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The CHAP domain of Cse functions as an endopeptidase that acts at mature septa to promote *Streptococcus thermophilus* cell separation.

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SUMMARY

Cell separation is dependent on cell wall hydrolases that cleave the peptidoglycan shared between daughter cells. In *Streptococcus thermophilus*, this step is performed by the Cse protein whose depletion resulted in the formation of extremely long chains of cells. Cse, a natural chimeric enzyme created by domain shuffling, carries at least two important domains for its activity: the LysM expected to be responsible for the cell wall-binding and the CHAP domain predicted to contain the active center. Accordingly, the localization of Cse on *S. thermophilus* cell surface has been undertaken by immunogold electron and immunofluorescence microscopies using of antibodies raised against the N-terminal end of this protein. Immunolocalization shows the presence of the Cse protein at mature septa. Moreover, the CHAP domain of Cse exhibits a cell wall lytic activity in zymograms performed with cell walls of *Micrococcus lysodeikticus*, *Bacillus subtilis* and *S. thermophilus*. Additionally, RP-HPLC analysis of muropeptides released from *B. subtilis* and *S. thermophilus* cell wall after digestion with the CHAP domain shows that Cse is an endopeptidase. Altogether, these results suggest that Cse is a cell wall hydrolase involved in daughter cell separation of *S. thermophilus*.
INTRODUCTION

Cell separation can be defined as the mechanism needed to disconnect the two new daughter sacculi after the cell division is completed (or at the very late step of cell division). This is still a poorly understood process that requires the involvement of cell wall hydrolases. Cell-separating hydrolases were mostly identified by phenotypic analysis, their absence resulting in the formation of chains of unseparated cells (Borges et al., 2005; Buist et al., 1995; Fukushima et al., 2006; Garcia et al., 1999; Heidrich et al., 2001; Margot et al., 1998; Margot et al., 1999; Yoshimura et al., 2006) or in a highly aggregated phenotype (Kajimura et al., 2005). In Streptococcus thermophilus, the deletion of cse led to a dramatic increase of the chain length indicating that Cse is the key S. thermophilus cell-separating enzyme (Borges et al., 2005).

The Cse protein consists of four regions: a signal peptide and a LysM domain at its N-terminus, a catalytic domain at its C-terminus, both domains being separated by a central variable region (Borges et al., 2005; Borges et al., 2006). The LysM motif functions as a general peptidoglycan binding module (Eckert et al., 2006; Steen et al., 2005a) that may play important roles in appropriate localization and/or recognition of substrates (Fukushima et al., 2006; Kajimura et al., 2005; Steen et al., 2005a; Yamamoto et al., 2003).

The C-terminal catalytic domain of Cse carries a Cysteine, Histidine-dependent Amidohydrolases/Peptidases (CHAP) domain conferring to the protein a potential catalytic activity (Borges et al., 2005; Layec et al., 2008a). Point mutation of the conserved Cys and His residues or total deletion of this domain reveals that it retains cell wall hydrolase activity (Donovan et al., 2006; Navarre et al., 1999; Nelson et al., 2006; Pritchard et al., 2004; Yokoi et al., 2005).

Among the Firmicutes, few bacterial and phage-encoded CHAP proteins have been characterized and participate either in cell division (Chia et al., 2001; Mattos-Graner et al., 2006; Ng et al., 2004; Reinscheid et al., 2001) daughter cell separation (Borges et al., 2005; Kajimura et al., 2005) or in cell lysis (Donovan et al., 2006; Huard et al., 2003; Navarre et al., 1999; Pritchard et al., 2004; Yokoi et al., 2005).
By analogy with other cell-separating enzymes and taking into account the involvement of Cse in cell disconnection, we postulate that Cse is a cell wall hydrolase probably acting at cell separation sites. However, there has been no direct evidence concerning either the Cse catalytic activity or its localization at the Streptococcus thermophilus cell surface. In this study, we provide genetic and biological evidence that Cse separates daughter cells by cleaving the bond between D-Ala of the stem peptide and L-Ala of the cross-bridge in S. thermophilus peptidoglycan at mature septa.
RESULTS

The LysM and CHAP domains are essential for Cse cell separating activity.

To get more insight into the biological role of the LysM and CHAP domains of the Cse protein, in frame deletion mutants were constructed resulting in the cseΔLysM and cseΔCHAP mutant strains (Fig. 1A). Semi-quantitative RT-PCR was used to quantify the level of expression of cse alleles in the wild-type and the mutant strains. The level of expression of gyrA was included an internal housekeeping control to ensure that equivalent amounts of RNA were used for the RT-PCR analysis. As shown in Fig. 1B, there is no significant difference between the levels of expression of the different alleles.

Visual inspection of overnight standing HJL cultures revealed that turbidity in the cultured medium was only observed for the CNRZ368 control strain whereas the cells of the Δcse, cseΔLysM and cseΔCHAP mutants all flocculate at the bottom of the tube (Fig. 2A). To determine if the sedimentation properties of these mutants correlate with an increase of their chain length, aliquots of each culture were observed under light microscopy followed by the counting of the number of cells per chain. As observed in Fig. 2B, chains of the wild-type strain were rather small since 70% of them were constituted by less than 40 cells. By contrast, chains of the 3 cse deletion mutants were mostly composed of more than 201 cells. Complementation experiments were performed by transformation of the cseΔLysM and cseΔCHAP mutants with pNST260⁺::cse, a vector containing an intact copy of cse that we developed previously (Borges et al., 2005). Complemented strains exhibited a wild-type phenotype with respect to the number of cells per chain and to the sedimentation property while the negative control maintained a mutant phenotype (data not shown). Thus, deletion of the LysM or the CHAP domain of the cse gene had a clear effect on the chain length indicating that both domains were required for Cse efficient cell separation activity.
Cse is involved in late stage of cell division.

Transmission electron microscopy of both the wild-type and the ∆cse mutant revealed that they formed linear chains that appeared like linear necklaces of diplococcal cells (Fig. 3). As observed for *S. pneumoniae* (Morlot *et al.*, 2003), progression of the *S. thermophilus* septum synthesis is materialized by three kinds of septa (Fig. 3C and F): (i) nascent septum (a) located equidistant from the cell poles and initiated by the constriction ring, (ii) intermediate septum (b) generated by further constriction of the cell walls and (iii) mature septum (c) observed at the end of the division process and corresponding to an extension of the septum holding daughter cells together. No impairment of the septum formation was observed in the ∆cse mutant suggesting that Cse had no role in cell wall synthesis at septa. The only difference between the ∆cse mutant (Fig. 3A to C) and the wild-type strains (Fig. 3D to F) is the regularity of the cell morphology. Within the mutant, the chains of cells consist of a regular succession of diplococcus, a phenotype that corresponds to fully divided cells. A more irregular phenotype is observed in the wild-type suggesting that the processes of cell division and of disconnection of daughter cells could interfere.

