

## Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus*/*Lactobacillus bulgaricus* mixed cultures in milk

P. Courtin, V. Monnet and F. Rul

Author for correspondence: F. Rul. Tel: +33 1 34 65 21 48. Fax: +33 1 34 65 21 63.  
e-mail: rul@jouy.inra.fr

Unité de Biochimie et  
Structure des Protéines,  
INRA, 78352 Jouy-en-Josas  
Cedex, France

**The manufacture of yoghurt relies on the simultaneous utilization of two starters: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*). A proto-cooperation usually takes place between the two species, which often results in enhanced milk acidification and aroma formation compared to pure cultures. Cell-wall proteinases of *Lactococcus lactis* and lactobacilli have been shown to be essential to growth in milk in pure cultures. In this study, the role of proteinases PrtS from *S. thermophilus* and PrtB from *Lb. bulgaricus* in bacterial growth in milk was evaluated; a negative mutant for the *prtS* gene of *S. thermophilus* CNRZ 385 was constructed for this purpose. Pure cultures of *S. thermophilus* CNRZ 385 and its PrtS-negative mutant were made in milk as well as mixed cultures of *S. thermophilus* and *Lb. bulgaricus*: *S. thermophilus* CNRZ 385 or its PrtS-negative mutant was associated with several strains of *Lb. bulgaricus*, including a PrtB-negative strain. The pH and growth of bacterial populations of the resulting mixed cultures were followed, and the *Lactobacillus* strain was found to influence both the extent of the benefit of *Lb. bulgaricus*/*S. thermophilus* association on milk acidification and the magnitude of *S. thermophilus* population dominance at the end of fermentation. In all mixed cultures, the sequential growth of *S. thermophilus* then of *Lb. bulgaricus* and finally of both bacteria was observed. Although proteinase PrtS was essential to *S. thermophilus* growth in milk in pure culture, it had no effect on bacterial growth and thus on the final pH of mixed cultures in the presence of PrtB. In contrast, proteinase PrtB was necessary for the growth of *S. thermophilus*, and its absence resulted in a higher final pH. From these results, a model of growth of both bacteria in mixed cultures in milk is proposed.**

Keywords: bacterial growth, milk fermentation, thermophilic bacteria

### INTRODUCTION

*Streptococcus thermophilus* is a thermophilic lactic acid bacterium (LAB), widely used as a starter to produce fermented dairy products. It is generally used in association with other micro-organisms, in particular with *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) for the manufacture of yoghurt. For this application, the fast-growing capacity of these bacteria in milk is crucial to enable intense and rapid acidification

of milk. LAB are fastidious micro-organisms, which have in particular several amino acid auxotrophies. Most *S. thermophilus* strains are stimulated by the supply of two to five amino acids (Bracquart & Lorient, 1977; Letort & Juillard, 2001; Shankar & Davies, 1977), whereas lactobacilli require between three and 14 amino acids (Hebert *et al.*, 2000; Ledesma *et al.*, 1977; Morishita *et al.*, 1981). The optimal growth of LAB in milk thus depends on their proteolytic system, which hydrolyses milk caseins into peptides and amino acids (Thomas & Mills, 1981). The cell-wall proteinases of LAB are of major importance in this process, as they are responsible for the first step of casein breakdown. They

**Abbreviations:** FSDA, Fast Slow Difference Agar; LAB, lactic acid bacterium/bacteria.

belong to the same multi-domain proteinase family and show significant homologies, even though differences in specificity, bacterial anchor and domain organization have been described (Fernandez-Esplá *et al.*, 2000; Siezen, 1999). The cell-wall proteinase of *Lactococcus lactis* (PrtP), which is very frequent in this species, has been extensively studied. In milk, *Lc. lactis* PrtP-negative strains only reach 10% of the cell densities observed with PrtP-positive strains (Thomas & Mills, 1981). In *S. thermophilus*, the presence of a cell-wall proteinase, PrtS, recently characterized, is less common than in *Lc. lactis*. In this species, high cell-wall proteinase activities are associated with high milk-acidifying capacities (Shahbal *et al.*, 1991). In *Lb. bulgaricus*, the cell-wall proteinase, PrtB, is also essential for optimal growth in milk; a proteinase-negative strain reaches only 22% of the final biomass of a proteinase-positive strain when grown in milk (Gilbert *et al.*, 1997).

In yoghurt, *S. thermophilus* and *Lb. bulgaricus* are grown in association, which often results in a positive interaction. This relationship, called proto-cooperation, has a beneficial effect on growth of both species and on acid and aroma production. *S. thermophilus* indeed produces pyruvic acid, formic acid and CO<sub>2</sub> (for reviews, see Tamine & Robinson, 1999; Zourari *et al.*, 1992), which stimulate the growth of *Lb. bulgaricus*. In turn, *Lb. bulgaricus* produces peptides and amino acids that stimulate *S. thermophilus* growth (Accolas *et al.*, 1971; Bautista *et al.*, 1966; Higashio *et al.*, 1977; Pette & Lolkema, 1950b; Radke-Mitchell & Sandine, 1984), which correlates with a lower proteolytic capacity of *S. thermophilus* compared to *Lb. bulgaricus* (Hamdy *et al.*, 1955; Hickey *et al.*, 1983; Rajagopal & Sandine, 1990; Shankar & Davies, 1978).

In the present study, we wished to determine the role of the cell-wall proteinases PrtS from *S. thermophilus* and PrtB from *Lb. bulgaricus* in bacterial growth in milk. We therefore constructed a negative mutant for the cell-wall proteinase gene (*prtS* gene) of *S. thermophilus* CNRZ 385, which was recently sequenced and characterized in our laboratory (Fernandez-Esplá *et al.*, 2000). The latter mutant was used to study the role of PrtS on the growth of *S. thermophilus* in pure culture in milk. We also took advantage of the availability of a PrtB-negative mutant of *Lb. bulgaricus* (Gilbert *et al.*, 1997) to evaluate the role of cell-wall proteinases PrtS and PrtB on growth and thus on acidification in *S. thermophilus*/*Lb. bulgaricus* mixed cultures.

## METHODS

**Plasmids, bacterial strains, culture conditions and bacterial enumeration.** The bacterial strains and plasmids used for this study are presented in Table 1.

Strains of *S. thermophilus* and *Lb. bulgaricus* were grown in three different media: reconstituted skim milk (Nilac Low Heat Milk powder, NIZO) heated for 10 min at 95 °C, supplemented with yeast extract (3 g l<sup>-1</sup>; Difco) when required, M17 medium (Terzaghi & Sandine, 1975) supplemented with 20 g lactose l<sup>-1</sup>, and MRS medium (De Man *et al.*, 1960) supplemented with 20 g lactose g l<sup>-1</sup> and acidified at

pH 5.2, supplemented with streptomycin (Sigma) (2 mg ml<sup>-1</sup>) when required. The *Escherichia coli* strain was grown at 37 °C on Luria-Bertani (Difco) medium (Sambrook *et al.*, 1989) with shaking, in the presence of erythromycin (Ery) (150 µg ml<sup>-1</sup>) when required.

