Another Breaker of the Wall: the Biological Function of the Usp45 Protein of *Lactococcus lactis*

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ABSTRACT *Lactococcus lactis* is a Gram-positive bacterium that is widely used as a cell factory for the expression of heterologous proteins that are relevant in the pharmaceutical and nutraceutical fields. The signal peptide of the major secreted protein of *L. lactis*, Usp45, has been employed extensively in engineering strategies to secrete proteins of interest. However, the biological function of Usp45 has remained obscure despite more than 25 years of research. Studies on Usp45 homologs in other Gram-positive bacteria suggest that Usp45 may play a role in cell wall turnover processes. Here, we show the effect of inactivation and overexpression of the *usp45* gene on *L. lactis* growth, phenotype, and cell division. Our results are in agreement with those obtained in streptococci and demonstrate that the *L. lactis* Usp45 protein is essential for proper cell division. We also show that the *usp45* promoter is highly activated by galactose. Overall, our results indicate that Usp45 mediates cell separation, probably by acting as a peptidoglycan hydrolase.

IMPORTANCE The cell wall, composed mainly of peptidoglycan, is key to maintaining the cell shape and protecting the cell from bursting. Peptidoglycan degradation by peptidoglycan hydrolysis and autolysins occurs during growth and cell division. Since peptidoglycan hydrolases are important for virulence, envelope integrity, and regulation of cell division, it is valuable to investigate their function and regulation. Notably, PcsB-like proteins such as Usp45 have been proposed as new targets for antimicrobial drugs and could also be target for the development of food-grade suicide systems. In addition, although various other expression and secretion systems have been developed for use in *Lactococcus lactis*, the most-used signal peptide for protein secretion in this bacterium is that of the Usp45 protein. Thus, elucidating the biological function of Usp45 and determining the factors affecting its expression would contribute to optimize several applications.

KEYWORDS *Lactococcus lactis*, Usp45, cell morphology, cell separation, peptidoglycan synthesis

Lactococcus lactis has proven to be a suitable bacterial host for the expression and secretion of heterologous proteins (1). The most used signal peptide (SP) for protein secretion in *L. lactis* is that of the native Usp45 protein (2).

Previous studies have attempted to characterize Usp45 and have led to the use of the *usp45* promoter (P_{usp45}) for gene expression and to the use of the Usp45 export signal (SP_{Usp45}) for protein secretion in *L. lactis* (3–5). A role of Usp45 in the proteolytic system of *L. lactis* was excluded, and it did not possess any antimicrobial activity against Gram-positive bacteria (3). Thus, its biological function remained elusive (6).

A significant body of research on the PcsB protein in *Streptococcus pneumoniae*, which is homologous to Usp45 in *L. lactis* (7), has shown that it is required for normal growth and cell division (8). Importantly, comparative genomic analysis reveals that homologs of PcsB are widely distributed in Gram-positive bacteria (9). Among these proteins, PcsB protein has 41.8% similarity to Usp45 from *L. lactis* and 28.3% similarity

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to P45 from *Listeria monocytogenes* (7, 10). Although muralytic activity of the purified catalytic domain (cysteine, histidine-dependent amidohydrolases/peptidases [CHAP]) of PcsB was recently demonstrated (11), the full-length P45 protein from *L. monocytogenes* is the only protein exhibiting murein hydrolase activity *in vitro* (7, 12). P45 from *L. monocytogenes* does not contain a CHAP domain; instead, it contains an NLPC/P60 domain (13). Moreover, recent studies have shown that PcsB is recruited to the septum during the bacterial cell division process, where its muralytic activity is triggered by an ATP-driven conformational change, which might explain the nondetectable catalytic activity of recombinant PcsB *in vitro* (11).

Studies in *S. pneumoniae* and *Streptococcus pyogenes* show that the two-component system (TCS) WalkR is essential and that it positively regulates the PcsB protein (14, 15). Two-component systems are commonly used by bacteria to sense and respond to environmental signals (16). The WalkR system, originally described in *Bacillus subtilis*, is highly conserved and specific to low-G+C-content Gram-positive bacteria. It is responsible for the coordination of cell wall metabolism and cell division (17). Interestingly, the WalR orthologue, LlcC, is not essential in *L. lactis* (18). Considering all of these observations, the differences in the regulation or function of the PcsB-like proteins might indicate subtle differences in cell division in Gram-positive bacteria. In the current report, we investigated the biological function of the Usp45 protein of *L. lactis* and its contribution to the cell division process. These findings now explain the problem of making knockouts of the *usp45* gene in previous studies and its essentiality under certain conditions.

Since *usp45* encodes the major extracellular protein from *L. lactis* and its SP has been extensively used to drive the secretion of proteins and peptides in *L. lactis* (4), we also studied growth conditions to enhance the *usp45* promoter activity. Our results show that the *usp45* promoter is induced by galactose.

RESULTS

Bioinformatic analysis identifies *mreCE* genes upstream of *usp45*. Analysis of the *Lactococcus lactis* MG1363 genome reveals that the two genes upstream from *usp45* encode homologs of the cell shape-determining proteins MreC and MreD found in other Gram-positive bacteria (Fig. 1a). Previous studies identified the *mreCD* genes in ovococcus species always upstream from an *usp45* orthologue (*pcsB*) (19). The conservation of this genomic organization in several Gram-positive bacteria suggests a relationship between the biological function of these proteins in cell wall metabolism and cell shape. We performed an analysis of the amino acid sequence of Usp45 using the Conserved Domains Database (CDD-NCBI) and InterProScan software to identify protein domains and to structurally predict protein motifs (see Fig. 1a) (20). We identified the Cwl01 and CHAP domains, which suggest that Usp45 has an amidase function (21). Cwl01 is a domain described as uncharacterized N-terminal domain of peptidoglycan hydrolase, and the CHAP domain is present in proteins involved in cell wall metabolism of bacteria (22). The structural prediction identified the Usp45 export signal (SP_{Usp45}) and three coil motifs. Since a previous study of PcsB from *S. pneumoniae* describes key protein motifs for the protein activity, such as a coiled coil (CC) motif (11), we performed a sequence and structure alignment of the Usp45 protein sequence and determined the three-dimensional (3D) structural information of PcsB using the PROMALS3D software (see Fig. S1 in the supplemental material) (23). This alignment reveals the presence of the CC domain in Usp45 and corroborates the presence of the CHAP domain, including the conservation of three key amino acid residues of the catalytic CHAP domain (C292, H343, and E360). The results of the analysis of Usp45 protein domains and motifs are consistent with the structure of PcsB (11).