Since deletion of *cse* led to a dramatic reduction of daughter cell separation and because the material linking daughter cells was expected to be peptidoglycan, we postulate that Cse acts as a late cell wall hydrolase that is able to bind *S. thermophilus* cell surface and separate divided cells.

Localization of Cse and its derivatives at *S thermophilus* cell surface.

In a previous study, we demonstrate that Cse is an extracellular protein whose export is signal peptide dependent (Borges *et al.*, 2005). Moreover, a search of the Conserved Domain Database shows that the N-terminal parts of Cse contain a LysM domain potentially involved in Cse attachment to the cell wall. Thus, to visualize the specific localization of Cse on the *S. thermophilus* cell surface, we utilized immunogold electron microscopy. To avoid the simultaneous detection of Cse and PcsB sharing the same C-terminal end (Borges *et al.*, 2005), antibodies were raised against a specific region (from 1 to 183 aa) of the Cse protein. As shown in Fig. 4, the labelled gold particles were localized on the fine link connecting daughter cells and within adjacent cell poles.
(Fig. 4A and B). However, because of the darkness of the cells, we could not determine if labelled particles were present elsewhere. We used next immunofluorescence microscopy on the wild-type strain, and confirmed that Cse binding was highly localized at mature septa with no or little Cse protein at nascent septa (Fig. 5A2 and E2). Staining of the wild-type *S. thermophilus* cells with calcofluor fluorescent dye (Fig. 5, E4) which stains cell walls blue confirmed the specific binding of Cse to the peptidoglycan of the mature septa. No fluorescence was detected on the cseΔlysM mutant (Fig. 5C) or on the wild-type strain stained with preimmune serum (Fig. 5B) used as negative controls. Fluorescence analysis of the cseΔVar and cseΔCHAP mutants (Fig. S1C and Fig. 5D) showed that the Cse derivatives were capable of recognizing the same targets but surprisingly the patterns of fluorescence was more diffuse than that of the wild-type strain.

**Cse is an endopeptidase.**

(i) The CHAP domain of Cse confers the cell wall hydrolytic activity.

In a previous study, we tried to overexpress the full length Cse protein but our attempts failed. This time, only the CHAP domain of Cse (from position in aa 341 to 461) was expressed in *E. coli*. To facilitate the purification of the CHAP protein, a hexa-histidine tag was introduced at its N-terminus. On SDS-PAGE, the purified CHAP-His6-tagged protein gave a band of about 15 kDa estimated to be more than 96% pure (Fig. S2). This band corresponded to the cell-wall hydrolyzing band observed on zymogram and was also revealed by Western immunoblot analysis using an anti-His tag monoclonal antibody (Fig. S2). Zymograms were done using as substrates cells from *Micrococcus lysodeikticus* (data not shown), purified peptidoglycan from *B. subtilis* 168 HR (Fig. S2 and Fig. 6A) and from *S. thermophilus* Δcse mutant (Fig. S2 and Fig. 6B) and revealed that the CHAP domain carries a cell wall hydrolytic activity. The CHAP-His6-tagged protein activity was revealed with incubation buffer containing 0.2% Triton_X100 at pH 4.0 or pH 4.5. No activity was detected at other pH (data not shown).
(ii) Determination of the hydrolytic specificity of the CHAP domain of Cse on *B. subtilis* peptidoglycan.

In the literature, the CHAP domain of several phage-encoded lytic proteins displays an endopeptidase activity (Baker *et al.*, 2006; Donovan *et al.*, 2006; Navarre *et al.*, 1999; Nelson *et al.*, 2006; Pritchard *et al.*, 2004; Yokoi *et al.*, 2005), but this domain is also reported to function as N-acetylmuramoyl-L-alanine amidase (Kajimura *et al.*, 2005; Llull *et al.*, 2006). To determine the hydrolytic specificity of Cse, the purified CHAP-His<sub>6</sub>-tagged protein was used to digest purified peptidoglycan of *B. subtilis* whose structure was established previously (Atrih *et al.*, 1999). After incubation, the digestion mixture was centrifuged and both soluble and insoluble materials were analyzed. No muropeptide or peptide could be detected in the soluble material when analyzed by RP-HPLC (data not shown) whereas an N-acetylmuramoyl-L-alanine amidase is expected to release peptide moieties from peptidoglycan as observed previously (Bourgeois *et al.*, 2009), this suggests that Cse does not present such specificity. The insoluble material was further digested with mutanolysin and the digestion products were analyzed by RP-HPLC. Comparison of the muropeptide profile of the peptidoglycan double digest with the muropeptide profile obtained by mutanolysin digestion of *B. subtilis* peptidoglycan (Fig. 6A), revealed a large increase of monomers in parallel with a clear decrease of dimers and trimers in the CHAP-His<sub>6</sub>-tagged protein treated sample. Especially, the amount of the major dimers, peaks 2 and 3, decreased strikingly in *B. subtilis* peptidoglycan treated with the CHAP-His<sub>6</sub>-tagged protein whereas the amount of several monomers in peaks 1, 4 and 5, increased clearly (Fig. 6A). These results indicate that Cse CHAP-domain is able to cleave bonds inside insoluble native *B. subtilis* peptidoglycan. Cleavages occur most probably inside the peptide moieties since the decrease of dimers and trimers is accompanied with an increase of monomers suggesting that the Cse CHAP-domain has endopeptidase activity (Table 1, Fig. 6A).
We then characterized further the hydrolytic bond specificity of Cse CHAP-domain on muropeptides derived from *S. thermophilus* peptidoglycan by mutanolysin digestion. Peptidoglycan was digested by mutanolysin and the resulting digestion products were separated by RP-HPLC. Three muropeptides characterized by MALDI-TOF mass spectrometry (Table 1, peaks 8, 9, 10 and Fig. S3) and purified by RP-HPLC (Fig. S4) were digested with the CHAP-His$_6$-tagged protein to determine its cleavage site on *S. thermophilus* peptidoglycan. The resulting digestion products were analyzed by RP-HPLC. In Fig. 6B, the peaks with the highest retention times [peak 8 in (a); peak 9 in (b) and peak 10 in (c)] correspond to the intact muropeptides indicating partial digestion. However, the others peaks [peak 6 in (a); peaks 6 and 7 in (b) and peaks 6 to 9 in (c)] are consistent with being the CHAP-His$_6$-tagged protein digestion products. According to the retention times of each peak and their masses determined by MALDI-TOF MS (Table 1), muropeptide structures were proposed (Fig. S3). These results indicate that the CHAP-His$_6$-tagged protein cleaved the peptidic bond between the D-Ala residue of the stem peptide and the L-Ala residue of the cross-bridge of *S. thermophilus* peptidoglycan (Fig. 6B). Thus, Cse has D,L-endopeptidase activity on *S. thermophilus* peptidoglycan.
DISCUSSION

Within the Firmicutes, only a few CHAP proteins have been characterized that function as cell wall hydrolases. Among them are PcsB/GbpB from Streptococcus involved in cell division (Chia et al., 2001; Mattos-Graner et al., 2006; Ng et al., 2004; Reinscheid et al., 2001), Sle1 from Staphylococcus aureus (Kajimura et al., 2005) and Cse from S. thermophilus that are necessary for daughter cell separation (Borges et al., 2005). AcmB from Lactococcus lactis contributes to cellular autolysis (Huard et al., 2003). Six other phage-encoded CHAP proteins are involved in bacterial cell lysis (Donovan et al., 2006; Llull et al., 2006; Navarre et al., 1999; Nelson et al., 2006; Pritchard et al., 2004; Rashel et al., 2008; Yokoi et al., 2005). Besides the variety of their roles, these proteins differ by the cleavage specificity of their CHAP domains that present either an endopeptidase or an N-acetylmuramoyl-L-alanine amidase activity.

