IS3th1 or IS1167 flanking genes, and the prtS locus (Additional file 1, Table S1). A random insertion of the prtS locus in both
| Strains     | Putative insertion sites and their surrounding genes | prtS insertion number |
|------------|------------------------------------------------------|----------------------|
|            | ciaH  | blpT  | galU  | msrA1 | topA  | STND0510 | stu0861 | stu1089 | STND0227 | STND0823 | STND0900 | STND1130 | STND1212 |
|            | rspt   | stu1680 | stu1836 | brnQ | stu0900 | STND0513 | stu0868 | stu1075 | STND0229 | STND-0825 | STND0902 | STND1132 | STND1214 |
| DGCC7666 PrtS+ |       |         |       |       |        |         |         |         |         |          |          |          |          |
| CL1        | -     | +      | -     | +     | /      | /       | -       | +       | /       | /         | /         | /         | /         |
| CL2        | +     | +      | -     | -     | /      | /       | +       | +       | /       | /         | /         | /         | /         |
| CL3        | +     | -      | -     | +     | /      | /       | -       | -       | /       | /         | /         | /         | /         |
| CL4        | -     | -      | +     | -     | /      | /       | -       | -       | /       | /         | /         | /         | /         |
| CL6        | -     | -      | -     | +     | /      | /       | -       | -       | /       | /         | /         | /         | /         |
| DGCC7891 PrtS+ |       |         |       |       |        |         |         |         |         |          |          |          |          |
| CL1A, 19A  | +     | +      | +     | /     | /       | -       | /       | +       | +       | +         | -         | +         | 7         |
| CL1B       | +     | +      | +     | /     | /       | -       | /       | +       | +       | -         | -         | +         | 6         |

Symbols: /, IS element not detected in the chromosome; -, prtS locus not detected; +, prtS locus detected
ISSth1 and IS1167 loci was detected among strains and isolated clones (Table 2). Multiple insertions were also observed, reaching in some cases up to seven copies of prtS in the same clone (Table 2). The stable maintenance of prtS copies in three clones per strain was then studied in liquid milk-based medium. After ~160 generations, the copy number and the insertion profile determined by PCR mapping remained unchanged in all isolates (Data not show).

Stable acquisition of the prtS genomic island improves the acidification activity of S. thermophilus

To further characterise the phenotype of PrtS+ derivative strains, their performance was analysed by determining kinetics parameters of acidification in milk. We tested culture conditions that are mimicking those encountered in industrial fermentation processes i.e. in the 10% UHT skimmed milk (vol/vol), and the 9% (wt/vol) reconstituted skimmed milk. The results are presented in Fig. 3 and in Table 3. Compared to the parental strains, acidification rates of all derivatives were remarkably improved (2.4 fold) in both milks. In addition, we observed a reduction of the latency period i.e. the time before the pH begins to decrease (Fig. 3 and Data not shown). Consequently, a significantly shorter fermentation time is needed to obtain pH 5.2 (2 to 3-fold) (Table 3). Interestingly, the acidification curves obtained in both milks were almost identical among all PrtS+ clones deriving from the same parental strain. They were also similar to natural fast acidifying strains DGCC7710 or LMD-9 (Table 3 and Data not shown).

Altogether, our results show that the transfer of the prtS island between S. thermophilus strains is sufficient as such to convert slow acidifying strains into transformants displaying fast activities similar to PrtS+ natural isolates. In addition, acquisition of this property was shown to be independent of the prtS copy number or the chromosomal integration locus.

Conclusion

To our knowledge, we performed for the first time a stable and fully functional transfer of the prtS locus in PrtS-deficient backgrounds of S. thermophilus. By comparing the PrtS+ derivative transformants isolated in this study to (i) natural fast and slow acidifying isolates and (ii) prtS and srtA mutants, we have ultimately shown that PrtS is the most relevant trait responsible for rapid milk acidification. In monoculture, acquisition of a proteolytic system capable of producing short oligopeptides from the casein matrix fulfils thus optimally the nutritional requirements of S. thermophilus in milk. The housekeeping sortase A is not required for full PrtS activity and optimal milk acidification. However, we can not exclude that it could be required in other culturing conditions or for the anchorage and activity of others putative sortase substrates. In absence of sortase A, it is probable that most of active PrtS proteins remain anchored in the cell membrane through their C-terminal hydrophobic domain (downstream of the LPXTG motif).
Further studies would be required to fully explore the relation between the SrtA protein and its PrtS substrate in *S. thermophilus*.

The PrtS\(^+\) phenotype was previously reported to be only present in a few strains of *S. thermophilus*. However, since a decade the proportion of strains isolated from industrial dairy products displaying this phenotype has sharply increased, indicating a significant interest of the food industry for this infrequent adaptation [11-15]. The transfer protocol developed in this work is applicable to all transformable *S. thermophilus* strains, provided the insertion sites for *prtH* island i.e. ISSth1 or IS1167 elements are present in their genome. It will thus provide the dairy industry with novel and improved starter strains of *S. thermophilus*, which performed better under fermentation processes and may have a non-Genetically Modified Microorganisms (GMM) status according to the European Legislation [20,30].

### Additional material

Additional file 1: supplementary material – Table S1, Figs S1-S5.

List of abbreviations used

CEP: Cell-Envelope Proteinases; FSDA: Fast Slow Difference Agar; GMM: Genetically Modified Microorganisms; IS: Insertion Sequence; LAB: Lactic Acid Bacteria; ORF: Open Reading Frames; PCR: Polymerase Chain Reaction; WT: Wild type.

### Acknowledgements

This research was carried out with financial support from Danisco and FNRS. N. F. is postdoctoral researcher at FNRS. P. Hols is research associate at FNRS.