To obtain insight into the proteins related to Usp45, a protein-protein interaction network analysis was performed with the STRING database (24) (Fig. 1b). Proteins identified in the network have a role in cell division or cell lysis, except for Llmg_0506, the function of which is unclear. For instance, the enzymes AcmA and AcmD have been extensively characterized in *L. lactis* (25, 26). AcmA participates in cell division and

FIG 1 Features of the *L. lactis* protein Usp45. (a) Genetic organization of the *lmg_2508*, *lmg_2509* and *usp45* genes and protein features (domain and predictions) of Usp45 of *L. lactis*. The products of *lmg_2508* and *lmg_2509* show homology to MreD and MreC, which were described as cell shape-determining proteins in previous studies (19). The arrows indicate transcription start sites. The domain features and predictions were identified with InterProScan software (EMBL-EBI) (<https://www.ebi.ac.uk/interpro/>) and the Conserved Domains Database (CDD-NCBI) (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Cw101, uncharacterized N-terminal domain of peptidoglycan hydrolase (COG3883); CHAP, amidase function (pfam05257). Structural predictions identified a signal peptide (SP; highlighted in light orange color) and three coil motifs (indicated in orange). (b) The interaction network (as displayed by EMBL-STRING) for genetically interacting proteins possibly related in function with *L. lactis* Usp45 is shown. Protein-protein interaction network analysis obtained from the STRING database (https://string-db.org/cgi/input.pl?sessionId=LiYGICgSH5Uw&input_page_show_search=on). Usp45 is illustrated as a red node. The thickness of the network edges indicates the strength of data support. *lmg_2005*, cell wall-associated hydrolase; *acmA*, N-acetylmuramidase; *acmD*, N-acetylmuramidase; *mreD*, cell shape-determining protein; *mreC*, cell shape-determining protein; *ftsX*, cell division protein; *ps356*, endolysin; *lmg_0505*, uncharacterized protein.

autolysis, whereas *AcuD* is also involved in cell separation and contributes to autolysis when *AcmA* is present (25).

The *usp45* gene is essential for growth of *L. lactis*. Genes homologous to lactococcal *usp45* are essential in serotype 2 *S. pneumoniae* (*pcsB*) (8) and *Enterococcus faecium* (*sagA*) (27), but not in *Staphylococcus aureus* (*ssa*) (28), serotype 4 *S. pneumoniae* (29), or *Streptococcus mutans* (*gsp-781*) (30). None of the strategies employed by van Asseldonk et al. (3) to inactivate the chromosomal *usp45* gene of *L. lactis* by homologous recombination were successful, suggesting that *usp45* is essential under the conditions used. We constructed the pCSP_{*usp45*}-*usp45* *L. lactis* strain and tried several times to delete *usp45*. Homologous recombination in two steps was attempted, using the pCS1966 double crossover (DCO) strategy (31), by growing *L. lactis* cells on synthetic amino acid (SA) medium plates (32) supplemented with 30 $\mu\text{g} \cdot \text{ml}^{-1}$ 5-fluoroorotic acid hydrate. No *usp45* deletion mutant *L. lactis* strain was obtained. A second strategy entailed the replacement of P_{*usp45*} by P_{*nisA*} (the native *usp45* gene driven by the *nisA* promoter). The DCO strategy was attempted in the pCS1966-P_{*nisA*}-*usp45* strain by growing this strain on SA medium plates with 30 $\mu\text{g} \cdot \text{ml}^{-1}$ 5-fluoroorotic acid hydrate and nisin at different concentrations (10, 20, 30, 40, and 50 ng $\cdot \text{ml}^{-1}$). Again, no *L. lactis* colonies were obtained when P_{*usp45*} was replaced by P_{*nisA*}. These results clearly indicate that *usp45* is an essential gene in *L. lactis*.

Use of the CRISPR-Cas9 system to target the *usp45* gene. To repress *usp45* by CRISPR interference (CRISPRi), the *L. lactis* sgRNA(*usp45*) strain was constructed. The production of a single guide RNA (sgRNA) against *usp45* is induced with nisin (10 ng $\cdot \text{ml}^{-1}$). Repression of *usp45* in *L. lactis* was performed in rich GM17 medium. Figure 2 shows that induction of sgRNA(*usp45*) results in a growth defect, i.e., a lower growth rate compared to that under noninduced conditions is observed when the cells are grown in GM17. Remarkably, Fig. 3a shows the effect of knocking down *usp45* by the CRISPRi system on the cell phenotype in *L. lactis*. Cells grown under nisin-induced

FIG 2 Effect of induction of the single guide RNA against the *usp45* gene on growth of *Lactococcus lactis*. Growth curves of the *L. lactis* sgRNA(*usp45*) strain in GM17 medium over 20 h, nisin-induced single guide RNA (induced; nisin was added at a concentration of $10 \text{ ng} \cdot \text{ml}^{-1}$) and control sample (noninduced). Points are means of 3 replicates for each growth curve. Data are presented as mean $\pm$ standard deviation (SD). Error bars represent SD.

conditions show a variety of aberrant cell shapes, including small and large cells, clumps, and chains (Fig. 3b). Staining with fluorescent vancomycin (FI-Van) was performed to visualize accumulation of peptidoglycan precursors during the cell division process (33). Besides the observation of the aberrant phenotype, some cells were stained entirely with FI-Van. This accumulation of peptidoglycan precursors suggests that the cells failed to divide. Moreover, Fig. 3b highlights a cell division defect (blue arrow), where septum formation was not evident with the fluorescent vancomycin staining. In agreement with the results of inactivation of homologs to *usp45* in *Streptococcus agalactiae* (7), the repression of *usp45* in *L. lactis* cells shows irregular cell division compared to that in noninduced cells.