The CHAP domains of the φ11, φ12, B30, LysWYM and Cse proteins (Donovan et al., 2006; Navarre et al., 1999; Pritchard et al., 2004; Sass and Bierbaum, 2007; Yokoi et al., 2005) and potentially that of AcmB have an endopeptidase activity (Huard et al., 2003). Alternatively, the CHAP domain of the Sle1 and Skl proteins were demonstrated to function as N-acetylmuramoyl-L-alanine amidase (Kajimura et al., 2005; Llull et al., 2006). This indicates that there is no correlation between the cleavage specificity of the CHAP proteins and their biological functions.

In a previous study, the C-terminal end of Cse, including the CHAP domain, was demonstrated to be homologous with that of PcsB/GbpB (Borges et al., 2005). Many attempts were done to demonstrate the potential cell wall hydrolytic activity of these enzymes, but all failed (Borges et al., 2005; Ng et al., 2004; Reinscheid et al., 2001). This time, by over expressing the CHAP domain of Cse and by searching for optimal experimental conditions, we succeeded to demonstrate its endopeptidase activity. By zymogram, Cse activity was only detected at pH 4 - 4.5. Such low pH could exist in the cell wall matrix if we consider that like B. subtilis (Calamita et al., 2001), the cell wall of S. thermophilus could be protonated during growth.
The process of daughter cell separation can be viewed as an active process that takes place at the end or after the completion of the cell division process. Indeed, cells lacking cell-separating enzymes are inhibited only in cell separation and are not inhibited in cell division, with normal septa still being formed (Ohnishi et al., 1999). The cell separation process requires dedicated cell wall hydrolases that cleave the sacculi connecting daughter cells. In their vast majority, cell-separating enzymes are composed of a unique CHAP, NLPC/P60 or Glucosaminidase (more rarely Glyco_hydro_25) catalytic domain (Layec et al., 2008b). The catalytic domain diversity is correlated with the diversity of the peptidoglycan linkage that they cleave. For instance, AcmA from *L. lactis* (Buist et al., 1995; Steen et al., 2005a) and LytB from *S. pneumoniae* (De Las Rivas et al., 2002; Garcia et al., 1999) carry a glucosaminidase domain conferring the N-acetylglucosaminidase activity. Ami A, B and C from *E. coli* and Sle1 from *S. aureus* are N-acetylmuramoyl-L-alanine amidases. Aml from *S. mutans*, carrying a “Glyco_hydro_25” domain, presents a N-acetylmuramidase activity (Yoshimura et al., 2006). At last, several cell-separating enzymes display an endopeptidase activity. That is the case of Cse from *S. thermophilus* (this study) and of LytE, LytF and CwlS (YolJ) from *B. subtilis* (Fukushima et al., 2006; Margot et al., 1998; Margot et al., 1999). Collectively, all these data clearly show that evolution has selected several alternative enzymes exhibiting distinct catalytic activity to perform the daughter cell separation process.

All the cell-separating enzymes described so far are cell surface proteins and Cse does not escape this rule. Cse is thought to bind peptidoglycan via its LysM domain. The LysM domain is one of the most common attachment modules in bacterial cell-separating enzymes. Thus, AcmA from *L. lactis*, AltA from *E. faecalis*, Sle1 from *S. aureus*, LytE, LytF and CwlS from *Bacillus* contain 2 to 6 tandem repeats of the LysM motif (Eckert et al., 2006; Fukushima et al., 2006; Ishikawa et al., 1998; Kajimura et al., 2005; Margot et al., 1998; Margot et al., 1999; Ohnishi et al., 1999; Steen et al., 2003). In addition to its role in cell wall-binding, LysM is important for biological functioning (Buist et al., 2008). The chaining phenotype of the cseΔlysM mutant
corroborated these results since LysM was found to be necessary for efficient Cse cell-separating activity.

The location of only a few cell-separating enzymes has been studied so far but reveals that all of them are positioned only at cell separation sites (Fukushima et al., 2006; Kajimura et al., 2005; Steen et al., 2003) and questions about the reasons leading to such exclusive location.

In the case of Cse, one explanation is to consider that the restricted binding of the enzyme would be ensured by its LysM domain. Another possibility is to consider that a specific mechanism targets Cse to its site of action. Recently, Carlsson et al (2006) (Carlsson et al., 2006) demonstrated that the signal sequence of M protein promotes secretion at the division septum, whereas that of PrtF preferentially promotes secretion at the old pole. Thus, the possibility that the Cse signal peptide (and potentially its LysM domain) governs its secretion to mature septum is not excluded.

Within Cse protein, other regions were found to be important for the correct and specific location of the Cse protein. Indeed, depletion of the CHAP domain (Fig. 5D) or of the Var region (Fig. S1C) led to a modification of the Cse localization on S. thermophilus cell walls. Although we cannot exclude a direct role for these regions on cell wall attachment and/or substrate recognition, it is also possible that the CseΔvar and CseΔCHAP recombinant proteins make inadequate conformations resulting in an altered localization.

At last, not only the structure protein but also the cell wall constituents and probably its architecture possibly interfere with protein attachment. Indeed, studies of AcmA led to the conclusion that the binding of this autolysin over the entire surface is hindered by lipoteichoic acid a cell wall compound associated with glycan molecules (Steen et al., 2005b).

Data from this study indicate that Cse is not involved in cell wall synthesis but only takes part in the very late stage of (or after) the cellular division to catalyse the separation of daughter cells. It demonstrates that cell separation is an active process that requires the action of dedicated cell wall hydrolases.
EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, media and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table S1. *S. thermophilus* CNRZ368 and its derivatives were cultivated in milk medium, M17 (Terzaghi and Sandine, 1975) or HJL (Stingele and Mollet, 1996). Milk medium was used for strain storage, M17 for mutant generation, and Hogg Jago Lactose (HJL) for phenotypic analysis. When required, erythromycin (final concentration 2 µg.ml⁻¹) was added. *S. thermophilus* strains containing pGh9 (Maguin et al., 1992) or pNST260+ derivatives were cultivated at 30°C when plasmid self-maintenance was required and at 42°C for selection of clones with the chromosome’s integrated plasmid. Phenotypic analyses were all performed at 42°C. *S. thermophilus* CNRZ368Δcse mutant was cultured in HJL at 42°C and was used (i) as a substrate in renaturing SDS-PAGE and (ii) to prepare cell-wall extracts for the zymography assays. *E. coli* strains were grown in Luria-bertani (LB) medium (Sambrook et al., 1989) at 37°C with shaking. When required erythromycin or ampicillin was added to a final concentration of 150 µg/ml. *E. coli* EC101 (Leenhouts, 1995) was used as a host of recombinant plasmids derived from pGh9. *E. coli* strain DH5α was used as a host of pET15b derivatives, and *E. coli* strain BL21(DE3) (Stratagene) was used as a host to overexpress the LysM<sub>cse</sub> and CHAP fusion (His<sub>6</sub>-tagged) proteins. *B. subtilis* 168 HR was cultured in LB medium at 37°C and was used to prepare cell-wall extracts for the study of (i) lytic activity by zymogram and (ii) hydrolytic activity.