Stock cultures of each strain of *S. thermophilus* and *Lb. bulgaricus* were prepared after growth at 42 °C on skim milk, supplemented with yeast extract for proteinase-negative strains, from overnight skim milk cultures, supplemented with yeast extract when required. The pH was then measured, and bacterial numbers were estimated by plating, with an automatic spiral plater (AES Laboratory), appropriate dilutions of the culture on agar medium: M17Lac was used for specific enumeration of *S. thermophilus* cells, and MRSLac pH 5.2, supplemented with streptomycin when required, for specific enumeration of *Lb. bulgaricus* cells. For *S. thermophilus* strains and before dilution, chains of cells were disrupted for 30 s in a mechanical blender (Turax X620, Labo-Moderne). After 48 h incubation at 42 °C in anaerobic jars (Anaerocult A, Merck), cells were enumerated with the EC1 colony counter software (AES Laboratory). At the end of culture, bacteria were directly frozen in liquid nitrogen and kept at -80 °C.

Growth rates of *S. thermophilus* 385 and 385-PrtS strains were determined in M17 at 42 °C using a Microbiology Reader Bioscreen C (Labsystems) in 100-well, sterile, covered microplates. Each well, containing 200 µl M17Lac, was inoculated at 1% with overnight M17Lac cultures of *S. thermophilus* and covered with one drop of paraffin oil. The optical density was measured at 600 nm every 20 min, after gentle shaking. The apparent growth rate ( $\mu_{\max}$ ) was defined as the maximum slope of semi-logarithmic representation of growth curves, assessed by optical density measurements.

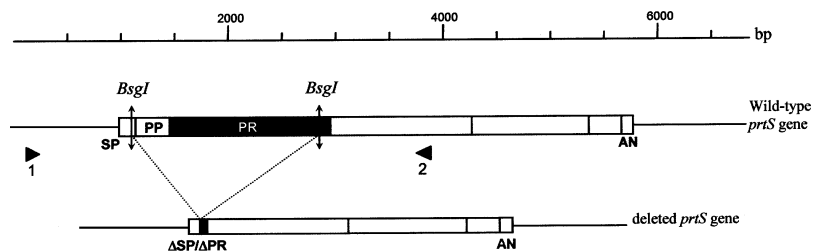
Mixed cultures of *S. thermophilus* and *Lb. bulgaricus* strains were performed at 42 °C by inoculating skim milk with  $5 \times 10^6$  c.f.u. ml<sup>-1</sup> of stock cultures of each strain. For proteinase-negative strains, cells from stock culture were washed three times in 50 mM Tris buffer (pH 7) before inoculation to avoid peptides and/or amino acids being supplied in the mixed culture. Every 20 min, the pH of the culture was measured, and bacteria were enumerated as described above. Total bacterial populations were estimated by addition of data from enumerations of each bacterial species on specific medium to the others, as indicated above.

**Proteinase assay.** The PrtS proteinase phenotype of *S. thermophilus* strains was determined on bacterial colonies in two ways. First, bacteria were grown on FSDA medium (Fast Slow Difference Agar) (Huggins & Sandine, 1984). This milk-based agar medium made it possible to differentiate bacteria exhibiting slow or limited growth in milk from those exhibiting rapid growth; in particular, bacteria possessing a cell-wall proteinase activity appeared as white, opaque, rounded colonies, whereas proteinase-negative colonies were small, flat and translucent. Second, bacteria from an overnight skim milk culture were diluted and plated on agar skim milk plates (cell culture dishes, 35 mm in diameter). After 24–48 h incubation at 42 °C in anaerobic jars, colonies were covered by a solution containing Tris buffer (50 mM, pH 7), a chromogenic substrate of proteinase PrtS (Suc-Ala-Ala-Pro-Phe-βNA, 10 mg ml<sup>-1</sup>; Novabiochem), 10 mg ml<sup>-1</sup> Fast-Garnet (GBC, Sigma) and 10–50 mM CaCl<sub>2</sub>. PrtS-positive clones appeared as red colonies, whereas PrtS-negative clones remained white.

Proteinase activity was measured on cellular suspensions using [<sup>14</sup>C]casein as the substrate according to the method of Monnet *et al.* (1987), modified as follows. Cell suspensions

**Table 1.** Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
<b>Strains</b>		
<i>S. thermophilus</i> CNRZ 302	Protease-negative (PrtS <sup>-</sup> )	Shahbal <i>et al.</i> (1991)
<i>S. thermophilus</i> CNRZ 385	Protease-positive (PrtS <sup>+</sup> )	Shahbal <i>et al.</i> (1991)
<i>S. thermophilus</i> CNRZ 703	Protease-positive (PrtS <sup>+</sup> )	Shahbal <i>et al.</i> (1991)
<i>S. thermophilus</i> CNRZ 385-PrtS	Protease-negative (PrtS <sup>-</sup> ) mutant of CNRZ 385	This work
<i>Lb. bulgaricus</i> CNRZ 397	Protease-positive (PrtB <sup>+</sup> )	Laloi <i>et al.</i> (1991)
<i>Lb. bulgaricus</i> 1159 = 397-PrtB	Protease-negative (PrtB <sup>-</sup> ) mutant of CNRZ 397	Gilbert <i>et al.</i> (1997)
<i>Lb. bulgaricus</i> 1038	Protease-positive (PrtB <sup>+</sup> ), streptomycin-resistant mutant of CNRZ 208 (ATCC11842)	M. Van de Guchte (personal communication)
<i>Lc. lactis</i> MG1363	Plasmid-free derivative of wild-type strain NCDO712	Gasson (1983)
<i>E. coli</i> TOP10	<i>recA1</i>	Invitrogen
<b>Plasmids</b>		
pCR-XL-TOPO	TOPO-Cloning site; Kan <sup>R</sup>	Invitrogen
pCR-XL-TOPO- $\Delta$ <i>prtS</i> -1	3.776 kb fragment <i>prtS</i> in pCR-XL-TOPO; Kan <sup>R</sup>	This work
pCR-XL-TOPO- $\Delta$ <i>prtS</i> -2	Blunt-ended and circularized 5.4 kb <i>Bgl</i> I fragment of pCR-XL-TOPO- $\Delta$ <i>prtS</i> -1; Kan <sup>R</sup>	This work
pG + host9	Ery <sup>R</sup>	Maguin <i>et al.</i> (1996)
pG + h9:: $\Delta$ <i>prtS</i>	2.078 kb <i>Not</i> I and <i>Spe</i> I fragment of pCR-XL-TOPO- $\Delta$ <i>prtS</i> -2 ligated to pG + host9; Ery <sup>R</sup>	This work