This article has been published as part of Microbial Cell Factories Volume 10 Supplement 1, 2011: Proceedings of the 10th Symposium on Lactic Acid Bacterium. The full contents of the supplement are available online at http://www.microbialcellfactories.com/supplements/10/51.

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### Authors’ contributions

Phols, LF and DD designed the project; DD, LF, MH performed the experimental work; DD, LF, PHorvath, CF, PHols and PB analysed the data; DD and LF wrote the paper; PHorvath, CF, Phols and PB critically reviewed the paper. All authors approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

Published: 30 August 2011

### References

1. Courtin P, Monnet V, Rul F: Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus*/*Lactobacillus bulgaricus* mixed cultures in milk. Microbiology 2002, 148:3413-3421.

2. Christensen JE, Dudley EG, Pederson JA, Steele JL: Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 1999, 76:217-246.

3. Savijoki K, Ingmer H, Varmanen P: Peptidolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol 2006, 73:394-406.

4. Genay M, Sadat L, Gagnaire V, Lortal S: prth2, not *prtH*, is the ubiquitous cell wall proteinase gene in *Lactobacillus helveticus*. Appl Environ Microbiol 2009, 75:3238-3249.

5. Pederson JA, Mileski GJ, Weimer BC, Steele JL: Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. J Bacteriol 1999, 181:4592-4597.

6. Sadat-Mekmene L, Jardin J, Corre C, Molle D, Richoux R, Delage MM, et al: Simultaneous presence of PrtH and PrtH2 proteins in *Lactobacillus helveticus* strains breaks down of the pure alpha1-casein. Appl Environ Microbiol 2011, 77:179-186.

7. Fernandez-Espia MD, Graulat P, Monnet V, Rul F: Streptococcus thermophilus cell wall-anchored proteinase: release, purification, and biochemical and genetic characterization. Appl Environ Microbiol 2000, 66:4772-4778.

8. Clancy KW, Melvin JA, McCafferty DG: Sortase transpeptidases: insights into mechanism, substrate specificity, and inhibition. Biopolymers 2010, 94:385-396.

9. Paterson GK, Mitchell TJ: The biology of Gram-positive sortase enzymes. Trends Microbiol 2004, 12:89-95.

10. Hols P, Hancy F, Fontaine L, Grossiord B, Prozzi D, Leblond-Bourget N, et al: New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. FEMS Microbiol Rev 2003, 29:435-463.

11. Shahbali S, Hemme D, Desmazeaud M: High cell wall-associated proteinase activity of some *Streptococcus thermophilus* strains (H-strains) correlated with a high acidification rate in milk. Lait 1991, 71:351-357.

12. Shahbali S, Hemme D, Renault P: Characterization of a Cell Envelope-Associated Proteinase Activity from *Streptococcus thermophilus* H-Strains. Appl Environ Microbiol 1999, 55:171-182.

13. Delorme C, Bartholini C, Bolotin A, Ehrlich SD, Renault P: Emergence of a cell wall protease in the *Streptococcus thermophilus* population. Appl Environ Microbiol 2010, 76:451-460.
14. Galia W., Perrin C, Dary A: Variability and molecular typing of Streptococcus thermophilus strains displaying different proteolytic and acidifying properties. Lait 2009, 19:89-95.
15. Rasmussen TB, Danielsen M, Valina O, Garrigues C, Johansen E, Pedersen NB: Streptococcus thermophilus core genome: comparative genome hybridization study of 47 strains. Appl Environ Microbiol 2008, 74:4703-4710.
16. Letort C, Juillard V: Development of a minimal chemically-defined medium for the exponential growth of Streptococcus thermophilus. J Appl Microbiol 2001, 91:1023-1029.
17. Huggins AR, Sandine WE: Differentiation of Fast and Slow Milk-Coagulating Isolates in Strains of Lactic Streptococci. Journal of Dairy Science 1984, 67:1674-1679.
18. Fonseca F, Beal C, Corrieu G: Method of quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. J Dairy Res 2000, 67:83-90.
19. Pernoud S, Fremaux C, Sepulchre A, Corrieu G, Monnet C: Effect of the metabolism of urea on the acidifying activity of Streptococcus thermophilus. J Appl Microbiol 2001, 91:1023-1029.
20. Fontaine L, Dandoy D, Boutry C, Delplace B, de Frahan MH, Fremaux C, et al: Development of a Versatile Procedure Based on Natural Transformation for Marker-Free Targeted Genetic Modification in Streptococcus thermophilus. Appl Environ Microbiol 2010, 76:7332-3337.
21. Fontaine L, Bouty C, de Frahan MH, Delplace B, Fremaux C, Horvath P, et al: A novel pheromone quorum-sensing system controls the development of natural competence in Streptococcus thermophilus and Streptococcus salivarius. J Bacteriol 2010, 192:1444-1454.
22. Sambrook J, FEaMT: Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press NY; 1989.
23. Nobbs AH, Vajna RM, Johnson JR, Zhang Y, Erlandsen SL, Oli MW, et al: Consequences of a sortase A mutation in Streptococcus gordonii. Microbiology 2007, 153:4088-4097.
24. Lalioui L, Pellegrini E, Dramsi S, Baptista M, Bourgeois N, Doucet-Populaire F, et al: The SrtA Sortase of Streptococcus agalactiae is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. Infect Immun 2005, 73:3342-3350.
25. Sun Z, Chen X, Wang J, Zhao W, Shao Y, Wu L, et al: Complete genome sequence of Streptococcus thermophilus strain ND03. J Bacteriol 2011, 193:793-794.