DNA and RNA manipulations

Plasmids and primers used in this study are listed in Table S1 and Table S2, respectively.

(i) Construction of *S. thermophilus* CNRZ368 cseΔLysM and cseΔCHAP mutant strains.

The LysM and the CHAP modules of the *cse* gene were deleted by allelic replacement as previously described (Thibessard et al., 2004). To make deletions, the two regions flanking the LysM or CHAP
domains were independently amplified by PCR using the following primers pairs: del_lysME5’/del_lysMI5’ and del_lysMI3’/del_lysME3’ or del_CHAPE5’/del_CHAPI5’ and del_CHAPI3’/del_CHAPE3’, respectively (Table S2). The PCR products were digested by appropriate restriction enzymes, joined together and ligated with the plasmid pGh9 (Maguin et al., 1992). The ligation products were used to transform E. coli EC101. After transformation of S. thermophilus with the recombinant plasmid, deleted mutants displaying an EcoRI restriction site replacing 459 pb of the LysM module or 318 bp of the CHAP module were selected. Deletion mutants were checked by PCR, Southern hybridization and sequencing (data not shown).

(ii) cseΔLysM and cseΔCHAP complementation.

The complementation of cseΔlysM or cseΔCHAP mutants was carried out by inserting the pNST260+::cse plasmid in their chromosome as previously described (Borges et al., 2005).

(iii) RNA extraction and RT-PCR experiments

Total RNA was isolated from bacterial cultures according to the protocol described earlier (Kieser et al., 2000). The quantity and quality of the RNA samples were verified by agarose gel electrophoresis and by measuring their absorbance at 260 and 280 nm. Isolated RNAs were stored at -80°C. Reverse transcription was performed according to the manufacturer’s instructions (MMLV-reverse transcriptase, Fermentas). RNA samples isolated from S. thermophilus cultures were used for semi-quantitative RT-PCR analysis of the cse and gyrA transcript levels followed by separation of the PCR products on 1.5% gel agarose electrophoresis and quantitation using Quantity One software (Bio-Rad).

(iv) Construction of the pET15b::lysM_{cse} and pET15b::chap plasmids for LysM_{Cse−} and CHAP-His$_6$-tagged proteins overexpression.

S. thermophilus CNRZ368 DNA fragments encoding (i) the 5’ end of cse including the cell wall-binding LysM module (from 1 to 549 bp) and (ii) the catalytic CHAP module (from 1021 to
1383 bp) of cse were amplified by PCR. The amplified DNA fragments were purified, double
digested with NdeI and BamHI, repurified, and fused in-frame to the 3’end His<sub>6</sub>-tagged of pET15b
(Novagen), generating the pET15b::lysM<sub>cse</sub> and pET15b::chap plasmids, respectively (Table S1).
The in-frame fusion was confirmed by DNA sequencing.

**Overexpression, purification of His<sub>6</sub>-tagged proteins and western blotting.**

Expression of the His<sub>6</sub>-tagged proteins was induced in *E. coli* BL21(DE3) containing
pET15::lysM<sub>cse</sub> or pET15b::chap by adding IPTG (to 1 mM final concentration) at OD<sub>600</sub> = 0.5 for
4h at 28°C. For extraction, cells were harvested by centrifugation and disrupted on ice with a
microtip of Sonifier 250 (Branson Ultrasonics). The soluble fraction containing the recombinant
protein was collected by centrifugation at 14,000 rpm for 30 min at 4°C. The His<sub>6</sub>-tagged proteins
were purified by a HiTrap chelating HP column according to the manufacturer’s instructions (5ml,
Amersham Biosciences). The LysM<sub>cse</sub>- and CHAP-His<sub>6</sub>-tagged proteins have a predicted molecular
mass of 21.5 kDa and 15.1 kDa, respectively. The purity of the His<sub>6</sub>-tagged proteins was confirmed
by SDS-15% [wt/vol] polyacrylamide gel electrophoresis (PAGE) and was estimated >96% by
Quantity One software (Bio-Rad). The LysM<sub>cse</sub>-His<sub>6</sub>-tagged protein was used for antibodies
generation; the CHAP-His<sub>6</sub>-tagged protein was used to demonstrate Cse enzymatic properties.

For Western blotting, proteins were transferred onto PVDF membrane (Roche) using a semi-dry
blotting system (Bio-Rad) following standard protocols (Towbin et al., 1992). To control the
presence of the His-tag on the fusion proteins, immunodetection assays were performed using rabbit
IgG against RGS-His tag (Qiagen, at dilution 1:2000). The ECL Western blotting detection was
done according to the manufacturer’s instructions (Roche) and visualized by FluorS’max (Bio-Rad).
Cse immunolocalization.

(i) Production of anti-LysM<sub>Cse</sub> antibodies.

Polyclonal anti-LysM<sub>Cse</sub> were obtained from New Zealand White Rabbit inoculated with a mixture of 500 µg of purified LysM<sub>Cse</sub>-His<sub>6</sub>-tagged protein and Freund’s complete adjuvant (Sigma-Aldrich) followed by booster two times with LysM<sub>Cse</sub>-His<sub>6</sub>-tagged protein and incomplete adjuvant every three weeks. The purified LysM<sub>Cse</sub>-His<sub>6</sub>-tagged protein was separated by SDS-15% [wt/vol] polyacrylamide gel, cut from the gel, fragmented and used for immunization. The specificity of the LysM<sub>Cse</sub> antibodies was verified on the purified LysM<sub>Cse</sub>-His<sub>6</sub>-tagged protein (at dilution 1:10,000) and <i>S. thermophilus</i> total proteins extract (at dilution 1:5000) by Western blotting analysis. In the soluble fraction of <i>S. thermophilus</i> CNRZ368 cells extract, one approximately 45.5 kDa band compatible with Cse molecular mass without its signal peptide (theoretical molecular mass with signal peptide, 48.5 kDa) was detected with polyclonal anti-LysM<sub>Cse</sub> antibodies (data not shown). No band was discovered in <i>S. thermophilus</i> CNRZ368<sup>Δ</sup>cse mutant cells extract, used as a negative control (data not shown).