Overexpression of the *usp45* gene. Overexpression of the *usp45* gene was performed by nisin induction in the *L. lactis* pNZ-P_{nisA}-*usp45* strain. Figure 4 shows that overexpression of *usp45* driven by nisin induction results in a lower growth rate and lower cell density compared to those of the control. Next, we evaluated the effect on

FIG 3 Induction of the single guide RNA against the *usp45* gene of *Lactococcus lactis* results in aberrant cell shape phenotype. Growth of the *L. lactis* sgRNA(*usp45*) strain was performed in GM17 medium. (a) Cells were grown in two conditions, nisin-induced single guide RNA (induced; nisin was added at a concentration of $10 \text{ ng} \cdot \text{ml}^{-1}$) and control sample (noninduced). (b) Accumulation of fluorescent vancomycin (FI-Van) is indicated with a green arrow, and disturbed cell separation is indicated with a blue arrow. Samples of each bacterial culture were taken at exponential growth phase (optical density at 600 nm of 0.5). Representative images of fluorescent vancomycin (FI-Van) and phase-contrast are shown. Bars, 10 μm .

FIG 4 Effect of overexpression of *usp45* on the growth of *L. lactis*. Growth curves of the *L. lactis* P_{nisA} -*usp45* strain in GM17 medium over 20 h, nisin-induced *usp45* expression (induced; nisin was added at a concentration of $10 \text{ ng} \cdot \text{ml}^{-1}$) and control sample (noninduced). Points are means of 3 replicates for each growth curve. Data are presented as mean $\pm$ standard deviation (SD). Error bars represent SD.

the phenotype of cells at exponential growth phase (Fig. 5). The overexpression of *usp45* causes no visible effect on cell shape, nor does it have an effect on the localization of cell wall synthesis. Moreover, no irregularities in septum formation during cell division were found.

The *usp45* promoter is induced by galactose. Although the *usp45* promoter has been previously described as a strong promoter in *L. lactis* (34), we investigated whether the activity of the *usp45* promoter is affected when *L. lactis* is grown with a carbon source other than glucose. Previous studies have shown that the binding of the major autolysin AcmA to the peptidoglycan is reduced when *L. lactis* cells are grown on galactose (26). Therefore, we tested the activity of both the *acmA* and *usp45* promoters in *L. lactis* P_{usp45} -*gfp* and P_{acmA} -*gfp* strains grown in chemically defined medium (CDM)

FIG 5 Effect of overexpression of *usp45* on phenotype of *L. lactis*. Growth of the *L. lactis* P_{nisA} -*usp45* strain was performed in GM17 medium. Cells were grown under the following two conditions: nisin-induced single guide RNA (induced; nisin was added at a concentration of $10 \text{ ng} \cdot \text{ml}^{-1}$) and control sample (noninduced). Samples of each bacterial culture were taken in the exponential growth phase (optical density at 600 nm [OD_{600}] of 0.5). Peptidoglycan synthesis was observed by staining the cells with fluorescent vancomycin (FI-Van). Representative fluorescent vancomycin and phase-contrast images are shown. Bars, $10 \mu\text{m}$.

FIG 6 The *usp45* and *acmA* promoters are induced by sugars. (a) The *usp45*, *acmA*, and *fbA* promoters were fused to the green fluorescent protein reporter gene (*gfp*). *L. lactis* strains bearing each construct (*P_{usp45}-gfp*, *P_{acmA}-gfp*, and *P_{fbA}-gfp*) were grown in chemically defined medium (CDM) in the presence of different sugars (glucose, galactose, fructose, and maltose) at concentrations of 0.5% (wt/vol). Population-level normalized green fluorescent protein (GFP) expression (relative fluorescence units [RFU]/OD₆₀₀) of bacterial cultures. Data are presented as mean $\pm$ standard deviation (SD). Dots represent the single values of independent experiments ($n = 3$). Error bars represent SD. (b) Single-cell fluorescence measurements by flow cytometry in the presence of different sugars at concentrations of 0.5% (wt/vol). Fluorescence measurements were taken at the beginning of the stationary-growth phase. Ten thousand ungated events for each sample are shown. A control sample of a *L. lactis* strain bearing an empty vector (promoterless pSEUDO-*gfp*) was used to compare background fluorescence of the bacterial cells.

supplemented with different carbon sources. Figure 6a shows that both promoters are highly induced in the presence of galactose, and to a lesser extent when maltose is present. An ~ 4 -fold increase of the *usp45* promoter activity and an ~ 3.5 -fold increase of the *acmA* promoter activity is observed in cells grown on galactose compared to that in cells grown on glucose (Fig. 6a). To validate our results, we aimed to test the activity of a constitutive promoter to the presence of various sugars. Thus, we constructed another transcriptional fusion with the promoter *P_{fbA}*. The *fbA* gene encodes the fructose-bisphosphate aldolase, a key enzyme in the glycolysis pathway, i.e., it has a housekeeping role in metabolism (35). The green fluorescent protein (GFP) expression values of the *P_{fbA}-gfp* strain were more homogenous in the presence of different

FIG 7 Effect of sugars on cell separation. The nisin-induced single guide RNA against *usp45* affects the cell phenotype of *L. lactis* sgRNA(*usp45*) when it is grown in M17 containing 0.5% (wt/vol) galactose. Cells were grown under two conditions, nisin-induced single guide RNA (induced; nisin was added at a concentration of $10 \text{ ng} \cdot \text{ml}^{-1}$) and control sample (noninduced). Representative images of the control sample (noninduced) are shown. Peptidoglycan synthesis was observed by staining the cells with fluorescent vancomycin (FI-Van). Representative fluorescent vancomycin (FI-Van) and phase-contrast images are shown. Bars, $10 \mu\text{m}$.