**Fig. 1.** Construction of the PrtS-negative mutant of *S. thermophilus* CNRZ 385: structure of the deleted *prtS* gene compared to the wild-type *prtS* gene. AN, cell-wall anchor; PP, propeptide; PR, catalytic domain; SP, signal peptide. ►◄, oligonucleotides 1 and 2 used for first PCR amplification.

were prepared from 4 ml overnight M17 cultures; cells were recovered by centrifugation (20 min, 8000 g, 4 °C) and washed three times in Tris buffer (50 mM, pH 7). The last pellet was suspended in 150 µl Bistris buffer (50 mM, pH 6.5) containing 10 mM CaCl<sub>2</sub>. Fifty microlitres of cell suspension was incubated with 50 µl of <sup>14</sup>C casein solution (0.1%) at 37 °C for 15, 60 and 120 min. Enzyme reactions were stopped by the addition of 100 µl TCA (12%), left for 30 min at room temperature and centrifuged for 2 min at 10000 g, and the radioactivity was then measured in the supernatants. Protease activity corresponded to the percentage of casein hydrolysis in 10 min.

#### DNA manipulations and sequencing

**Total DNA preparation.** Total DNA of *S. thermophilus* CNRZ 385 was prepared as described by Pospiech & Neumann (1995).

**Preparation of electrocompetent cells of *S. thermophilus* and *Lc. lactis*.** Electrocompetent cells of *S. thermophilus* CNRZ 385 and *Lc. lactis* MG1363 were prepared according to the method of Holo & Nes (1989), modified as follows. From an overnight culture in M17Lac, a culture was performed at 37 °C (*S. thermophilus*) or at 30 °C (*Lc. lactis*) by 1% inoculation of M17Lac containing DL-Thr (100 mM) for *S. thermophilus* or Gly (1.5%) for *Lc. lactis* until the OD<sub>600</sub> reached 0.6–1. Cells were collected by centrifugation at 5000 g for 5 min and washed four times in 0.5 M sucrose/10% glycerol solution.

They were then resuspended in 10% glycerol/30% PEG2000 solution for *S. thermophilus* or in 0.5 M sucrose/10% glycerol solution for *Lc. lactis* and immediately frozen in liquid N<sub>2</sub> and stored at –80 °C.

**DNA sequencing.** The Sanger method of DNA sequencing was carried out on double-strand DNA plasmids and on PCR products with the BigDye Terminator cycle sequencing ready reaction kit (370A DNA sequencer, Applied Biosystems).

**Construction of a negative mutant for PrtS.** A 3776 bp PCR product containing part of the *prtS* gene was amplified using oligonucleotides 1 (5' CAT CAC GGA AAG TCT AGG 3') and 2 (5' AAC GTA TTG ATA CTT ACC 3') from total DNA of *S. thermophilus* CNRZ 385 strain (Fig. 1). Streptococcal DNA (100 ng) was added to a PCR mixture containing 2.5 U of *Taq* polymerase (Quantum Appligene) and 0.26 µM of each oligonucleotide (Life Technology). After 5 min of denaturation at 94 °C, 30 cycles of 30 s annealing at 50 °C and 3 min of elongation at 72 °C were carried out using a Perkin-Elmer DNA thermal cycler (model 480). The amplified fragment was purified from 0.7% agarose gel with the QIAquick gel-extraction kit (Qiagen). It was then ligated to pCR-XL-TOPO vector (Invitrogen) and cloned by transformation of electrocompetent TOP10 *E. coli* cells (Invitrogen) according to the manufacturer's protocol. The recombinant vector, pCR-XL-TOPO- $\Delta$ *prtS*-1, was purified with QIAprep Spin Miniprep Kit (Qiagen) from the recombinant cells and digested with *Bgl*I (New England Biolabs). A 5.4 kb fragment containing the

TopoXL vector and part of the *prtS* gene was then purified from 0.7% agarose gel with QIAquick gel extraction kit (Qiagen) and blunt-ended with T4 polymerase 3'→5' exonuclease (Life Technologies) according to the supplier's protocol. It was then circularized by self-ligation with Fast-link DNA ligation kit (Epicentre Technology); the resulting plasmid, pCR®-XL-TOPO-Δ*prtS*-2, was produced by transformation of electrocompetent TOP10 *E. coli* cells and purified as described above. It was then digested with *NotI* and *SpeI* (Eurogentec), and the resulting 2.078 kb fragment was purified as already described above. The 2.078 kb fragment (~200 ng) was ligated to pGhost9 vector (~100 ng) (Maguin *et al.*, 1996), digested with *NotI* and *SpeI*. The ligation mix was used to electrotransform 100 µl of electrocompetent cells of *Lc. lactis* MG1363, as described by Holo & Nes (1989). Recombinant clones were selected on M17Lac Ery plates after incubation at 28 °C. The recombinant vector, pG<sup>+</sup>h9::Δ*prtS*, was purified as described above, and 20 µg was used to transform electrocompetent cells of *S. thermophilus* CNRZ 385, as previously described (Garault *et al.*, 2000). Integration of pG<sup>+</sup>h9::Δ*prtS* into the streptococcal chromosome was performed as described by Garault *et al.* (2000) with the following modification: to induce chromosomal integration of the plasmid, the culture was diluted and plated on M17Lac Ery plates. Finally, the mutant for PrtS was obtained by successive incubations of the culture containing the chromosomal integration at 37 °C to favour the excision of the pGhost9 plasmid.

## RESULTS

### PrtS is essential to *S. thermophilus* growth in milk

***S. thermophilus* PrtS-negative mutant construction.** We have described here for the first time the construction of a targeted negative mutant for *S. thermophilus* cell-wall proteinase PrtS. This mutant of *S. thermophilus* CNRZ 385 was constructed by gene replacement using a truncated copy of *prtS* gene cloned in pGhost9 plasmid. DNA sequencing confirmed that this copy was inserted at the *prtS* locus and that pGhost9 was subsequently excised, resulting in a truncated *prtS* gene. As expected, the truncated gene was deprived of part of the signal sequence, all the pro-region (removed after maturation of the protein in the parental strain), and almost all of the region encoding the catalytic domain of the enzyme (Fig. 1). Only the region encoding the six C-terminal amino acids among the 495 constituting the catalytic domain (PR domain) was still present in the mutant and did not include the sequence encoding the residues involved in the catalytic activity of the proteinase (Fernandez-Espla *et al.*, 2000). Furthermore, protein exportation signals were no longer present in the mutant; the signal sequence was truncated, and the expected peptide cleavage site was excised.

**Cell-wall proteinase activity of the wild-type and PrtS-negative mutant of *S. thermophilus*.** Using two different methods, we checked that the *S. thermophilus* PrtS-negative mutant lacked cell-wall proteinase activity. First, using <sup>14</sup>C-labelled casein as a substrate, we observed that cell suspensions of the PrtS-positive strain were capable of hydrolysing casein (12.5% of total casein hydrolysed within 10 min), whereas PrtS-negative cells had no detectable caseinolytic activity. Second, we

set up a rapid test on colonies using a chromogenic substrate of PrtS. Three strains of *S. thermophilus* were used: the proteinase-negative strain CNRZ 302 as negative control and the two proteinase-positive strains CNRZ 385 and CNRZ 703, which have a high cell-wall proteinase activity (Shahbal *et al.*, 1991, 1993). After growing on milk agar plates, colonies were covered with a solution containing the substrate Suc-A-A-P-F-βNA, Fast-Garnet and different concentrations of CaCl<sub>2</sub>, the latter being an activator of PrtS proteinase (Fernandez-Espla *et al.*, 2000). Whatever the CaCl<sub>2</sub> concentration (10, 20 or 50 mM), colonies of strains 703 and 385 rapidly became red, whereas those of the negative strain 302 remained white. Using this test, we confirmed that the mutant strain was PrtS-negative, as colonies remained white even after several hours of contact with the substrate solution. This test functioned on milk plates but not on rich medium M17 plates for strain 703, which confirmed a probable regulation of *prtS* expression by the growth medium as already observed for this strain (Shahbal *et al.*, 1993). This test will be useful to screen for *S. thermophilus* PrtS-negative strains in milk and also for PrtS-deregulated strains in M17.

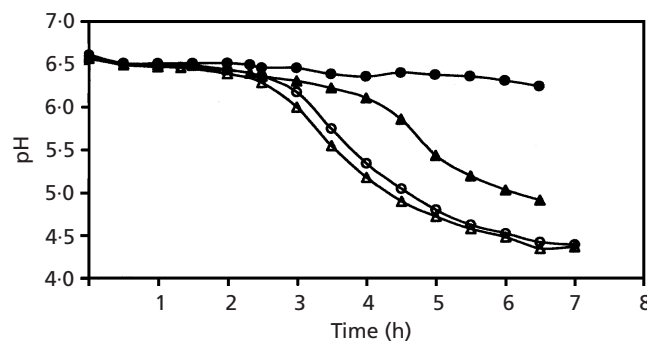
**Growth characteristics of the wild-type and PrtS-negative mutant of *S. thermophilus*.** By comparing the phenotypes of the parental strain 385 and its PrtS<sup>-</sup> mutant on FSDA, and their growth curves in liquid M17 and milk, we showed that proteinase PrtS was essential to the growth of *S. thermophilus* in milk.

The PrtS<sup>-</sup> mutant, plated on FSDA, appeared as flat and translucent colonies, as expected for PrtS<sup>-</sup> bacteria, whereas the PrtS<sup>+</sup> parental strain appeared as white, opaque, rounded colonies.

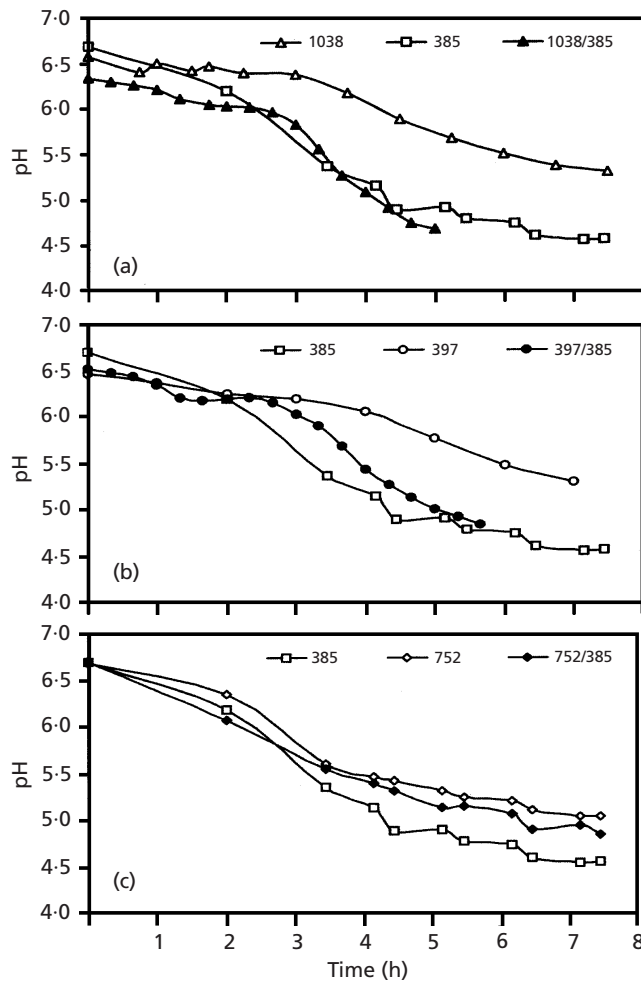
In M17, both strains had similar growth curves with a  $\mu_{\max}$  of 0.89 and 0.85 h<sup>-1</sup> for the parental strain and the mutant strain, respectively. In milk, streptococcal growth was determined indirectly by pH measurement. Growth of the PrtS<sup>-</sup> mutant was severely impaired in milk, as indicated by the reduced acidification of milk by this strain compared to the parental strain 385 (Fig. 2). For the PrtS<sup>-</sup> strain, milk acidification, and thus bacterial growth, was restored to the same extent as that for the wild-type strain, after the addition of yeast extract to milk (Fig. 2).

### The *Lactobacillus* strain influences the extent of the positive effect of *S. thermophilus*/Lb. *bulgaricus* association

Mixed cultures of *S. thermophilus* and *Lb. bulgaricus* were made using two different strains of *Lb. bulgaricus*. To choose the last two strains, we first determined the effect of the co-culture of *Lb. bulgaricus* strains with the *S. thermophilus* CNRZ385 strain on milk acidification, compared to the pure culture of *Lb. bulgaricus* (Fig. 3). Among the three strains of lactobacilli tested, the effect of adding strain 385 on the acidification was greatest with strains *Lb. bulgaricus* 397 and 1038; indeed, for these two *Lactobacillus* strains, the addition of the *Streptococcus* highly enhanced the acidification rate



**Fig. 2.** Acidification curves of *PrtS*<sup>+</sup> *S. thermophilus* strain 385 (▲, △) and its *PrtS*<sup>-</sup> mutant grown in milk (●, ○), in the presence (○, △) or absence (●, ▲) of yeast extract.



**Fig. 3.** Milk acidification curves of *S. thermophilus* 385, and *Lb. bulgaricus* (1038) (a), 397 (b) and 752 (c) in the presence or absence of *S. thermophilus* 385.

compared to the *Lb. bulgaricus* strain alone (Fig. 3a, b). Furthermore, the positive effect of the bacterial association on milk acidification was more intense for strain 1038, as, for this strain, the acidification rate and the final pH were higher and lower, respectively, in the mixed culture than in the pure culture. In contrast, addition of *S. thermophilus* strain 385 had no significant effect on milk acidification by *Lb. bulgaricus* strain 752

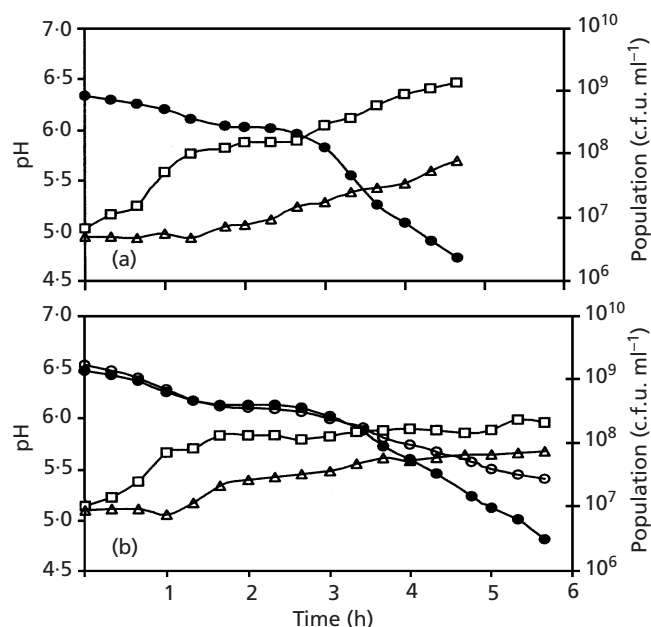
(Fig. 3c). Thus, strains 397 and 1038 of *Lactobacillus* were kept for the following study. In addition, a proteinase-negative mutant of strain *Lb. bulgaricus* CNRZ 397 was available and was used for the following experiments.

#### In mixed cultures, proteinase *PrtS* has no effect on final pH and bacterial populations, but *PrtB* affects both

The effect of proteinases *PrtS* and *PrtB* on acidification of mixed cultures and bacterial populations was estimated by measuring the final pHs, final total bacterial populations and final individual populations of cultures performed with a strain of *S. thermophilus* *PrtS*<sup>+</sup> (strain 385) or *PrtS*<sup>-</sup> (strain 385-*PrtS*) and a strain of *Lb. bulgaricus* *PrtB*<sup>+</sup> (strains 397 and 1038) or *PrtB*<sup>-</sup> (strain 397-*PrtB*).

The presence of proteinase *PrtS* had no effect either on the final bacterial populations or on the final pHs of mixed cultures involving *PrtB*<sup>+</sup> *Lactobacillus* strains. The final total populations were always similar in the presence or not of proteinase *PrtS*:  $1.39$  and  $1.36 \times 10^9$  c.f.u. ml<sup>-1</sup>, respectively, for cultures involving *Lb. bulgaricus* strain 1038, and  $7.05$  and  $6.27 \times 10^8$  c.f.u. ml<sup>-1</sup>, respectively, with strain 397. The absence of any differences in final total populations corresponded to similar final individual populations of *S. thermophilus* and *Lb. bulgaricus*, regardless of the presence of *PrtS*:  $1.3 \times 10^9$  c.f.u. ml<sup>-1</sup> for strains 385 and 385-*PrtS* and  $8.9 \times 10^7$  c.f.u. ml<sup>-1</sup> for strain 1038 for mixed cultures involving strain 1038,  $5.5 \times 10^8$  c.f.u. ml<sup>-1</sup> for strains 385 and 385-*PrtS* and  $1.1 \times 10^8$  c.f.u. ml<sup>-1</sup> for strain 397 in mixed cultures involving strain 397. This correlated well with the similar final pH obtained: 4.72 and 4.85 for mixed cultures involving, respectively, strain 1038 and strain 397.

It is noteworthy that both the final total bacterial populations and the acidification rates varied according to the *Lactobacillus* strain associated with *S. thermophilus* strain 385. The final total population when using *Lactobacillus* strain 1038 ( $1.38 \times 10^9$  c.f.u. ml<sup>-1</sup>) was twice as high as that of strain 397 ( $6.67 \times 10^8$  c.f.u. ml<sup>-1</sup>), because the *Streptococcus* populations were more than twice as high with strain 1038, *Lactobacillus* populations remaining constant. Final pHs were not significantly different, but the time required to reach these pHs was



**Fig. 4.** Variation of pH and specific bacterial populations during mixed cultures in milk of *S. thermophilus* and *Lb. bulgaricus*. (a) Mixed culture 385/1038: ●, pH; △, population of 1038; □, population of 385. (b) Mixed cultures 385/397 and 385/397-PrtB: ●, pH of culture 385/397; ○, pH of culture 385/397-PrtB; □, population of 385 in mixed culture 385/397-PrtB; △, population of 397-PrtB in mixed culture 385/397-PrtB.

shorter for mixed cultures, including strain 1038, than those including strain 397 (4.66 h with strain 1038 versus 5.66 h with strain 397).

In contrast, the presence of proteinase PrtB affected both the final bacterial populations and the final pHs. Final total bacterial populations were threefold higher in the presence of PrtB than in its absence ( $7.05 \times 10^8$  versus  $2.8 \times 10^8$  c.f.u. ml<sup>-1</sup>). This resulted from higher final populations of *S. thermophilus* in the presence of PrtB ( $6.1 \times 10^8$  versus  $2.06 \times 10^8$  c.f.u. ml<sup>-1</sup>) and led to a significantly better acidification in the presence of PrtB (final pH 4.86 versus 5.42).

In our conditions of inoculation (*Streptococcus/Lactobacillus* ratio of 1:1), *S. thermophilus* was systematically predominant in the total final populations, regardless of the strain of *Lactobacillus* and the presence of proteinases PrtS and PrtB. The magnitude of this predominance depended on the *Lb. bulgaricus* strain used: with strain 1038, *S. thermophilus* populations were 15-fold higher than *Lactobacillus* populations and fivefold higher with strain 397, when PrtB was present. This predominance was less marked in the absence of PrtB, as the *S. thermophilus* populations were threefold lower ( $6.1 \times 10^8$  c.f.u. ml<sup>-1</sup>) than populations reached in the presence of PrtB ( $2.06 \times 10^8$  c.f.u. ml<sup>-1</sup>).

#### Variation of individual populations of *S. thermophilus* and *Lb. bulgaricus* throughout mixed cultures in milk

Proteinase PrtS had no significant effect on the variation of pH and of individual populations throughout the culture and, regardless of the mixed culture considered (except that involving strain PrtB<sup>-</sup>), the variation of these two parameters remained similar. Fig. 4a gives an example of this variation (a mixed culture made of *S. thermophilus* 385 and *Lb. bulgaricus* 1038); for mixed

cultures including *Lb. bulgaricus* strain 397, we observed the same behaviour (data not shown). During the first 60–90 min, which corresponded to the first acidification phase, *S. thermophilus* grew exponentially, whereas *Lb. bulgaricus* did not grow significantly. Then, as the pH remained constant, the streptococcal population stabilized for about 60–90 min, whereas *Lb. bulgaricus* started to grow regularly and continuously. Finally, during the last 2 or 3 h of fermentation, when the acidification rate was the highest, both the *Lactobacillus* and the *Streptococcus* grew regularly.

In contrast, proteinase PrtB was clearly involved in the variation of bacterial populations and of pH, as demonstrated with mixed cultures involving strain 397-PrtB (Fig. 4b). In fact, regardless of the presence of PrtB, the first two phases of acidification corresponding to the sequential growth of *S. thermophilus* and *Lb. bulgaricus* were similar. However, during the third acidification phase, the growth of *S. thermophilus* slowed down in the absence of PrtB, the bacterial populations remaining almost constant during the last 2 h of fermentation. This reduced growth resulted in a reduced acidification rate and an increased final pH (pH 5.42 in the absence of PrtB and 4.86 in the presence of PrtB).

#### DISCUSSION

The present work aimed at evaluating the role of proteinase PrtS from *S. thermophilus* in the growth in milk of *S. thermophilus* in a pure culture. We also determined the effect of the presence of both PrtS and PrtB from *Lb. bulgaricus* on *S. thermophilus/Lb. bulgaricus* mixed cultures. For this purpose, we constructed a targeted negative mutant of proteinase PrtS from *S. thermophilus* CNRZ 385 and performed pure cultures of *S. thermophilus* and mixed cultures with *Lb. bulgaricus* in milk.

### In milk, the extent of the beneficial effect of the *S. thermophilus*/*Lb. bulgaricus* association varies

We observed that the effect of the co-culture of *Lb. bulgaricus* strains with *S. thermophilus* strain 385 on the acidification of milk, and thus the benefit of the bacterial association, depends on the strain of *Lb. bulgaricus* used. In fact, with *Lb. bulgaricus* strain 752, we did not obtain a marked beneficial effect of the association with *S. thermophilus* 385 as already observed by several authors with other strains (Accolas *et al.*, 1977; Bautista *et al.*, 1966; Sodini *et al.*, 2000). In contrast, mixed cultures of strains 1038 and 397 resulted in a higher acidification than pure cultures. Acidification was higher with strain 1038 than with strain 397 due to higher *S. thermophilus* populations, *Lb. bulgaricus* populations being similar. These higher *S. thermophilus* populations probably resulted from a better peptide and/or amino acid supply by one *Lactobacillus* strain compared to the other as these nitrogen compounds are growth-limiting for *S. thermophilus* in milk. The two strains of *Lb. bulgaricus* thus probably differ in their proteolytic potential, which is in agreement with the differences observed in the final quantities of free amino acids and free NH<sub>2</sub> groups in the supernatants of mixed cultures including these two strains (data not shown). Some authors have also reported a variability in the *Lb. bulgaricus* proteolytic potential (El-Soda *et al.*, 1986; Rajagopal & Sandine, 1990; Singh & Sharma, 1983). This variability could be related to the presence of one cell-wall proteinase in *Lb. bulgaricus*, which is the case of strain 397 (Gilbert *et al.*, 1997), or of two proteinases, as reported for other strains (Pederson *et al.*, 1999; Stefanisti *et al.*, 1995).

### PrtS is essential to the growth of *S. thermophilus* in milk in pure culture but not in mixed culture

Proteinase PrtS is essential to the growth of *S. thermophilus* in milk as its PrtS<sup>-</sup> mutant was unable to grow efficiently in milk until a nutritional complement [yeast extract or bactotryptone (data not shown)] was added. This indicated that proteinase PrtS was involved in nitrogen supply to the cell, via casein hydrolysis, which is consistent with data previously obtained with cell-wall proteinases of other lactic acid bacteria (Exterkate, 1990; Gilbert *et al.*, 1997; Thomas & Pritchard, 1987).

However, we demonstrated here that proteinase PrtS had no significant effect on the growth of *S. thermophilus* in mixed cultures in milk with *Lb. bulgaricus*; the growth of the parental strain 385 and of the PrtS<sup>-</sup> mutant in mixed culture was similar when *Lb. bulgaricus* proteinase PrtB was present. This indicates that assimilable nitrogen compounds necessary for *S. thermophilus* growth are supplied by PrtB, as confirmed by the fact that the absence of PrtB led to lower streptococcal populations. Furthermore, as the streptococcal population was higher in the presence of PrtB than in the presence of PrtS, we can assume that PrtB is more efficient in the supply of peptides to *S. thermophilus*

than PrtS. This can be explained by a more active proteinase PrtB compared to PrtS, as previous studies reported that the global proteolytic activities of *Lb. bulgaricus* strains were 25–70 times higher than that of *S. thermophilus* strains (Rajagopal & Sandine, 1990; Shankar & Davies, 1978). We cannot rule out the possibility that PrtS and PrtB have different substrate specificity, which leads to the production of different peptides, some being more assimilable than others. Indeed, PrtS is capable of hydrolysing MS-Arg-Pro-Tyr-pNA (Fernandez-Espla *et al.*, 2000), a substrate also hydrolysed by lactococcal proteinase PrtP (Exterkate, 1990) but not by PrtB (Laloi *et al.*, 1991). Furthermore, when comparing the substrate-binding region of proteinases PrtS and PrtB, in particular the residues 138, 166, 748, which have been identified as being directly involved in substrate specificity in lactococci (Siezen *et al.*, 1993), we noticed that they are totally different in PrtS (Thr, Ala, Asp) (Fernandez-Espla *et al.*, 2000) and PrtB (Gly, Val, Thr) (Gilbert *et al.*, 1996).

### Model of growth of *S. thermophilus* associated with *Lb. bulgaricus* and effect on acidification

In all the mixed cultures performed in milk, we observed the sequential development of *S. thermophilus* and then of *Lb. bulgaricus*, which is in agreement with previous studies (Beal & Corrieu, 1991; Pette & Lolkema, 1950a; Puhon & Banhegyi, 1974; Tamine & Robinson, 1999). Recently, the growth of *S. thermophilus* in pure culture in milk has been characterized, in particular with regard to nitrogen nutrition (Letort *et al.*, 2002); it consists of two exponential growth phases, interrupted by a non-exponential growth phase. From these latter results and those of the present work, we propose the following model of growth of *S. thermophilus* in mixed cultures with *Lb. bulgaricus* with three *S. thermophilus* growth phases corresponding to three acidification steps.

During the first acidification step, characterized by a small decrease in pH (<0.5 pH units), *S. thermophilus* grows exponentially, whereas *Lb. bulgaricus* does not grow; *S. thermophilus* is thus responsible for this first acidification, as first observed by Pette & Lolkema (1950c). The preferential growth of *S. thermophilus* can be explained first by the fact that *S. thermophilus* has fewer nutritional requirements than lactobacilli in milk (Desmazeaud, 1983). In particular, *S. thermophilus* requires few amino acids and is capable of synthesizing branched-chain amino acids (Garault *et al.*, 2000); its growth can probably be supported by free amino acids and peptides present in milk, as previously demonstrated in pure culture, regardless of the presence of PrtS (Letort *et al.*, 2002). In contrast, *Lb. bulgaricus* is much more demanding from a nutritional point of view than *S. thermophilus* (Letort, 2001); its optimal growth relies on the supply of essential factors (CO<sub>2</sub>, pyruvate, formate) produced by *S. thermophilus* (for reviews, see Tamine & Robinson, 1999; Zourari *et al.*, 1992). Second, in our study, mixed cultures were performed at



42 °C, a temperature more favourable for *S. thermophilus*, whose optimal growth temperature ranges between 40 and 45 °C, versus 45–50 °C for *Lb. bulgaricus*.

Then, the *S. thermophilus* exponential growth pauses and, concomitantly, the acidification, while *Lb. bulgaricus* begins to grow slowly and regularly until the end of fermentation. This pause probably corresponds to depletion of amino acids and peptides in milk, due to their consumption by *S. thermophilus*, as shown recently by Letort *et al.* (2002) in pure culture, and the absence of compensatory production by cell-wall proteinases. These authors actually demonstrated that proteinase PrtS synthesis starts in the middle of this phase and is maximal during the second exponential growth phase in pure culture. Concerning the growth of *Lb. bulgaricus*, we assume that as *S. thermophilus* reaches a high cellular density during its first growth phase, it probably produces enough growth-stimulating factors to favour the growth of *Lb. bulgaricus*.

Finally, during the following acidification phase, which leads to a high pH decrease (about 1.5 pH units), *Lb. bulgaricus* continues to grow; at the same time, *S. thermophilus* starts a second exponential growth phase. We suggest that this acidification results not only from the growth of *Lb. bulgaricus* but also from that of *S. thermophilus*. This acidification phase is indeed greatly improved by the addition of *S. thermophilus* to a *Lb. bulgaricus* culture; furthermore, in the absence of PrtB, acidification is reduced, while only *S. thermophilus* populations significantly decrease. The growth of *S. thermophilus* probably occurs because of the utilization of peptides produced by PrtS (when PrtB is absent) but also mainly by PrtB. No differences in the growth of *S. thermophilus* were observed in the presence or absence of PrtS when PrtB was present, and *S. thermophilus* populations were significantly reduced in the absence of PrtB, i.e. when PrtS was the sole source of peptide production.

In conclusion, we have determined the role of cell-wall proteinases PrtS and PrtB in the growth of *S. thermophilus* and *Lb. bulgaricus* in mixed cultures. We have shown that PrtB is involved in the optimal growth of *S. thermophilus*, whereas PrtS does not play a significant role when PrtB is present. Studies of the effect of these proteinases on the free amino acid and peptide contents as well as on the aroma profiles of mixed cultures are in progress. As precursors, amino acids are involved in the formation of aroma in dairy products, and variations in their composition can affect aroma development. However, the different pH values observed in the present study at the end of fermentation, when varying the presence of proteinase PrtB, can modify the yoghurt flavour (Ott *et al.*, 2000).

## ACKNOWLEDGEMENTS

We gratefully acknowledge D. Atlan and M. van de Guchte for the supply of *Lb. bulgaricus* strains 1159 and 1038, respectively. We thank M. Nardi, for her helpful suggestions

concerning mutant construction, and M. Diard for his technical assistance.

## REFERENCES

- Accolas, J. P., Veaux, M. & Auclair, J. (1971). Etude des interactions entre diverses bactéries lactiques thermophiles et mésophiles, en relation avec la fabrication des fromages à pâte cuite. *Lait* 57, 1–23.
- Accolas, J. P., Bloquel, R., Didienné, R. & Regnier, J. (1977). Propriétés acidifiantes des bactéries lactiques thermophiles en relation avec la fabrication du yoghourt. *Lait* 57, 249–272.
- Bautista, E. S., Dahiya, R. S. & Speck, M. L. (1966). Identification of compounds causing symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in milk. *J Dairy Res* 33, 299–307.
- Beal, C. & Corrieu, G. (1991). Influence of pH, temperature, and inoculum composition on mixed cultures of *Streptococcus thermophilus* 404 and *Lactobacillus* 398. *Biotechnol Bioeng* 38, 90–98.
- Bracquart, P. & Lorient, D. (1977). Effet des acides aminés sur la croissance de *Streptococcus thermophilus*. *Milchwissenschaft* 32, 221–224.
- De Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol* 23, 130–135.
- Desmazeaud, M. (1983). L'état des connaissances en matière de nutrition des bactéries lactiques. *Lait* 63, 267–316.
- El-Soda, M. A., Desmazeaud, M. J., Le Bars, D. & Zevaco, C. (1986). Cell-wall-associated proteinases in *Lactobacillus casei* and *Lactobacillus plantarum*. *J Food Prot* 49, 361–365.
- Exterkate, F. A. (1990). Differences in short peptide-substrate cleavage by two cell-envelope-located serine proteinases of *Lactococcus lactis* subsp. *cremoris* are related to secondary binding specificity. *Appl Microbiol Biotechnol* 33, 401–406.
- Fernandez-Espla, M.-D., Garault, P., Monnet, V. & Rul, F. (2000). *Streptococcus thermophilus* cell wall-anchored proteinase, release, purification, and biochemical and genetic characterization. *Appl Environ Microbiol* 66, 4772–4778.
- Garault, P., Letort, C., Juillard, V. & Monnet, V. (2000). Branched-chain amino acid biosynthesis is essential for optimal growth of *Streptococcus thermophilus* in milk. *Appl Environ Microbiol* 66, 5128–5133.
- Gasson, M. J. (1983). Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* 154, 1–9.
- Gilbert, C., Atlan, D., Blanc, B., Portalier, R., Germond, J. E., Lapierre, L. & Mollet, B. (1996). A new cell surface proteinase, sequencing and analysis of the *prtB* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *J Bacteriol* 178, 3059–3065.
- Gilbert, C., Blanc, B., Frot-Coutaz, J., Portalier, R. & Atlan, D. (1997). Comparison of cell surface proteinase activities within the *Lactobacillus* genus. *J Dairy Res* 64, 561–571.
- Hamdy, M. K., Harper, W. J. & Weisier, H. H. (1955). Acidic free amino compounds formed in various lactic acid starter cultures as measured by ion exchange chromatography. *Appl Microbiol* 3, 221–226.
- Hebert, E. M., Raya, R. R. & De Giori, G. S. (2000). Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. *Appl Environ Microbiol* 66, 5316–5321.
- Hickey, M. W., Hillier, A. J. & Jago, G. R. (1983). Peptidase activities in lactobacilli. *Aust J Dairy Technol* 38, 118–123.



- Higashio, K., Yoshioka, Y. & Kikuchi, T. (1977). Isolation and identification of growth factor of *Streptococcus thermophilus* by *Lactobacillus bulgaricus*. *J Agric Chem Soc Jpn* 51, 203–208.
- Holo, H. & Nes, I. F. (1989). High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol* 55, 3119–3123.
- Huggins, A. M. & Sandine, W. E. (1984). Differentiation of fast and slow milk coagulating isolates in strains of streptococci. *J Dairy Sci* 67, 1674–1679.
- Laloi, P., Atlan, D., Blanc, B., Gilbert, C. & Portalier, R. (1991). Cell-wall-associated proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397, differential extraction, purification and properties of the enzyme. *Appl Microbiol Biotechnol* 36, 196–204.
- Ledesma, O. V., de Ruiz Holgado, A. P., Oliver, G., de Giorgi, G. S., Raibaud, P. & Galpin, J. V. (1977). A synthetic medium for comparative nutritional studies of lactobacilli. *J Appl Bacteriol* 42, 123–133.
- Letort, C. (2001). *Relation entre croissance et nutrition azotée de deux bactéries lactiques thermophiles, Streptococcus thermophilus et Lactobacillus delbrueckii subsp. bulgaricus*. PhD thesis, University of Poitiers, France.
- Letort, C. & Juillard, V. (2001). Development of a minimal chemical defined medium for the exponential growth of *Streptococcus thermophilus*. *J Appl Microbiol* 91, 1–7.
- Letort, C., Nardi, M., Garault, P., Monnet, V. & Juillard, V. (2002). Casein utilization by *Streptococcus thermophilus* results in a diauxic growth in milk. *Appl Environ Microbiol* 68, 3162–3165.
- Maguin, E., Prevost, H., Ehrlich, D. & Gruss, A. (1996). Efficient insertional mutagenesis in lactococci and other Gram-positive bacteria. *J Bacteriol* 178, 931–935.
- Monnet, V., Le Bars, D. & Gripon, J. C. (1987). Partial characterization and comparison of cell wall proteinases from 5 strains of *Streptococcus lactis*. *Lait* 67, 51–61.
- Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T. & Yura, T. (1981). Multiple nutritional requirements of lactobacilli, genetic lesions affecting amino acid biosynthetic pathways. *J Bacteriol* 148, 64–71.
- Ott, A., Hugli, A., Baumgartner, M. & Chaintreau, A. (2000). Sensory investigation of yogurt flavour perception, mutual influence of volatiles and acidity. *J Agric Food Chem* 48, 441–450.
- Pederson, J. A., Mileski, G. J., Weimer, B. C. & Steele, J. L. (1999). Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. *J Bacteriol* 181, 4592–4597.
- Pette, J. W. & Lolkema, H. (1950a). Symbiosis and antibiosis in mixed cultures *Lb. bulgaricus* and *S. thermophilus*. *Neth Milk Dairy J* 4, 197–208.
- Pette, J. W. & Lolkema, H. (1950b). Growth stimulating factors for *Streptococcus thermophilus*. *Neth Milk Dairy J* 4, 209–224.
- Pette, J. W. & Lolkema, H. (1950c). Acid formation and aroma formation in yoghurt. *Neth Milk Dairy J* 4, 261–273.
- Pospiech, A. & Neumann, B. (1995). A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet* 11, 217–218.
- Puhan, Z. & Banhegyi, M. (1974). Effect of incubation temperature on the ratio of *Lactobacillus bulgaricus* to *Streptococcus thermophilus* in yoghurt. *Schweiz Milchwirtsch Forsch* 3, 9–13.
- Radke-Mitchell, L. & Sandine, W. E. (1984). Associative growth and differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, a review. *J Food Prot* 47, 245–248.
- Rajagopal, S. N. & Sandine, W. E. (1990). Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. *J Dairy Sci* 73, 894–899.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shahbal, S., Hemme, D. & Desmazeaud, M. (1991). High cell wall-associated proteinase activity of some *Streptococcus thermophilus* strains (H-strains) correlated with a high acidification rate in milk. *Lait* 71, 351–357.
- Shahbal, S., Hemme, D. & Renault, P. (1993). Characterization of a cell envelope-associated proteinase activity from *Streptococcus thermophilus* H-strains. *Appl Environ Microbiol* 59, 177–182.
- Shankar, P. A. & Davies, F. L. (1977). Associative bacterial growth in yoghurt starters; initial observations on stimulatory factors. *J Soc Dairy Technol* 30, 31–32.
- Shankar, P. A. & Davies, F. L. (1978). [Conference paper] XX International Dairy Congress, Paris, vol. E, pp. 467–468.
- Siezen, R. J. (1999). Multi-domain, cell-envelope proteinases of lactic acid bacteria. *Antonie van Leeuwenhoek* 76, 139–155.
- Siezen, R. J., Bruinenberg, P. G., Vos, P., Van Allen-Boerrigter, I. J., Nijhuis, M., Alting, A. C., Exterkate, F. A. & De Vos, W. M. (1993). Engineering of the substrate-binding region of the subtilisin-like, cell envelope proteinase of *Lactococcus lactis*. *Protein Eng* 6, 927–937.
- Singh, J. & Sharma, D. K. (1983). Proteolytic breakdown of casein and its fraction by lactic acid bacteria. *Milchwissenschaft* 38, 148–149.
- Sodini, I., Latrille, E. & Corrieu, G. (2000). Identification of interacting mixed cultures of lactic acid bacteria by their exclusion from a model predicting the acidifying activity of non-interacting mixed cultures. *Appl Microbiol Biotechnol* 54, 715–718.
- Stefanitsi, D., Sakellaris, G. & Garel, J. R. (1995). The presence of two proteinases associated with the wall of *Lactobacillus bulgaricus*. *FEMS Microbiol Lett* 128, 53–58.
- Tamine, A. Y. & Robinson, R. K. (1999). *Yoghurt Science and Technology*, 2nd edn. Cambridge: Woodhead.
- Terzaghi, B. E. & Sandine, W. E. (1975). Improved medium for lactic streptococci and their bacteriophages. *Appl Microbiol* 29, 807–813.
- Thomas, T. D. & Mills, O. E. (1981). Proteolytic enzymes of starter bacteria. *Neth Milk Dairy J* 35, 255–273.
- Thomas, T. D. & Pritchard, G. G. (1987). Proteolytic enzymes of dairy starter cultures. *FEMS Microbiol Rev* 46, 245.
- Zourari, A., Accolas, J. P. & Desmazeaud, M. J. (1992). Metabolism and biochemical characteristics of yogurt bacteria. A review. *Lait* 72, 1–34.

Received 4 April 2002; revised 20 June 2002; accepted 15 July 2002.