(ii) Preparation of <i>S. thermophilus</i> samples for Cse immunolocalization.

After overnight growth at 42°C in HJL medium, samples (2 ml) of <i>S. thermophilus</i> were pelleted by centrifugation (3,000 rpm for 20 min at 4°C) and washed two times with 2 ml phosphate buffered saline (81 mM NaCl, 1.62 mM KCl, 0.9 mM KH<sub>2</sub>PO<sub>4</sub>, 4.8 mM K<sub>2</sub>HPO<sub>4</sub> [pH 7.2]; PBS buffer). The cell pellets were resuspended in 2 ml PBS buffer and fixed by addition of 4% [vol/vol] final concentration paraformaldehyde and 0.2% [vol/vol] final concentration glutaraldehyde for 3 h of incubation on ice. Cells were harvested by centrifugation and washed four times in 2 ml PBS buffer containing 0.05 M glycine. Cells were resuspended in 1 ml PBS buffer. A sample of 100 µl was centrifugated and resuspended in 50 µl PBS buffer containing polyclonal anti-LysM<sub>Cse</sub> primary antibodies and incubated for 12 h at 4°C with gentle rolling. After washing, cells were resuspended in 50 µl of PBS buffer containing Alexa fluor 488-conjugated anti-rabbit IgG antibodies (1:400)
(Molecular-Probes) and observed by Fluorescence Microscopy. For Transmission Electron Microscopy (TEM), cells were resuspended in 50 µl of PBS buffer containing anti-rabbit IgG conjugated with 10 nm colloidal gold secondary antibodies (1:50) (Sigma-Aldrich). The fluorochrome calcofluor (Sigma-Aldrich) was used on *S. thermophilus* cells with a 0.01% [wt/vol] solution to reveal peptidoglycan.

(iii) Microscopy.

Immunofluorescence microscopy was performed with a Bio-Rad LRC Radiance 2100 (Elexience, Paris, France) confocal laser scanning microscope (CLSM), attached to Nikon TE2000-U inverted microscope, using an excitation at 488 nm from 100 mW argon ion laser and appropriate barrier filters. Cells were observed through x60 Nikon immersion oil plan apo (NA 1.40). Images were contrast enhanced in Adobe Photoshop (Adobe Systems Inc., Mountain View, USA). A sample of the bacterial cell suspension was deposited onto formvar carbon-coated copper nickel grids. Cells were examined with a Zeiss 902A transmission electron microscope at 80kV (magnification x 20,000 or x 30,000). *S. thermophilus* chaining phenotypes were observed with a Nikon OPTIPHOT microscope mounted with phase-contrast equipment “Ph”. Cells were photographed with a Nikon coolpix 5400 digital camera.

Cell wall hydrolase activity.

(i) SDS-PAGE and zymography.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out as previously described with a protean II minigel system (Bio-Rad). Zymograms were performed with 15% [wt/vol] polyacrylamide separating gels containing either lyophilized of autoclaved cells (from *M. lysodeikticus* ATCC4698 (Sigma, 0.2% [wt/vol]) or from *S. thermophilus* CNRZ368Δcse mutant (0.4% [wt/vol]) or purified peptidoglycan 0.08% [wt/vol] (from *S. thermophilus* CNRZ368Δcse mutant or *B. subtilis*). Peptidoglycan from *S. thermophilus* and *B. subtilis* 168 HR vegetative cells were prepared as described previously (Courtin *et al.*, 2006; Meyrand *et al.*, 2007).
After electrophoresis, gels were washed two times for 30 min. in 3% [vol/vol] Triton X-100 and then incubated in renaturation buffer overnight at 30°C. Proteins renaturation was performed with:

- 25 mM sodium citrate/ 50 mM sodium phosphate (Huard et al., 2003) or 50 mM KH₂PO₄ buffer containing 0.2% [vol/vol] Triton X-100 at pH 3 to 7. The gels were stained with 0.1% [wt/vol] methylene blue in 0.01% [wt/vol] KOH for 4 h at room temperature and destained in deionized H₂O. Gel images were generated with GS-800 Calibrated Densitometer Scanner (Bio-Rad).

(ii) Determination of CHAP cell wall hydrolytic specificity.

Peptidoglycan from *B. subtilis* 168 HR and *S. thermophilus* was extracted as described previously (Meyrand et al., 2007). *B. subtilis* peptidoglycan (4 mg) was incubated overnight at 30°C with purified CHAP-His₆-tagged protein (500 µg) in a final volume of 500 µl buffer (25 mM sodium citrate/ 50 mM sodium phosphate containing 0.2% Triton X-100 at pH 4.0). The sample was boiled for 3 min to stop the reaction and then centrifuged at 14,000 g for 15 min. The insoluble material as well as half of the soluble fraction, both recovered after incubation with CHAP-His₆-tagged protein, were further treated with mutanolysin (2,500 U.ml⁻¹) overnight at 37°C. A control digest of *B. subtilis* peptidoglycan was obtained by mutanolysin digestion as described (Courtin et al., 2006).

The soluble muropeptides obtained after the digestions were reduced with sodium borohydride and separated by reverse phase HPLC using a Hypersil ODS column (C18, 250 x 4.6 mm; 5 µm; ThermoHypersil-Keystone) at 50°C using ammonium phosphate buffer and a methanol linear gradient as described previously (Atrih et al., 1999; Courtin et al., 2006). Peaks were analysed without desalting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry with a Voyager DE STR mass spectrometer (Applied Biosystems) as described previously (Courtin et al., 2006).

*S. thermophilus* peptidoglycan was digested with mutanolysin overnight at 37°C and the resulting soluble muropeptides were reduced and separated by RP-HPLC as described previously (Hebert et al., 2007). Three muropeptides were collected, dried in a Speed-vac concentrator under vacuum. The purified muropeptides were incubated with 50 µg of purified CHAP-His₆-tagged
protein in a total volume of 200 µl in the conditions described above for *B. subtilis* peptidoglycan. The reaction products were then analyzed by RP-HPLC and MALDI-TOF mass spectrometry as described above.
ACKNOWLEDGMENTS

This study is supported by grants from the Ministère de l’Éducation Nationale de l’Enseignement Supérieur et de la Recherche.