sugars than those of the P_{usp45} -*gfp* and P_{acmA} -*gfp* strains. Although all of the strains were inoculated with the same dilution (see Materials and Methods), they differed in growth rate when grown in CDM supplemented with different carbon sources. This growth effect was taken into account to correct the GFP expression values at different optical density values (see Materials and Methods and Fig. S2 in the supplemental material). Moreover, we performed single-cell GFP measurements by flow cytometry to corroborate the effects of sugars on the activity of the *usp45* and *acmA* promoters. Accordingly, Fig. 6b shows a shift in the GFP expression levels of the P_{usp45} -*gfp* and P_{acmA} -*gfp* strains grown in the presence of galactose and maltose compared to the GFP expression levels when grown in glucose. We also measured the GFP expression of the P_{fbaA} -*gfp* strain and observed homogenous GFP expression when it was grown in the presence of different sugars. We added a second control for this experiment by constructing a *L. lactis* strain (indicated as the control strain in Fig. 6b) bearing the empty vector pSEUDO-*gfp* (promoterless) to distinguish the background fluorescence values. Together, these findings confirm that both promoters, *acmA* and *usp45*, are highly induced in the presence of galactose.

Similarly to what was seen when the cells are grown on glucose, different cell shapes were observed when *usp45* was inactivated in cells grown on galactose (Fig. 7). There was a reduced variation in cell shape, with long-chain formation being the predominant phenotypic change. This observation could be due to the fact that when the galactose-mediated induction results in large amounts of Usp45, the repression of *usp45* by the nisin-induced CRISPRi system might not reach the same repression levels as those when cells are grown with glucose.

DISCUSSION

We report here that the long-known Usp45 protein of *L. lactis* is essential for proper cell division. Usp45 from *L. lactis* has catalytic domains similar to that of PcsB in other streptococcal species (36). Inactivation of the *usp45* gene affects cell wall synthesis and cell shape in *L. lactis*, which is consistent with a role of Usp45 acting as a cell wall hydrolase, and a function that is attributed to the cysteine, histidine-dependent ami-

dohydrolases/peptidases (CHAP) domain (Pfam identifier PF05257). Moreover, sequence and structure alignment of the Usp45 protein sequence and 3D structural information of PcsB reveals the conservation of three key amino acid residues of the catalytic CHAP domain (C292, H343, and E360; see Fig. S1 in the supplemental material). The essentiality of the Usp45 in *L. lactis* is comparable to that of the PcsB in *S. pneumoniae*, in which the cysteine and histidine residues from the CHAP domain were shown to be required for viability (22, 37). Since previous studies performed by van Asseldonk et al. reported that the purified Usp45 protein has no function in the proteolytic system of *L. lactis*, nor antimicrobial activity against other Gram-positive bacteria (3), we speculate that Usp45 requires an ATP-driven conformational change to activate the catalytic domains *in vivo*, as is predicted for its homolog PcsB in *S. pneumoniae* (11). The VicRK TCS regulates *pcsB* in *S. pneumoniae*, and the VicR regulator is essential for viability in this bacterium (38). The essentiality of VicR is caused by regulation of multiple genes, including genes that mediate wall teichoic acid biosynthesis, virulence, or exopolysaccharide production (17, 39). Conversely, in the homologous TCS in *L. lactis* (KinC and LlrC), VicR (KinC) is not essential, but its deletion results in a clumping phenotype (18). In addition, *L. lactis* strains where either the *kinC* or *llrC* gene is disrupted show a similar phenotype to that of the wild type (WT) (see Fig. S3 in the supplemental material). These findings suggest that either the lactococcal *usp45* may not be completely regulated by the homologous TCS in *L. lactis* (KinC and LlrC) or that this TCS only indirectly affects Usp45 expression (15).

The CRISPRi-mediated knockdown of *usp45* led to aberrantly shaped cells, variation in cell size, and different patterns of septum formation (Fig. 3a). The formation of long chains of cells is a phenotype often described for strains deficient in cell wall hydrolytic activity (40). Thus, inactivation of *usp45* causes a cell separation defect and unusual cell wall synthesis. Accordingly, we suggest that Usp45 acts as a cell wall hydrolase that participates in cell separation and cell wall synthesis.

The *usp45* gene product is a secreted 45-kDa protein. There is a discrepancy in the literature regarding the description of Usp45 as a surface protein with two possible types of surface display, either as a lipoprotein or anchored by transmembrane helices (TMH). This description is based on a study that combined software prediction and proteomic data, which suggested that the Usp45 protein might exist in an equilibrium between unbound and bound states (41). However, recent studies of the PcsB protein provide experimental evidence of the PcsB anchoring to the cell via its interaction with the membrane-embedded protein FtsX (11, 42). Our study further supports the supposition of Usp45 being a surface protein that plays an important role in cell division.

In a previous study, the *usp45* gene was used as an internal standard because it is presumably constitutively expressed (43). Here, we reveal that the *usp45* promoter is strongly induced by galactose. Previous studies on the major autolysin AcmA of *L. lactis* have shown that the nature of the carbon source influences binding of AcmA to peptidoglycan (26). The authors confirmed that growth of *L. lactis* on galactose affects the carbohydrate composition in the lipoteichoic acids (LTAs) in the cell wall in such a way that AcmA binding is decreased compared to that when cells are grown on glucose. As alanylation or galactosylation of the LTAs affects the function of AcmA, these changes in cell wall composition, when *L. lactis* is grown on galactose, could diminish efficient peptidoglycan hydrolysis by Usp45. Therefore, large amounts of Usp45 are required for efficient cell division when galactose is present, and thus the *usp45* promoter is highly induced. In support of this hypothesis, Fig. 6a shows that repression of *usp45*, when the cells are grown on galactose, causes the formation of long chains of cells. We speculate that, although cell separation is impaired, the lesser variation in cell shape and cell size compared to those when *usp45* is inactivated in cells grown on glucose (Fig. 3) might result from a higher expression of *usp45* that counterbalances the inactivation.

The present results are significant in at least two major respects. First, we provide evidence for a role of *L. lactis* Usp45 in cell shape and division. Second, since the *usp45* promoter and Usp45 signal peptide are commonly used for heterologous protein

TABLE 1 Strains and plasmids used in this study

Strain name/genotype or plasmid ^a	Relevant genotype and/or description ^b	Source or reference
<i>L. lactis</i>		
NZ9000 (WT)	MG1363 <i>pepN::nisRK</i>	50
sgRNA(<i>usp45</i>)	Ery ^r Cm ^r , NZ9000 carrying pNZ- <i>P_{nisa}-dcas9</i> and pTLR- <i>P_{usp45}-sgRNA(usp45)</i>	This work
<i>P_{nisa}-usp45</i>	Cm ^r , NZ9000 carrying pNZ- <i>P_{nisa}-usp45</i>	This work
<i>P_{usp45}-gfp</i>	Ery ^r , NZ9000 carrying pSEUDO:: <i>P_{usp45}-gfp</i>	53
<i>P_{acmA}-gfp</i>	Ery ^r , NZ9000 carrying pSEUDO:: <i>P_{acmA}-gfp</i>	This work
pCSP _{<i>usp45</i>} - <i>usp45</i>	Ery ^r , NZ9000 carrying pCS1966:: <i>P_{usp45}-usp45</i>	This work
pCSP _{<i>nisa</i>} - <i>usp45</i>	Ery ^r , NZ9000 carrying pCS1966:: <i>P_{nisa}-usp45</i>	This work
<i>P_{fbaA}-gfp</i>	Ery ^r , NZ9000 carrying pSEUDO:: <i>P_{fbaA}-gfp</i>	This work
pSEUDO vector	Ery ^r , NZ9000 carrying pSEUDO:: <i>gfp</i> (promoterless)	This work
$\Delta kinC$	Ery ^r derivative of MG1363 with 800-bp disruption in the <i>kinC</i> gene; MGKinC	18
$\Delta llrC$	Ery ^r derivative of MG1363 with 500-bp disruption in the <i>llrC</i> gene; MGRrC	18
<i>E. coli</i>		
DH5 α	F ⁻ Φ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ^- <i>thi-1 gyrA96 relA1</i>	Laboratory stock
Plasmids		
pCS1966	Ery ^r , <i>oroP</i> integration vector	31
pSEUDO- <i>sfgfp</i> (Bs)	Ery ^r , pCS1966 derivative for integration in <i>llmg_pseudo10</i> locus	56
pNZ8048	Cm ^r , <i>P_{nisa}</i> nisin-inducible gene expression vector	51
pJWV102- <i>P_{lac}-dcas9sp</i>	Amp ^r , <i>P_{lac}-dcas9sp</i>	54
pNZ- <i>P_{nisa}-sgRNA(usp45)</i>	Cm ^r , pNZ8048 carrying <i>P_{nisa}-sgRNA(usp45)</i>	This work
pSEUDO:: <i>P_{usp45}-dcas9</i>	Ery ^r , pSEUDO carrying <i>P_{usp45}-dcas9</i>	This work
pNZ- <i>P_{nisa}-usp45</i>	Cm ^r , pNZ8048 carrying <i>P_{nisa}-usp45</i>	This work
pSEUDO:: <i>P_{usp45}-gfp</i>	Ery ^r , pSEUDO carrying <i>P_{usp45}-sfgfp</i> (Bs)	53
pSEUDO:: <i>P_{acmA}-gfp</i>	Ery ^r , pSEUDO carrying <i>P_{acmA}-sfgfp</i> (Bs)	This work
pSEUDO:: <i>P_{fbaA}-gfp</i>	Ery ^r , pSEUDO carrying <i>P_{fbaA}-sfgfp</i> (Bs)	This work
pCS1966:: <i>P_{usp45}-usp45</i>	Ery ^r , pSEUDO carrying <i>P_{usp45}-usp45</i>	This work
pCS1966:: <i>P_{nisa}-usp45</i>	Ery ^r , pSEUDO carrying <i>P_{nisa}-usp45</i>	This work
pNZ- <i>P_{nisa}-dcas9</i>	Cm ^r , pNZ8048 carrying <i>P_{nisa}-dcas9</i>	This work
pTLR- <i>P_{usp45}-sgRNA(usp45)</i>	Ery ^r , pTLR carrying <i>P_{usp45}-sgRNA(usp45)</i>	This work

^aWT, wild type; sgRNA, single guide RNA.^bCm^r, chloramphenicol resistant; Ery^r, erythromycin resistant; Amp^r, ampicillin resistant.

secretion, induction of *P_{usp45}* by galactose might be further developed for novel engineering strategies. Moreover, based on our results, food-grade suicide systems can be developed, which would remove recombinant *L. lactis* cells from food products after they have performed their function.

Further research should be undertaken to investigate whether one of the TCSs in *L. lactis* is responsible of the regulation of *usp45*. Since all known homologs of Usp45 in other Gram-positive bacteria are regulated by the WalkR (VicRK) two-component system, it would be relevant to determine what makes *L. lactis* an exception to this rule. One of the issues that emerges from previous studies and our findings is that PcsB-like proteins might not only be typical hydrolases but also be enzymes that organize the cell division process (42), a role that would be consistent with the essential nature of the *usp45* gene and its homologs (44).

The present study supports a model in which Usp45 mediates cell separation by its catalytic activity as peptidoglycan hydrolase. Gram-positive bacteria produce several enzymes that hydrolyze peptidoglycan (45, 46). *Lactococcus lactis* produces three different types of peptidoglycan hydrolases (47, 48). According to our data, when Usp45 is expressed at low levels, the balance between cell wall synthesis and cell division is affected. Usp45 may affect the activity of other cell wall hydrolases such as AcmA and AcMD. The great variation in aberrant cell shapes caused by irregular cell wall synthesis is a remarkable sign of the consequences of a perturbation in the peptidoglycan synthesis and turnover harmony.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *L. lactis* was grown as standing cultures at 30°C in M17 broth (Difco BD, NJ, USA) with 0.5% (wt/vol)

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5'–3')
usp45-Rv_NcoI	AGAACAGCCATGGAAAAAAGATTATCTCAG
usp45-Fw_KpnI	TAAGACGGTACCCTAGTTTGGCATCAAGAAAGTAAC
PacmA-Fw	GGCATACTCGAGTACCTTTCTAAAGATTACAAA
PacmA-Rv	TATCCAGCATGCTCATTTACATCATCTATTCTATC
PfbaA-Fw	GGGTCGATCGAATTCGGTCCTCGGGATATG
PfbaA-Rv	GACTTTGCAAGCTTGCATGCCTGCAGGTCG
FwKI_A_XbaI	GCAATTGTCTAGAGCGCCGCCAACACCTTGTA
RvKI_A_KpnI	CAATTGGGTACCCAGCTTTTGTTCCTTTAGTGAGG
FwKI_B_KpnI	GTGCTTGGGTACCTTCTTTTGTGAGCTTCATAAGCC
RvKI_B_EcoRI	ATTAAAGAATTCCAGTTATGAAAAAAGATT
FwKI_C_BamHI	ACAGCTCGGATCCCTAGTCTTATAACTATACTG
RvKI_C_EcoRI	TGCAGTAGAATTCGTGAGTGCTCCTTATAATT
FwKI_D_BamHI	TCCAGCGATTAGTAAATATAGGATCCTGTATA
RvKI_D_XbaI	CTAACTCTAGATGAGGGTGGAACACCAAGTG
KOusp45_1Fw	GCATTCTAGATAAGTAGTGAGTCTGATTTAC
KOusp45_2Rv	GCATGGATCCTTAAATAGCTGAGATAATC
KOusp45_3Fw	GCATGGATCCGTGCGTCTGGTGTTACTTTC
KOusp45_4Rv	CGTACTCGAGAATGATCGCGTGACCAAAAC
0310-sgRNA(usp45)_F	AGTATAATATGTTTAAGCGTAAACACCTGACAACGGTTTAAGAGC TATGC
0311-sgRNA(usp45)_R	GCATAGCTCTTAAACCGTTGTCAGGTGTTTACGCTTAAACATAT TATACT
0153-sgRNA_backbone_FW	GTTTAAGAGCTATGCTGGAACAG
0154-sgRNA_backbone_RV	TAAACATATTATACTATTCCTACCCAC
0217-pNZ8048_USER_F	AGCTTTATAAGUAATTACAGCAGTGTTGCTTTGATTG
0221-Pnis_pNZ8048_R	ATTTCTTATCCAUTGGTGAGTGCTCCTTATAATTT
0032-Pnis_dCas9_F	ATGGATAAGAAUACTCAATAGGCTTAG
0220-dCas9-R	ACTTATAAGCUCTCGAGGTCGACTTAGTCAC

glucose (GM17) or in chemically defined medium (CDM) (49) supplemented with glucose (GCDM), galactose, fructose, or maltose (Sigma-Aldrich, MO, USA). All sugars were added at concentrations of 0.5% (wt/vol). GM17-agar plates contained 1.5% (wt/vol) agar. When necessary, culture media were supplemented with erythromycin (Sigma-Aldrich, MO, USA) and/or chloramphenicol (Sigma-Aldrich, MO, USA), both at $5 \mu\text{g} \cdot \text{ml}^{-1}$.

Escherichia coli DH5 α (Life Technologies, Gaithersburg, MD) was used to perform all recombinant DNA techniques. Cells were grown at 37°C in Luria-Bertani (LB) broth or Luria-Bertani agar 1.5% (wt/vol) (Difco BD). For screening of colonies containing recombinant plasmids, chloramphenicol ($25 \mu\text{g} \cdot \text{ml}^{-1}$) or erythromycin ($150 \mu\text{g} \cdot \text{ml}^{-1}$) was added.

Nisin induction was performed by diluting an overnight culture of *L. lactis* 1:50 and adding nisin (Sigma-Aldrich, Munich, Germany) to a final concentration of $10 \text{ ng} \cdot \text{ml}^{-1}$.

For microscopy experiments and plate reader assays, *L. lactis* was grown in GCDM. Exponentially growing cells (optical density at 600 nm [OD_{600}] of 0.3) were collected by centrifugation in a Microfuge 16 centrifuge (Beckman Coulter, Woerden, The Netherlands) and washed three times with phosphate-buffered saline (PBS) (pH 7.2) solution containing KH_2PO_4 at $15.44 \mu\text{M}$, NaCl at 1.55 mM , and Na_2HPO_4 at $27.09 \mu\text{M}$.

Recombinant DNA techniques and oligonucleotides. DNA amplifications by PCR were performed using a PCR mix containing Phusion high-fidelity (HF) buffer (Thermo Fisher Scientific, Inc., MA, USA), 2.5 mM deoxynucleoside triphosphate (dNTP) mix, Phusion HF DNA polymerase (Thermo Fisher Scientific, Inc.), oligonucleotides ($0.5 \mu\text{M}$ each), and 50 ng of *L. lactis* chromosomal DNA as the template. Oligonucleotides (Table 2) were purchased from Biolegio (Nijmegen, The Netherlands). PCRs were performed in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). The DNA target sequence of interest was amplified by 35 cycles of denaturation (98°C for 30 s), annealing (5°C or more, lower than the melting temperature [T_m] for 30 s), and extension (70°C for 1 min per 1 kbp). Amplifications were confirmed by the 1% agarose gel electrophoresis method.

For DNA cloning, we used FastDigest restriction enzymes and T4 DNA ligase (Thermo Fisher Scientific, Inc.). Reactions were performed according to the manufacturer's recommendations. The ligation products were transformed into *E. coli* DH5 α (Life Technologies) competent cells by electroporation. Cells were plated on Luria-Bertani agar plates with appropriate antibiotics and grown overnight at 37°C. Screening of colonies to confirm the genetic construct was performed by colony PCR. Positive colonies with correct constructs were inoculated in Luria-Bertani broth with the appropriate antibiotic. Plasmid DNA and PCR products were isolated and cleaned up with a High Pure plasmid isolation kit (Roche Applied Science, Mannheim, Germany) according to the protocol of the manufacturer. DNA sequences of constructs were always confirmed by DNA sequencing (MacroGen Europe, Amsterdam, The Netherlands).

Construction of *L. lactis* strains. *Lactococcus lactis* NZ9000 was used throughout (50). To overexpress the Usp45 protein, plasmid pNZ-P_{nisa}-usp45 was constructed. It carries the *L. lactis* usp45 gene

under the control of the *nisA* promoter. The *usp45* coding region was amplified by PCR using the oligonucleotides Usp45_Fw and Usp45_Rv and chromosomal DNA of *L. lactis* NZ9000 as a template. The PCR fragment was cleaved with the enzymes NcoI and KpnI, after which it was inserted into the high-copy-number plasmid pNZ8048 digested with the same enzymes (51). Plasmid pNZ-*P_{nisA}-usp45* was introduced into *L. lactis* NZ9000 by electroporation as described by Holo and Nes (52). Transformants were selected on M17-agar plates supplemented with sucrose, glucose, and chloramphenicol ($5 \mu\text{g} \cdot \text{ml}^{-1}$), yielding *L. lactis* *P_{nisA}-usp45*.

To construct the plasmids pSEUDO::P_{acmA}-*gfp* and pSEUDO::P_{fbaA}-*gfp* carrying the *L. lactis* *acmA* and *fbaA* promoters upstream from the *gfp* gene, the *acmA* and *fbaA* promoters were amplified by PCR using the oligonucleotides PacmA_fw and PacmA_Rv, PfbA_fw, and PfbA_Rv and chromosomal DNA of *L. lactis* NZ9000 as a template. The PCR fragments were digested with PaeI and XhoI and inserted into pSEUDO-*sfGfp(Bs)* digested with the same enzymes. Plasmids pSEUDO::P_{acmA}-*gfp* and pSEUDO::P_{fbaA}-*gfp* were integrated into the silent *llmg_pseudo10* locus of *L. lactis* NZ9000 by single-crossover integration as described previously (53), yielding *L. lactis* *P_{acmA}-gfp* and *L. lactis* *P_{fbaA}-gfp*, respectively.

To obtain *usp45* double-crossover gene deletion mutants, upstream and downstream regions of *usp45* were selected and amplified using the oligonucleotides KOusp45_1Fw plus KOusp45_2Rv and KOusp45_3Fw plus KOusp45_4Rv, respectively. The upstream fragment was ligated into pCS1966 (31) using XbaI and BamHI restriction sites. The plasmid obtained was named pCS1966-A. Downstream fragment B was cloned into pCS1966-A using BamHI and XhoI restriction, and the resulting plasmid was named pCS1966::P_{usp45}-*usp45*. All pCS1966 derivatives were initially constructed in *E. coli* DH5 α and then introduced to *L. lactis* by electroporation. Homologous recombination in two steps was attempted by growing *L. lactis* cells on SA medium plates (32) supplemented with $30 \mu\text{g} \cdot \text{ml}^{-1}$ 5-fluoroorotic acid hydrate (Sigma-Aldrich, Munich, Germany). A second strategy entailed the replacement of P_{usp45} by P_{nisA}. Four PCR products were obtained: fragment A (oligonucleotides FwKI_A_XbaI and RvKI_A_KpnI amplify the backbone pCS1966), fragment B (oligonucleotides FwKI_B_KpnI and RvKI_B_EcoRI amplify the upstream region of the *usp45* promoter), fragment C (oligonucleotides FwKI_C_BamHI and RvKI_C_EcoRI amplify the *nisA* promoter from pNZ8048), and fragment D (oligonucleotides FwKI_D_BamHI and RvKI_EcoRI amplify the downstream region of the *usp45* promoter). The PCR products were ligated into pCS1966 via their corresponding restriction sites. The obtained vector was named pCS1966-*P_{nisA}-usp45* and was introduced by homologous recombination in *L. lactis* NZ9000. The strategy was then employed by using SA medium plates with $30 \mu\text{g} \cdot \text{ml}^{-1}$ 5-fluoroorotic acid hydrate and nisin (Sigma-Aldrich, Munich, Germany) at different concentrations (10, 20, 30, 40, and $50 \text{ ng} \cdot \text{ml}^{-1}$).

To obtain *L. lactis* sgRNA(*usp45*) (pNZ-*P_{nisA}-dcas9*; pTLR-*P_{usp45}-sgRNA-usp45*), the *dcas9* gene was amplified from plasmid pJWV102-*P_{lac}-dcas9sp* with the oligonucleotides 0217-pNZ8048_F and 0221-Pnis_pNZ8048_R. Plasmid pNZ8048 was amplified by PCR with oligonucleotides 0032-Pnis_dCas9_F and 0220-dCas9-R. The two fragments were ligated and used to transform *E. coli*, yielding pNZ-*P_{nisA}-dcas9*. This plasmid was introduced into *L. lactis* NZ9000. The second plasmid carries the sgRNA gene targeting *usp45*. It was obtained by the infusion cloning method (54). The set of oligonucleotides 0153-sgRNA_backbone_FW and 0154-sgRNA_backbone_RV were designed to obtain the linearized version of plasmid pTLR-*P_{usp45}-sgRNA(acmA)* (Chenxi Huang, unpublished data). The 20-nucleotide (nt) guide sequence targeting *acmA* was replaced by a sequence targeting *usp45* (AGCGTAAACACCTGACAACG). To this end, two 50-nt complementary oligonucleotides were designed [0310_sgRNA(*usp45*)_F and 0311-sgRNA(*usp45*)_R], with each oligonucleotide containing 15-nt overlaps with the linearized plasmid, one on each side. The plasmid obtained in *E. coli* pTLR-*P_{usp45}-sgRNA(usp45)* was introduced in *L. lactis* pNZ-*P_{nisA}-dcas9*.

Plate-reader assays. Cultures of *L. lactis* were grown and prepared as described above. For growth curves, *L. lactis* cells were diluted 1:50 in CDM or M17, both containing either glucose, maltose, fructose, or galactose. All sugars were added at concentrations of 0.5% (wt/vol). Growth was recorded in 0.2-ml cultures in 96-well microtiter plates and monitored using the microtiter plate reader VarioScan (Thermo Fisher Scientific, Inc.). The OD₆₀₀ was recorded every 10 min for 24 h. The signal was corrected for background noise of the medium.

The effect of sugars on promoter activities was determined as follows. All sugars were added at concentrations of 0.5% (wt/vol). Growth and GFP expression were monitored using the microtiter plate reader VarioScan (Thermo Fisher Scientific, Inc.) by measuring the optical density at 600 nm (OD₆₀₀) and the fluorescence signal (excitation, 485 nm; emission, 535 nm) every 10 min for 24 h. Both signals were corrected for the background values of the medium used for growth. The OD₆₀₀ values used were corrected for the background value of the corresponding medium used for growth (CDM). The calculation used for resolving the relative GFP measurements (relative fluorescence units [RFU]/OD₆₀₀) of the cultures is depicted by the following formula:

$$\frac{\text{GFP}_{\text{promoter}} - \text{GFP}_{\text{medium}}}{\text{OD}_{\text{promoter}} - \text{OD}_{\text{medium}}} - \frac{\text{GFP}_{\text{control}} - \text{GFP}_{\text{medium}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{medium}}}$$

GFP_{promoter} and OD_{promoter} are the fluorescence and optical density values of the *L. lactis* strain bearing the promoter of interest fused to the *gfp* gene. GFP_{medium} and OD_{medium} are the fluorescence and optical density values of the growth medium. GFP_{control} and OD_{control} are the fluorescence and optical density values of the control *L. lactis* strain (empty vector; see Table 1).

The maximum value of the fluorescence peak in each sample was considered to be the GFP value in all figures of this work and was corrected with the formula mentioned above, yielding the relative fluorescent values (RFU/OD₆₀₀).

Flow cytometry. *L. lactis* cultures were grown overnight in CDM as described above, washed three times in PBS, and transferred to fresh CDM supplemented with various carbon sources (glucose, galactose, fructose, and maltose). All sugars were added at a concentration of 0.5% (wt/vol). The cultures were incubated at 30°C, and samples were taken at the beginning of the stationary-growth phase. Thresholds for the forward scatter (FSC) and side scatter (SCC) parameters were set (200 in both) in a FACSCanto flow cytometer (BD Biosciences, CA, USA) to remove all events that did not correspond to cells. The GFP signal in all measured cells was recorded in 10,000 events and used for downstream analysis (ungated events shown in Fig. 6b). GFP signal measurements were obtained with a FACS Canto flow cytometer (BD Biosciences) using a 488-nm argon laser. Raw data were collected using FACSDiva Software 5.0.3 (BD Biosciences), and FlowJo software was used for data analysis.

Fluorescence microscopy. Washed, exponentially growing cells were transferred to a solidified thin layer of CDM with high-resolution agarose 1.5% (wt/vol) (Sigma-Aldrich). A standard microscope slide was prepared with a layer of solidified agar. Bacterial cells were spotted on the agar and covered with a standard microscope coverslip. For FI-Van experiments, 10 μ l of bacterial culture was taken at the end of the stationary phase and stained without fixation with FI-Van (a 1:1 mixture of vancomycin [Sigma-Aldrich]) and Bodipy FL-conjugated vancomycin (Sigma-Aldrich) at a final concentration of 2 μ g $\cdot$ ml⁻¹), followed by 5 min of incubation in the dark at room temperature.

Microscopy was performed with a temperature-controlled (Cube and Box incubation system; Life Imaging Services, Basel, Switzerland) DeltaVision (Applied Precision, WA, USA) IX71 microscope (Olympus, PA, USA) at 30°C. Images were obtained with a CoolSnap HQ2 camera (Princeton Instruments, NJ, USA) at 60-fold or 100-fold magnification using a 300-W xenon light source for bright-field and FI-Van detection (filter from Chroma; excitation, 470/40 nm, and emission, 525/50 nm). Snapshots in bright-field microscopy and for FI-Van detection were taken with 10% white light-emitting diode (LED) light (Applied Precision); 0.05-s exposure was used for bright-field detection and a 100% xenon light and 0.8 s of exposure were used for FI-Van signal detection. The raw data were stored using softWoRx 3.6.0 (Applied Precision) and analyzed using ImageJ software (55).

Statistics. Statistical analyses were performed using Prism 6.01 (GraphPad Software, Inc.). All experiments were repeated independently at least three times. All micrographs show representative images from three independent replicate experiments.

Bioinformatics. Protein structural predictions and domain features were identified using InterProScan software (20). Protein domain features were identified using the Conserved Domains Database (CDD-NCBI). Protein-protein interaction network analysis was performed by employing the STRING database (<https://string-db.org/>) (24). Protein alignments (sequences and structure) were performed by using the PROMALS3D multiple sequence and structure alignment server (23).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 3.9 MB.

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We declare no conflicts of interest.

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