We thank Amand Chesnel for polyclonal antibodies production and Nicolas Carraro for RT-PCR experiments.
REFERENCES


**LEGEND FIGURES**

**FIG. 1. RT-PCR detection of cse transcripts.**

**A.** *cse* modular structure. The positions in nucleotides delimit the signal peptide (SP), the LysM cell wall-binding module, the central variable region (Var) and the catalytic CHAP module of *S. thermophilus* CNRZ368 *cse* gene. Arrows and hairpin loops symbolize putative promoters and putative rho-independent transcriptional terminators, respectively. **B.** Semi-quantitative RT-PCR assays were carried out to compare the level of expression of the different *cse* alleles (*cse*, *Δcse*, *cseΔlysM*, *cseΔvar* and *cseΔchap*). Semi-quantitative RT-PCR reactions were performed with *cse* locus specific primers (surlysM5' and Del_lysMI5') referred in Table S2, yielding a product of 110 bp in size. The internal housekeeping gene *gyrA* served as a reference point to detect relative difference in the integrity of individual RNA samples and was amplified using the gyrA5' and gyrA3' primers (Table S2), which yielded a product of 140 bp in size. As a negative control, all DNAse RNA samples were subjected to PCR prior to RT analysis [- RT (gyrA)]. T(-), also corresponds to a negative control, using water as a PCR matrix. As positive control [T(+)], DNA samples extracted from the culture of each strains were subjected to PCR. For semi-quantitative analysis, the density ratios of the *cse* mutant allele versus the *cse* wild-type were calculated and used as an indication for the relative expression (*cse* = 100%; *Δcse* = 0%; *cseΔlysM* = 99.3+/− 2.9%; *cseΔvar* = 94.7+/− 6.2% and *cseΔchap* = 97.2+/− 8.2%).

**FIG. 2. Mutation of cse alters *S. thermophilus* cell separation.**

**A.** Picture of standing HJL cultures and phase contrast microscopy of the strains. Photographs were taken after 20 h of growth at 42°C in HJL medium. Magnification, x1,000. **B.** Chain length analysis. Cells were counted in stationary phase after 20 h of growth at 42°C in HJL medium. The error bars represent standard deviations of three independent experiments. The chain length analysis of the *cseΔVar* mutant was reported in the following reference (Borges *et al.*, 2006).
FIG. 3. *S. thermophilus* chaining phenotype observed by transmission electron microscopy (TEM).

The Δcse mutant chaining phenotype is shown in panels A to C, that of the wild-type in D to F. Cells were visualized after 20 h of growth at 42°C in HJL medium. Arrowheads in panels C and F pointed distinct septa. Diplococcal dividing cells are characterized by the in-growth of the septum (nascent septum, a) initiated by the constriction ring located equidistant from the cell poles. The progression of the septal peptidoglycan synthesis is materialized by the further constriction of the cell walls (intermediate septum, b). At the end of the division process, diplococcal cells are held together by a thin peptidoglycan filamentous corresponding to an extension of the septum (mature septum, c). Bars = 0.5 µm.

FIG. 4. Immunolocalization of Cse on *S. thermophilus* cell surface.

The TEM pictures of the wild-type strain are shown in A to D and that of the Δcse mutant in E and F. Cells were successively probed with anti-LysM<sub>Cse</sub> rabbit polyclonal antibodies (A, B, C, E and F) or with preimmun serum (D) and with anti-rabbit IgG conjugated with 10 nm colloidal gold secondary antibody. The localization of Cse protein is indicated by the arrowheads pointed on gold-conjugates secondary antibodies (A to C). No labelling was observed on the negative controls (D to F). Bars = 0.1 µm.

FIG. 5. Cse localizes on *S. thermophilus* CNRZ368 mature septa.

CNRZ368 wild-type (A, B and E), cseΔLysM (C) and cseΔCHAP (D) cells are shown. Surface bound Cse and derivatives proteins were detected by immunofluorescence microscopy using anti-LysM<sub>Cse</sub> antibodies. Phase-contrast images (A1 to E1) and fluorescence images (A2 to B2 and E2 to E4) of the bacterial cells in the same field are shown. The arrowheads pointed mature septa. No fluorescence staining on the wild-type cell surface was observed using the preimmun serum (B2), as
well as on the cseΔLysM mutant cells with the anti-LyM\textsubscript{Cse} antibodies (C2), as negative controls, (Bars = 25 µm).

E2 shows a single-labeled with anti-LysM\textsubscript{Cse} antibodies that indicates the localization of Cse exclusively on mature septa (Bars = 5 µm). E3 shows the fluorochrome calcofluor having an affinity for peptidoglycan. E4 is the result double-labelled.

**FIG. 6. Cse functions as an endopeptidase.**

A. RP-HPLC analysis of the muropeptides released from *B. subtilis* cell wall after incubation with mutanolysin (a) or the CHAP-His\textsubscript{6}-tagged protein and mutanolysin (b). B. RP-HPLC analysis of three different purified muropeptides from *S. thermophilus* (Table 1, Fig. S4) after digestion with the CHAP-His\textsubscript{6}-tagged protein. The numbers indicate the peaks analyzed by MALDI-TOF MS. The molecular ions \textit{m/z} values are indicated in Table 1. The CHAP domain activity is visible as a clear hydrolysis band observed on a renaturing SDS-PAGE with 8 µg of CHAP-His\textsubscript{6}-tagged protein in gel containing 0.08% [wt/vol] *B. subtilis* HR168 or *S. thermophilus* CNRZ368Δcse cell-wall as substrate.

The percentage of monomers corresponds to the sum of the area of the peaks corresponding to monomers over the sum of the areas of all peaks. The same was done for dimers and trimers. The percentage of each peak was calculated as the ratio of the peak area over the sum of areas of all the peaks identified by RP-HPLC.

Schematic structure of *S. thermophilus* cell-wall is illustrated. Arrow indicates the cleavage site of the CHAP-His\textsubscript{6}-tagged protein. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid.

**FIG. S1. Immunolocalization of Cse protein derivatives on the cell surface of *S. thermophilus*.**

*S. thermophilus* CNRZ368 cells of the Δcse (A), the cseΔCHAP (B) and the cseΔVar (C and D) mutants are shown. Cells were visualized with a laser scanning confocal microscope. Phase-constrat images (A1 to D1) and fluorescence images (A2 to D2) of the bacterial cells are shown in the same
field. No fluorescence was detected on negative controls: Δcse cells with anti-LysM Cse antibodies (A2); from cseΔCHAP to cseΔvar cells with preimmun serum (B2 and D2). C2 indicates the localization of Cse derivative protein using the LysM Cse antibodies. (Bars = 25 µm)

**FIG. S2.** Overexpression, purification, western-blotting and lytic activity of the CHAP-His₆-tagged protein.

SDS-15% polyacrylamide gel with Coomassie brilliant blue staining, Western blot obtained using RGS-His-tag antibodies raised against CHAP-His₆-tagged protein and Cell wall hydrolytic activity of the CHAP-His₆-tagged protein in renaturing SDS-15% polyacrylamide gel containing 0.08% [wt/vol] B. subtilis 168 HR or S. thermophilus CNRZ368Δcse peptidoglycan as substrate. Renaturing buffer contains 0.2% TritonX-100. The CHAP-His₆-tagged protein activity is visible as cleaning zones in the gel (lanes 3, 4 and 5). Lanes 1 and 2, cell extract and supernatant of E. coli BL21(DE3) containing pET15b. Lanes 3 and 4, cell extract and supernatant of E. coli BL21(DE3) containing pET15b::chap. Lane 5, purified CHAP-His₆-tagged protein. Lane MM, the molecular masses of standard proteins. The arrow indicates the position of purified CHAP-His₆-tagged protein.

**FIG. S3.** Schematic structure of the muropeptides from S. thermophilus obtained after mutanolysin digestion or after CHAP-His₆-tagged protein and mutanolysin digestion. Numbers peaks on the chromatograms presented in Fig. 6 and Table 1.

**FIG. S4.** Purity of Streptococcus thermophilus muropeptides.

Chromatogram of the three muropeptides from S. thermophilus peptidoglycan before CHAP-His₆-protein digestion. The molecular ions m/z values of peaks 8, 9 and 10 are indicated in Table 1 and their structures are illustrated in Fig. S3.
Table S1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/phenotype/ source</th>
<th>Origin/reference</th>
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<tr>
<td><strong>S. thermophilus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNRZ368</td>
<td>Wild-type; isolated from yogurt.</td>
<td>INRA-CNRZ, strain collection, France</td>
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<tr>
<td>CNRZ368∆cse</td>
<td>Derivative of CNRZ368 carrying a 1359 bp deletion in the cse gene</td>
<td>(Borges et al., 2005)</td>
</tr>
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<td>CNRZ368cse∆LysM</td>
<td>Derivative of CNRZ368 carrying a 459 bp deletion in the cse LysM Module</td>
<td>This study</td>
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<td>CNRZ368cse∆Var</td>
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<td><strong>E. coli strains</strong></td>
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<tr>
<td>EC101</td>
<td>supE hsd-5 thi Δ(lac-proAB) F’(traD6 proAB’ lacF’ lacZΔM15) repA’, derivative of TG1 strain (Sambrook et al., 1989)</td>
<td>(Leenhouts, 1995; Sambrook et al., 1989)</td>
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<td>DH5α</td>
<td>F’, endA1, gyrA96, thi-1, hsdR17(rK-,mK-), supE44, rel A1, M15, Δ(lacZYA-argF),U169</td>
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<td>BL21(DE3)</td>
<td>E. coli B, F’, dcm, ompT, hsdS(rK-,mK-), galD(TK)</td>
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<td><strong>B. subtilis</strong></td>
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<td></td>
</tr>
<tr>
<td>168 HR</td>
<td>trpC2</td>
<td>(Kunst et al., 1997)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pGh9</td>
<td>Thermosensitive plasmid, used for gene replacement; EryR</td>
<td>(Biswas et al., 1993; Maguin et al., 1992)</td>
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<td>pGh9 ::cseΔlysM</td>
<td>Derivative of pGh9 carrying a 1098 bp for LysM deletion</td>
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<td>pGh9 ::cseΔCHAP</td>
<td>Derivative of pGh9 carrying a 924 bp for CHAP deletion</td>
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<tr>
<td>pNST260+ ::cse</td>
<td>pNST260+ with CNRZ368 cse gene, its putative promoter and terminator</td>
<td>G. Guédon, personal communication</td>
</tr>
<tr>
<td>pET15b</td>
<td>Expression vector for N-terminal His6-tagged fusion; AmpR</td>
<td>Novagen</td>
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<td>pET15b :: lysM_cse</td>
<td>Derivative of pET15b carrying a 549 bp insert coding a LysM_cse His6-tagged protein</td>
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<tr>
<td>pET15b :: chap</td>
<td>Derivative of pET15b carrying a 369 bp insert coding a CHAP His6-tagged protein</td>
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### Table S2. Primers used in this study.

<table>
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<th>Name</th>
<th>Sequence (5’ to 3’)¹</th>
<th>Restriction sites</th>
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<tr>
<td>Construction of <em>S. thermophilus</em> (\text{cse}\Delta\text{lysM} ) mutant</td>
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<tr>
<td>Del_lysME5’</td>
<td>CCC CCC CCA <strong>AGC</strong> TTG ATG CTA TTA <strong>GAT</strong> CAG TTT GGA AC</td>
<td><strong>HindIII</strong></td>
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<tr>
<td>Del_lysMI5’</td>
<td>CCC CCC <strong>GAA</strong> TTTG TGA TTA <strong>GCT</strong> GAT CAT GGT AC</td>
<td><strong>EcoRI</strong></td>
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<tr>
<td>Del_lysMI3’</td>
<td>CCC <strong>GAA</strong> TTC GAT GCT ATG CAT GGT AC</td>
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<tr>
<td>Del_lysME3’</td>
<td>CCC <strong>CTG</strong> GCA GTC GAT ATG TAT TAG GAG TT</td>
<td><strong>PstI</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Construction of <em>S. thermophilus</em> (\text{cse}\Delta\text{chap} ) mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del_CHAPE5’</td>
</tr>
<tr>
<td>Del_CHAPI5’</td>
</tr>
<tr>
<td>Del_CHAPI3’</td>
</tr>
<tr>
<td>Del_CHAPE3’</td>
</tr>
</tbody>
</table>

| Construction of pET15b::\(\text{lysM} \)_{\text{out}} |                       |                   |
|------------------------------------------------------|
| surlysM5’                                           | CCC CCC CCA **TAT** GCT ATG AAA ATC TAA AAC | **NdI**           |
| surlysM3’                                           | CCC CCC **GG ATC** C TT AAG CTG GAG TAT CTG ATG C | **BamHI**         |

<table>
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<tr>
<th>Construction of pET15b::(\text{chap} )</th>
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<th></th>
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</thead>
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<tr>
<td>surchap5’</td>
<td>CCC CCC CCA <strong>TAT</strong> GCT ATG GCT ATG CTA CTA GGA ATA</td>
<td><strong>NdI</strong></td>
</tr>
<tr>
<td>surchap3’</td>
<td>CCC CCC <strong>GG ATC</strong> C TT ATG AAA TAT ATA CAG AA</td>
<td><strong>BamHI</strong></td>
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</table>

**gyrA** gene expression

<p>| | | |</p>
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<tr>
<td>gyrA5’</td>
<td>GATGCGTTAATTTGATGAT</td>
<td>/</td>
</tr>
<tr>
<td>gyrA3’</td>
<td>GAGCCTTTACCAGTTCGTA</td>
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*Restriction sites are in boldface and underlined.
Table 1: Observed m/z values for sodiated molecular ions of muropeptides obtained after hydrolysis of *B. subtilis* and *S. thermophilus* peptidoglycan.

<table>
<thead>
<tr>
<th>Peak</th>
<th>[M+Na]+ Muropeptide identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>852.3 DS-Tri (NH$_2$)</td>
</tr>
<tr>
<td>2</td>
<td>1815.7 DS-Tri-DS-Tetra (NH$_2$)</td>
</tr>
<tr>
<td>3</td>
<td>1814.7 DS-Tri (NH$_2$)-DS-Tetra (NH$_2$)</td>
</tr>
<tr>
<td>4</td>
<td>964.4 DS-Tetra</td>
</tr>
<tr>
<td>5</td>
<td>963.4 DS-Tetra (NH$_2$)</td>
</tr>
<tr>
<td>6</td>
<td>1061.3 DS-Tetra –Ala Ala (NH$_2$)</td>
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<tr>
<td>7</td>
<td>1132.4 DS- Penta – Ala Ala (NH$_2$)</td>
</tr>
<tr>
<td>8</td>
<td>2081.9 DS- Tetra (NH$_2$) – Ala Ala – DS Tetra (NH$_2$) – Ala Ala</td>
</tr>
<tr>
<td>9</td>
<td>2153.0 DS- Penta (NH$_2$) – Ala Ala – DS Tetra (NH$_2$) – Ala Ala</td>
</tr>
<tr>
<td>10</td>
<td>3173.3 DS- Penta (NH$_2$) – Ala Ala – DS Tetra (NH$_2$) – Ala Ala – DS Tetra (NH$_2$) – Ala Ala</td>
</tr>
</tbody>
</table>

*a* Peak numbers refer to the peaks on the chromatograms presented in Fig. 6, which were analyzed by mass spectrometry.

*b* DS, disaccharide GlcNAc-MurNAc (GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid). Tri, tripeptide (L-Ala-D-γGlu-mDAP) from *B. subtilis*; Tetra, tetrapeptide (L-Ala-D-γGlu-mDAP-D-Ala) from *B. subtilis* and (L-Ala-D-γGlu-L-Lys-D-Ala) from *S. thermophilus*; Penta, pentapeptide (L-Ala-D-γGlu-L-Lys-D-Ala-D-Ala) from *S. thermophilus*.

NH$_2$ indicates the presence of an amidation on the peptide chain on mDAP from *B. subtilis* according to (Atrih *et al.*, 1999) and most probably on D-γ-Glu from *S. thermophilus*.
Scanning electron photograph of Streptococcus thermophilus cse-deltaLysM mutant. The long chain phenotype indicates that the binding of Cse at cell surface is required for efficient cell separation.
Magnification, x 4,780
26009x17272mm (1 x 1 DPI)
FIG. 1. RT-PCR detection of cse transcripts. A. cse modular structure. The positions in nucleotides delimit the signal peptide (SP), the LysM cell wall-binding module, the central variable region (Var) and the catalytic CHAP module of S. thermophilus CNRZ368 cse gene. Arrows and hairpin loops symbolize putative promoters and putative rho-independent transcriptional terminators, respectively. B. Semi-quantitative RT-PCR assays were carried out to compare the level of expression of the different cse alleles (cse, Δcse, cseΔlysM, cseΔvar and cseΔchap). Semi-quantitative RT-PCR reactions were performed with cse locus specific primers (surlysM5' and Del_lysMI5') referred in Table S2, yielding a product of 110 bp in size. The internal housekeeping gene gyrA served as a reference point to detect relative difference in the integrity of individual RNA samples and was amplified using the gyrAS' and gyrA3' primers (Table S2), which yielded a product of 140 bp in size. As a negative control, all DNAse RNA samples were subjected to PCR prior to RT analysis [- RT (gyrA)]. T(-), also corresponds to a negative control, using water as a PCR matrix. As positive control [T(+)], DNA samples extracted from the culture of each strains were subjected to PCR. For semi-quantitative analysis, the density ratios of the cse mutant allele versus the cse wild-type were calculated and used as an indication for the relative expression (cse = 100% deltacse = 0%; cseΔlysM = 99.3 +/- 2.9% cseΔvar = 94.7 +/- 6.2% and cseΔchap = 97.2 +/- 8.2%).
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184x193mm (96 x 96 DPI)
FIG. 3. S. thermophilus chaining phenotype observed by transmission electron microscopy (TEM). The deltacse mutant chaining phenotype is shown in panels A to C, that of the wild-type in D to F. Cells were visualized after 20 h of growth at 42°C in HJL medium. Arrowheads in panels C and F pointed distinct septa. Diplococcal dividing cells are characterized by the in-growth of the septum (nascent septum, a) initiated by the constriction ring located equidistant from the cell poles. The progression of the septal peptidoglycan synthesis is materialized by the further constriction of the cell walls (intermediate septum, b). At the end of the division process, diplococcal cells are held together by a thin peptidoglycan filamentous corresponding to an extension of the septum (mature septum, c). Bars = 0.5 µm.

175x101mm (96 x 96 DPI)
FIG. 4. Immunolocalization of Cse on S. thermophilus cell surface. The TEM pictures of the wild-type strain are shown in A to D and that of the deltacse mutant in E and F. Cells were successively probed with anti-LysMCse rabbit polyclonal antibodies (A, B, C, E and F) or with preimmun serum (D) and with anti-rabbit IgG conjugated with 10 nm colloidal gold secondary antibody. The localization of Cse protein is indicated by the arrowheads pointed on gold-conjugates secondary antibodies (A to C). No labelling was observed on the negative controls (D to F). Bars = 0.1 µm.
FIG. 5. Cse localizes on S. thermophilus CNRZ368 mature septa. CNRZ368 wild-type (A, B and E), cseDeltaLysM (C) and cseDeltaCHAP (D) cells are shown. Surface bound Cse and derivatives proteins were detected by immunofluorescence microscopy using anti-LysMCse antibodies. Phase-contrast images (A1 to E1) and fluorescence images (A2 to B2 and E2 to E4) of the bacterial cells in the same field are shown. The arrowheads pointed mature septa. No fluorescence staining on the wild-type cell surface was observed using the preimmun serum (B2), as well as on the cseDeltaLysM mutant cells with the anti-LysMCse antibodies (C2), as negative controls, (Bars = 25 µm). E2 shows a single-labeled with anti-LysMCse antibodies that indicates the localization of Cse exclusively on mature septa (Bars = 5 µm). E3 shows the fluorochrome calcofluor having an affinity for peptidoglycan. E4 is the result double-labelled.
FIG. 6. Cse functions as an endopeptidase. A. RP-HPLC analysis of the muropeptides released from B. subtilis cell wall after incubation with mutanolysin (a) or the CHAP-His6-tagged protein and mutanolysin (b). B. RP-HPLC analysis of three different purified muropeptides from S. thermophilus (Table 1, Fig. S4) after digestion with the CHAP-His6-tagged protein. The numbers indicate the peaks analyzed by MALDI-TOF MS. The molecular ions m/z values are indicated in Table 1. The CHAP domain activity is visible as a clear hydrolysis band observed on a renaturing SDS-PAGE with 8 µg of CHAP-His6-tagged protein in gel containing 0.08% [wt/vol] B. subtilis HR168 or S. thermophilus CNRZ368deltacse cell-wall as substrate. The percentage of monomers corresponds to the sum of the area of the peaks corresponding to monomers over the sum of the areas of all peaks. The same was done for dimers and trimers. The percentage of each peak was calculated as the ratio of the peak area over the sum of areas of all the peaks identified by RP-HPLC. Schematic structure of S. thermophilus cell-wall is illustrated. Arrow indicates the cleavage site of the CHAP-His6-tagged protein. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid.