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Random Mutagenesis Identifies Novel Genes Involved in the Secretion of Antimicrobial, Cell Wall-Lytic Enzymes by *Lactococcus lactis*▽

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***Lactococcus lactis* is a gram-positive bacterium that is widely used in the food industry and is therefore desirable as a candidate for the production and secretion of recombinant proteins. Previously, we generated a *L. lactis* strain that expressed and secreted the antimicrobial cell wall-lytic enzyme lysostaphin. To identify lactococcal gene products that affect the production of lysostaphin, we isolated and characterized mutants generated by random transposon mutagenesis that had altered lysostaphin activity. Out of 35,000 mutants screened, only one with no lysostaphin activity was identified, and it was found to contain an insertion in the lysostaphin expression cassette. Ten mutants with higher lysostaphin activity contained insertions in only four different genes, which encode an uncharacterized putative transmembrane protein (llmg_0609) (three mutants), an enzyme catalyzing the first step in peptidoglycan biosynthesis (*murA2*) (five mutants), a putative regulator of peptidoglycan modification (*trmA*) (one mutant), and an uncharacterized enzyme possibly involved in ubiquinone biosynthesis (llmg_2148) (one mutant). These mutants were found to secrete larger amounts of lysostaphin than the control strain (MG1363[*lss*]), and the greatest increase in secretion was 9.8- to 16.1-fold, for the llmg_0609 mutants. The lysostaphin-oversecreting llmg_0609, *murA2*, and *trmA* mutants were also found to secrete larger amounts of another cell wall-lytic enzyme (the *Listeria monocytogenes* bacteriophage endolysin Ply511) than the control strain, indicating that the phenotype is not limited to lysostaphin.**

Lactococcus lactis is a gram-positive bacterium widely used in the dairy manufacturing industry. Due to its GRAS (generally regarded as safe) status for certain applications, it is often regarded as a promising candidate host for the production of recombinant proteins of therapeutic interest. One such protein is lysostaphin, an endopeptidase that is naturally produced by *Staphylococcus simulans* biovar staphylolyticus ATCC 1362 (34). Lysostaphin specifically cleaves the pentaglycine cross bridges of *Staphylococcus aureus* peptidoglycan and is able to cause cell wall breakdown and consequent cell death. Interest in this antimicrobial enzyme has increased in recent years due to the worsening problem of methicillin (meticillin)-resistant *S. aureus*, and several studies have demonstrated its usefulness in the treatment of infections (3, 4, 17, 27, 28). Recombinant lysostaphin was first produced in *Escherichia coli* in 1987 (32), and more recently, it has been expressed in *L. lactis* using the nisin-controlled gene expression system (21, 22). Recent work in our group has demonstrated the expression and secretion of active lysostaphin in several lactic acid bacteria, including *L. lactis* (39).

Levels of heterologous proteins secreted by *L. lactis* and other lactic acid bacteria are generally low, and efforts have been made to improve the secretion efficiency by modifying secretion signal sequences (5, 31), inactivating proteases (2, 23, 29), or supplying heterologous secretion machinery (26). Insights into protein secretion in *L. lactis* were gained when Nouaille et al. identified 13 genes that affect the secretion efficiency of a staphylococcal nuclease reporter enzyme (NucT) by using random mutagenesis (25). The inactivation of these genes resulted in either increased or decreased levels of secreted NucT. One gene of particular interest was *dltA*, whose product catalyzes the incorporation of D-alanine residues into lipoteichoic acids. The inactivation of *dltA* reduced NucT secretion efficiency due to modifications of the cell wall leading to negatively charged lipoteichoic acid interacting with the positively charged Nuc and physical entrapment of NucT in the peptidoglycan network.

In this study, we have used random transposon mutagenesis to identify genes that affect the extracellular activity of lysostaphin expressed by *L. lactis*. Four uncharacterized genes whose inactivation caused increases in the amounts of lysostaphin secreted by *L. lactis* were identified in this study. One gene encodes a predicted membrane-embedded protein; two genes are likely to affect cell wall peptidoglycan structure; and the fourth gene may be involved in ubiquinone biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The strains, plasmids, and oligonucleotides used are listed in Table 1. *Lactococcus lactis* subsp. *cremoris* MG1363 was grown using M17 (Oxoid, Basingstoke, United Kingdom) supplemented with 0.5% glucose (GM17) or 1% glucose (2GM17) and was incubated at 30°C or 37°C as indicated for the experiments described below. *Listeria monocytogenes* ATCC 19112 and *S. aureus* ATCC 49476 were grown in brain heart infusion medium (Oxoid) and incubated at 37°C. *Escherichia coli* JM109 (Promega, Madison, WI) was used in cloning experiments and was grown in either Luria-Bertani medium or brain heart infusion medium (both from Oxoid). Antibiotics were used at the following concentrations: ampicillin, 100

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Details	Source, reference, or purpose
Strains		
E. coli JM109	Cloning host	Promega
L. lactis MG1363	Plasmid-free *L. lactis* subsp. *cremoris*	10
L. lactis MG1363[*lss*]	MG1363 carrying the *lss* cassette on the chromosome	This study
L. monocytogenes ATCC 19112	Human spinal fluid isolate	ATCC
S. aureus ATCC 49476	Methicillin-resistant *S. aureus*	39
Plasmids		
pBluescript II KS (+)	Amp^r; 3.0 kb	Promega
pGEM-3Zf	Amp^r; 3.2 kb	Promega
pGEM-T Easy	Amp^r; 3.0 kb	Promega
pGhost9:IS*S1*	Em^r *ori* (Ts); 4.6 kb; nonreplicative in *L. lactis* at 37°C	19
pSep-6 × His-Lss	Amp^r; pGEM-3Zf containing the P_{Sep}–ss_{Sep}–six-histidine–*lss* expression construct (5.3 kb)	39
pBS-*his1*	Amp^r; pBS containing a fragment of the MG1363 *his* operon on the 5′ end of the *lss* cassette (4.2 kb)	This study
pGT-*his2*	Amp^r; pGEM-T Easy containing a fragment of the MG1363 *his* operon on the 3′ end of the *lss* cassette (4.2 kb)	This study
pGT-*his1-lss-his2*	Amp^r; pGEM-T Easy vector containing the *lss* cassette within the *his* operon (6.9 kb)	This study
pG9-*his1-lss-his2*	Em^r; pGhost9:IS*S1* containing the *lss* cassette within the *his* operon (7.6 kb)	This study
pSep511sec	Em^r; pGhost:IS*S1* containing the P_{Sep}–ss_{Sep}–six-histidine–Ply511 expression construct (5.5 kb)	39
Oligonucleotides		
His1Sal5	CAGGTCGACCTTTGGGAGTCGCCTTTGGCT	Amplification of *his1*
His1Xba3	TACTCTAGACTGAAACATCAGCCCAGTATA	
His2Xho5	AGACTCGAGGGCGCAAGCGACTATATCCGG	Amplification of *his2*
His2Eco3	TTCGAATTCTTCTTCGCGCTCCTTGCGGTG	
lss-Pst	AACAGCTGCAGGAGCTGCAACACATGAACATTC	39
His2-DS	TGTGATTTGGTACGACGCAGAATTCTAAAGT	Amplification of integrated *lss*
IS*S1*-seq1	CACGATAGCTTAGATTGTAACG	19
IS*S1*-seq2	GAACCGAAGAAATGGAACGCTC	19

μg/ml for *E. coli*; erythromycin, 300 μg/ml for *E. coli* and 5 and 2 μg/ml for plasmid- and chromosome-encoded resistance in *L. lactis*, respectively.

Construction of the *L. lactis* MG1363[*lss*] strain used for random insertional mutagenesis. We constructed an MG1363 strain carrying the lysostaphin expression cassette (*lss*) on the chromosome (Fig. 1). Previously, this cassette (containing the Sep promoter, P_{Sep}; the Sep secretion signal, ss_{Sep}; a six-histidine tag; and the gene encoding the mature lysostaphin protein) has been used to produce and secrete lysostaphin in MG1363 (39). The *lss* cassette was integrated into the same region of the inactive histidine biosynthesis (*his*) operon as previously described (25) (Fig. 1). The strategy used to produce the MG1363[*lss*] strain was as follows. The *lss* cassette was isolated as an XbaI/XhoI fragment from pSep-6 × His-Lss (39) (Table 1). This fragment was ligated to two *his* operon fragments in the same orientation (the upstream *his* fragment from pBS-*his1* digested using SalI/XbaI and the downstream *his* fragment from pGT-*his2* digested using XhoI/EcoRI) and PCR amplified using primers His1Sal5 and His2Eco3 (Table 1). The resulting 3.9-kb PCR fragment was first cloned into the pGEM-T Easy vector (Promega) for sequencing purposes before being cloned into pGhost9:IS*S1*. Both plasmids were transformed into *E. coli* JM109 before the pG9-*his1-lss-his2* (Lss^+ Em^r) plasmid was transformed into wild-type MG1363. Stable integration of *lss* was performed in two steps. First, strain MG1363 with plasmid pG9-*his1-lss-his2* was grown at 37°C and selected on a GM17 agar plate with 2 μg/ml erythromycin. This initial temperature-sensitive integrant, MG1363[pG9-*his1-lss-his2*], was confirmed by PCR using primers lss-Pst and His2-DS (Table 1; Fig. 1) to ensure that the *lss* cassette was inserted in the *his* operon. Primer His2-DS binds to the chromosome 26 bp downstream of the cloned *his2* fragment. It was also confirmed to have lysostaphin activity on GM17+SaB agar plates. These are GM17 agar plates containing 100 μl of autoclaved 100-fold-concentrated *S. aureus* ATCC 49476 cells, which are buffered with 0.2 M potassium phosphate buffer (pH 7.0) (39). The integrant was then made temperature stable by excision of pGhost9:IS*S1* at 30°C. This temperature-stable integrant, MG1363[*lss*] (Fig. 1), was then selected for lysostaphin activity and erythromycin sensitivity.

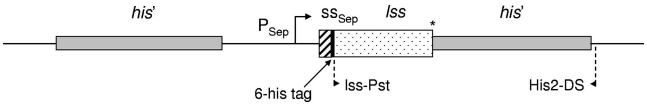

FIG. 1. Structure of strain MG1363 carrying the *lss* expression cassette. The mature lysostaphin gene was fused to the Sep promoter (P_{Sep}) and secretion signal (ss_{Sep}) (hatched box) with a six-histidine tag (filled box) at the N terminus of the lysostaphin gene. The asterisk indicates the lysostaphin stop codon. The *lss* expression cassette was integrated by single-crossover recombination into the middle of the *his* operon in MG1363. PCR primers lss-Pst and His2-DS (dashed arrows) were used to confirm that the *lss* expression cassette was integrated. The pGhost9:IS*S1* plasmid was excised from the chromosome by growing the single-crossover integrant at 30°C without erythromycin and selecting for clones with lysostaphin activity and erythromycin sensitivity.

Mutagenesis and screening conditions. Random insertional mutagenesis was performed on MG1363[*lss*] with pGhost9:IS*S1* as previously described (19). Approximately 50 CFU was plated onto each GM17+SaB or GM17+SaU (unbuffered GM17+SaB) agar plate with 2 μg/ml erythromycin and was incubated at 37°C for 48 h. The mutants were screened for the absence of halos or for halos significantly larger than that produced by the control strain, MG1363[*lss*]. The average diameter of the halo of the control strain, MG1363[*lss*], was 3.4 mm. Larger halos from selected mutants had diameters 0.8 mm to 2.8 mm greater than that produced by the control strain.

Characterization of the pGhost9:IS*S1* insertion sites and isolation of stable IS*S1* mutants. The sites where pGhost9:IS*S1* integrated into the chromosome were determined as previously described (19). The flanking chromosomal DNA

was sequenced using primer IS*S1*-seq1 for the EcoRI chromosomal junction or IS*S1*-seq2 for the HindIII junction (Table 1). The mutated genes were identified by sequence comparison with the recently sequenced genome of MG1363 (42). The number of pGhost9:IS*S1* integrations into the chromosome was determined by Southern hybridization of EcoRI-digested DNA using a digoxigenin-labeled IS*S1* fragment as a probe. For further experiments, we isolated stable IS*S1* mutants that grew at 30°C without erythromycin as previously described (19). These mutants were then retested for their lysostaphin activities by streaking a loopful of overnight cultures onto GM17+SaB agar and GM17+SaU agar and then incubating the cultures at 30°C (or 37°C where indicated) for 2 days.

Prediction of operon structures. To determine if genes containing IS*S1* insertions were cotranscribed with downstream genes, two operon prediction methods were used (9, 30). The accuracy of the method of Price et al. (30), which has been estimated based on the prediction of experimentally proven operons and from microarray expression data, is ≥82% for most genomes. We have identified likely cotranscribed downstream genes that were predicted by at least one of these two methods. The programs are available at http://www.microbesonline.org/operons/ and http://operondb.cbcb.umd.edu/cgi-bin/operondb/operons.cgi.

Prediction of subcellular locations of proteins. Protein sequences were entered into three different online programs: SOSUI, a predictor of transmembrane regions (http://bp.nuap.nagoya-u.ac.jp/sosui/) (14); TMpred, a predictor of transmembrane regions and protein orientation (http://www.ch.embnet.org/software/TMPRED_form.html) (15); and the PHD transmembrane helix predictor (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_htm.html) (33).

Cell fractionation, protein extraction, and Western blot analysis. Cell-associated fractions were prepared by boiling the cells from late-exponential- or early-stationary-phase cultures in 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins in the supernatants were concentrated using 5% trichloroacetic acid as described previously (37). Proteins were separated using SDS-PAGE, transferred to nitrocellulose filters (GE Healthcare), and blocked with 1% casein (Roche Applied Science, Mannheim, Germany). The membrane was probed with either an anti-His_5 monoclonal antibody (Qiagen, Hilden, Germany) at a 1:1,000 dilution or an anti-His_6 monoclonal antibody (Sigma-Aldrich, St. Louis, MO) at a 1:3,000 dilution, followed by a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) at a 1:1,000 dilution. The bound antibodies were detected using the Lumi-Light chemiluminescence kit (Roche Applied Science). To quantify the amounts of proteins, different nonsaturated film exposures were scanned (CanoScan800F; Canon), and the total amounts of proteins from the supernatant and cell-associated fractions were estimated with GeneTools software (Syngene, Frederick, MD). Statistical analysis was carried out using Student's *t* test.

Ply511 expression and secretion in [*lss*] mutant strains. The *L. monocytogenes* bacteriophage endolysin Ply511 was transformed into wild-type MG1363; the control strain, MG1363[*lss*]; and the *lom*[*lss*], *murA2*[*lss*], and *trmA*[*lss*] mutant strains. The Ply511 gene was introduced into these strains using the pGhost9:IS*S1* plasmid under the control of the Sep expression system (pSep511sec) (39). The transformed strains were tested for Ply511 activity against *L. monocytogenes* on GM17+LmB agar plates (GM17 with 5 μg/ml erythromycin and 300 μl of an autoclaved 100-fold concentrate of *L. monocytogenes* ATCC 19112 cells, buffered with 0.2 M potassium phosphate buffer [pH 7.0]) (39). Ply511 was also detected by Western blotting using an anti-His_6 monoclonal antibody (Sigma-Aldrich) as described above.

Lysozyme resistance analysis. The lysozyme resistance test was performed as described previously (40). Briefly, solutions of chicken egg white lysozyme (Sigma Aldrich) in GM17 were freshly prepared and diluted 10-fold into molten GM17 agar at 45°C. Then 5 μl of overnight cultures diluted 10-fold was spotted onto GM17 agar with various concentrations of lysozyme. The plates were then incubated at 30°C or 37°C as indicated.

Transmission electron microscopy. Bacterial cultures grown for 3, 6, or 20 h were fixed in 0.4% glutaraldehyde (ProSciTech)–0.1 M cacodylate buffer (pH 7.3). After overnight fixation, the cells were pelleted and washed in 0.1 M cacodylate buffer. Cells were postfixed in 1% osmium tetroxide and embedded in Spurr epoxy resin. Ultrathin sections (thickness, 50 to 100 nm) were cut and stained with uracyl acetate and lead citrate stains prior to examination and photography using the JEOL 1200 EX transmission electron microscope.

RESULTS AND DISCUSSION

Isolation and identification of mutants with altered lysostaphin activities. The purpose of this study was to identify host factors in MG1363 that affect the secretion efficiency of the peptidoglycan hydrolase lysostaphin, which has specific activity against *S. aureus*. We adapted a previously described method whereby random mutagenesis (using the IS*S1* element in plasmid pGhost9:IS*S1*) was used to identify host factors in the secretion of the staphylococcal nuclease reporter (NucT) in MG1363 (25). We screened more than 35,000 clones for significantly increased or decreased lysostaphin activity on GM17+SaB and GM17+SaU agar plates containing 2 μg/ml erythromycin. On that basis, 124 mutants were selected, and their lysostaphin activities were confirmed using this agar plate method by comparison to the control strain (MG1363[*lss*]). Ten of the 124 initial mutants were confirmed to have halo sizes significantly greater than that of MG1363[*lss*], while 1 mutant was confirmed as having no halo. The locations of inserted pGhost9:IS*S1* were identified by comparison of the flanking sequences with the MG1363 genome sequence (42) (Table 2; Fig. 2). The mutant with no lysostaphin activity resulted from an insertion of pGhost9:IS*S1* in the *lss* expression cassette. This was expected, because IS*S1* insertion is a random event (19). Three mutants had independent insertions in a gene encoding an uncharacterized putative transmembrane protein (llmg_0609); five mutants had independent insertions in the *murA2* gene; one mutant contained an insertion just upstream of *trmA*; and one mutant contained an insertion in a gene encoding an uncharacterized putative enzyme involved in ubiquinone biosynthesis (llmg_2148).

Examination of the MG1363 genome showed that it is possible that the llmg_0609, *murA2*, and llmg_2148 genes could be part of different operons. Downstream genes predicted to be located in these operons by at least one of two computational prediction programs (9, 30) are shown in Table 2. Transcript analysis by Dupont et al. (7) has shown that, due to the presence of a promoter in the IS*S1* element, IS*S1* (and pGhost9:IS*S1*) insertion does not lead to polar effects on downstream genes. Such effects are observed, however, when the IS*S1* sequence, which encodes a putative transposase, is orientated in the same direction as the interrupted genes. In our study, IS*S1* elements were orientated in the same direction as the interrupted genes in two llmg_0609 mutants (2190 and 24185) and two *murA2* mutants (18662 and 31397). In these mutants, it would be expected that the genes downstream would be transcribed and that the phenotypes would not be due to polar effects. In the llmg_2148 mutant, the IS*S1* element is in the opposite orientation to the interrupted gene and therefore may affect the transcription of the downstream *fmt* gene (Table 2). It should also be noted that the IS*S1* element in mutant 16270 has inserted 34 bp upstream of the *trmA* gene and in the opposite orientation and is therefore expected to prevent its transcription.

Plasmid pGhost9:IS*S1* was excised from all mutants to create temperature-stable mutants for further investigation. These temperature-stable mutants were streaked onto GM17+SaB agar (without erythromycin) to reconfirm the lysostaphin activity phenotype. Southern hybridization analysis showed that for each temperature-stable mutant, IS*S1* transposition occurred only at one site (data not shown). The llmg_0609 and llmg_2148 mutants retained their increased lysostaphin activities at 30°C, while large zones of activity were observed only at 37°C for the *murA2* and *trmA* mutants. The amount of lysostaphin secreted, the generation time, and the final culture pH were measured for all mutants (Table 2).

TABLE 2. Characteristics of mutants with lysostaphin activity greater than that of the wild type

Mutant	Gene[a]	Homologous protein	Predicted function[b]	Downstream gene possibly affected by IS*S1* insertion (predicted function)[c]	Proportion of lysostaphin[d] in the following fraction:		Generation time (min)	pH[e]
					Cell associated	Supernatant		
MG1363[*lss*]					1.0 (1.0)	1.0 (1.0)	67 (91)	4.3
2190	llmg_0609	Llmg_0609	Unknown (membrane location predicted)	*greA* (transcription elongation factor)	3.1	9.8	59	4.3
11135					3.1	10.1	61	4.5
24185					2.8	16.1	56	4.4
18662	llmg_0517 (*murA2*)	MurA2	UDP-*N*-acetylglucosamine enolpyruvyl transferase	llmg_0518 (unknown function)	3.0 (0.8)	3.4 (6.2)	63 (75)	4.3
24198					0.7 (2.0)	1.3 (5.4)	62 (76)	4.3
28801					1.3 (ND)[f]	1.3 (7.2)	59 (79)	4.3
31394					ND (ND)	2.4 (6.0)	57 (63)	4.3
31397					ND (ND)	2.8 (6.4)	64 (72)	4.3
16270	llmg_0640	TrmA (Spx)	Temperature resistance; transcription regulation	None	1.9 (1.2)	4.3 (7.2)	77 (55)	4.3
2194	llmg_2148	UbiG	3-Demethylubiquinone-9 3-methyltransferase	*fmt* (methionyl-tRNA formyltransferase)	3.3	6.6	56	4.4

[a] Gene in which pGhost9:IS*S1* integration occurred.
[b] As determined by the annotation of the MG1363 genome available on GenBank.
[c] Downstream genes that are likely located in an operon with the IS*S1*-containing gene were predicted by computational methods (9, 30).
[d] The amount of lysostaphin secreted was determined by Western blot analysis of the total amount of lysostaphin in the cell-associated or supernatant fraction in comparison to that for MG1363[*lss*], which is assigned the index value 1.0. Values in parentheses are measurements taken from cultures grown at 37°C.
[e] Measured from overnight cultures grown in 2GM17 at 30°C.
[f] ND, lysostaphin was not detected in the cell-associated fraction by Western blot analysis.

The increases in the lysostaphin activities of the 10 mutants were determined to be due to increases in the secretion of lysostaphin over that by the control strain, MG1363[*lss*], as determined by Western blot analysis (Fig. 2; Table 2). The levels were determined by direct comparison to the amount secreted by MG1363[*lss*], which was assigned a value of 1.0 (Table 2). The average increases (± standard deviations) in the lysostaphin levels in the cell extracts and supernatants of the three llmg_0609 mutants were 3.0-fold (±0.2-fold) and 12.0-fold (±3.6-fold), respectively; both levels were significantly higher than those of the control strain ($P < 0.01$). The average increase (± standard deviation) in the lysostaphin level in the supernatants of the five *murA2* mutants grown at 37°C was 6.2-fold (±0.7-fold), and the level was significantly higher than that of the control strain ($P < 0.01$).

Characterization of genes that affect lysostaphin secretion. The llmg_0609 gene has been annotated in the MG1363 genome as encoding PabC (42) but is unlikely to function as a 4-amino-4-deoxychorismate lyase like the PabC proteins of *E. coli* and *Bacillus subtilis* (11, 35). It has recently been proposed that the open reading frame of the true *pabC* gene in MG1363 is contained within the llmg_1154 open reading frame as a fusion to *pabB* (41). Therefore, we propose to rename llmg_0609 as *lom* (lysostaphin-oversecreting mutant), in order to avoid confusion. GenBank searches revealed that Lom has significant similarity only to hypothetical proteins or proteins with putative functions not yet experimentally tested. Protein sequence analysis of Lom revealed that a single transmembrane helix was predicted to lie in the central region (amino acids 177 to 195) (Fig. 2) and that the C-terminal region extends outside of the membrane.

MurA2 is a putative UDP-*N*-acetylglucosamine enopyruvyl transferase that catalyzes the first step in the biosynthesis of peptidoglycan (20). As with other low-G+C gram-positive bacteria (6), MG1363 has two MurA enzymes (MurA1 and MurA2) (42). MurA2 in MG1363 is more closely related to enzymes in related species that are annotated as the primary MurA enzyme. For example, MG1363 MurA2 is 61% and 40% identical to the *B. subtilis* MurAA and MurAB proteins, respectively. Similarly, MG1363 MurA2 is 70% and 44% identical to the *Streptococcus pneumoniae* MurA1 and MurA2 proteins, respectively. Interestingly, MG1363 MurA2, *B. subtilis* MurAA, and *S. pneumoniae* MurA1 are more similar to the MurA in *E. coli* and other species with just one MurA than are the MurA paralogs in the gram-positive species. The simplest explanation of this is that MG1363 MurA2, *B. subtilis* MurAA, and *S. pneumoniae* MurA1 are orthologs of *E. coli* MurA and may represent the primary MurA. Du et al. (6) demonstrated that MurA1 and MurA2 were both enzymatically functional and could substitute for each other in *S. pneumoniae*. In contrast, MurAB could not substitute for MurAA in *B. subtilis* (16). The lack of a growth defect in the MG1363 *murA2* mutants (Table 2) would suggest that the MG1363 *murA1* paralog is able to substitute functionally for *murA2*. The hypothesis that the role of MurA2 is distinct from that of MurA1 is supported by previous studies that identified MurA2 proteins in the cytoplasm of wild-type *Lactococcus lactis* subsp. *lactis* IL1403 (12) and, in particular, showed that MurA2 proteins were upregulated in response to acid stress in two MG1363 mutants (1). Since the lysostaphin oversecretion phenotype was observed primarily under heat stress conditions at 37°C, it is possible that MurA1 is not fully functional at this higher temperature and that this allowed lysostaphin to be readily exported through a defective cell wall. However, both exami-

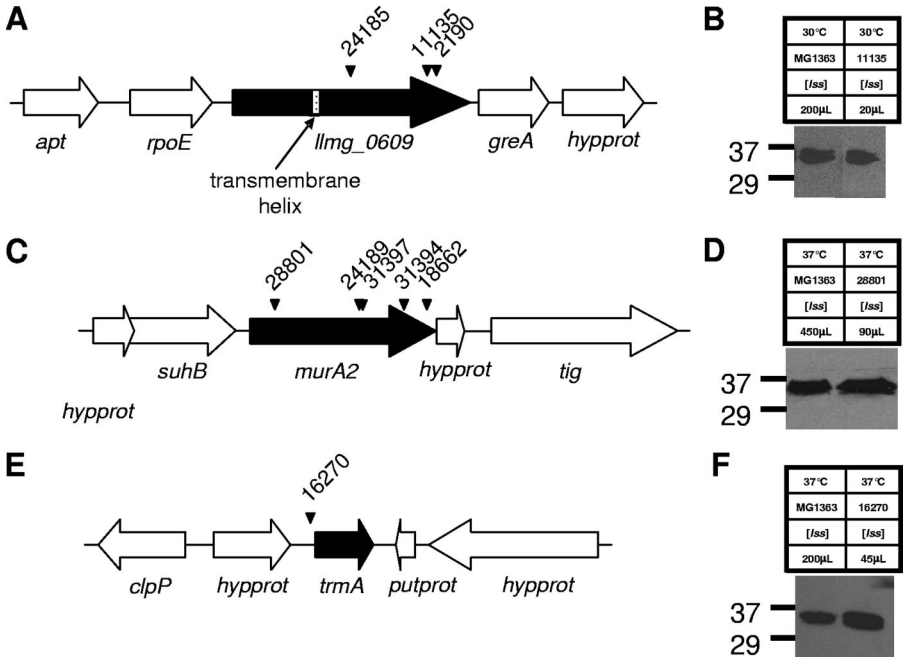

FIG. 2. Identification of the chromosomal loci of the nine lysostaphin-oversecreting mutants. (A) Locations of the three mutants with mutations in the llmg_0609 gene at nucleotide positions 818 (mutant 24185), 1354 (mutant 11135), and 1408 (mutant 2190) from the start of the gene. The predicted single transmembrane helix is located from amino acids 177 to 195 (dotted box). (B) Representative Western blot of lysostaphin in supernatant fractions from MG1363[*lss*] and mutant 11135[*lss*] grown at 30°C. (C) The five independent insertions in the *murA2* gene are located at nucleotide positions 116 (mutant 28801), 753 (mutant 24189), 766 (mutant 31397), 1050 (mutant 31394), and 1220 (mutant 18662). (D) Representative Western blot of lysostaphin in supernatant fractions from MG1363[*lss*] and mutant 28801[*lss*] grown at 37°C. (E) The single mutation in the *trmA* gene (mutant 16270) is located 34 bp upstream of the start of the gene. (F) Representative Western blot of lysostaphin in supernatant fractions from MG1363[*lss*] and mutant 16270[*lss*] grown at 37°C. The variable quantities of supernatants loaded in the Western blots are given in the tables above the blots (B, D, and F).

nation of cell morphology using light microscopy and determination of the thickness of cell walls using transmission electron microscopy failed to reveal any differences between the *murA2* mutants and the MG1363[*lss*] control strain grown at 30°C or 37°C (data not shown). SDS-PAGE analysis of Coomassie-stained proteins from supernatant fractions showed that the *murA2* mutants did not release greater amounts of intracellular proteins than the control strain (MG1363[*lss*]) (data not shown), suggesting that the former are not leaky.

TrmA has homology to Spx, an oxidative stress regulator in *B. subtilis* (24), and seven genes in the *trmA* family are encoded by the MG1363 genome (42). The inactivation of this gene has been reported to confer various stress-resistant phenotypes. Various random mutagenesis studies have identified *trmA* mutants as able to relieve temperature sensitivity in *recA* (8) and *clpP* (13) mutant strains and to confer resistance to tellurite and oxidative stress on the wild type (38). In addition, a *trmA* mutant has increased resistance against the hydrolytic activity of lysozyme over the wild-type strain (40), due to a modified cell wall. Veiga et al. hypothesized that TrmA competes with SpxB, one of its paralogs, for the binding of RpoA, the α subunit of RNA polymerase (40). The inactivation of *trmA* may allow SpxB to better bind RpoA, and this interaction activates the expression of *oatA*, encoding the lactococcal peptidoglycan *O*-acetylase, thus modifying the cell wall so that it becomes lysozyme resistant. The reason for the increased lysostaphin secretion levels of the *trmA* mutant is not clear, but as with the *murA2* mutant, they may be due to an altered cell wall structure.

Finally, one of the mutants selected from the screening had an inactivation in the gene annotated as llmg_2148. The basis for the increased lysostaphin secretion of this mutant remains unclear. This gene is annotated as a putative enzyme, 3-demethylubiquinone-9 3-methyltransferase (UbiG) (42), which in *E. coli* is involved in ubiquinone biosynthesis (36). However, llmg_2148 has no homology to functionally characterized UbiG enzymes in databases.

The *lom*, *murA2*, and *trmA* mutants oversecrete another hydrolytic enzyme, Ply511. To examine whether this increase in lysostaphin secretion is specific to lysostaphin, we compared the abilities of the wild-type MG1363, the control strain (MG1363[*lss*]), and the lysostaphin-secreting mutant strains (*lom*[*lss*], *murA2*[*lss*], and *trmA*[*lss*]) to express another heterologous protein, Ply511. Ply511 is a peptidoglycan-hydrolytic endolysin (*N*-acetylmuramoyl-L-alanine amidase) from an *L. monocytogenes* bacteriophage (18). All the strains transformed with the Ply511 expression cassette pSep511sec (Table 1) demonstrated activity against both *L. monocytogenes* and *S. aureus*, as evidenced by zones of clearing on agar plates containing autoclaved cells. We compared the zones of activity of Ply511 on GM17+LmB agar plates but observed no differences in the activity zones between the control strain and the mutant strains (data not shown). Western blot analysis, however, conclusively demonstrated that the amounts of Ply511 secreted into the

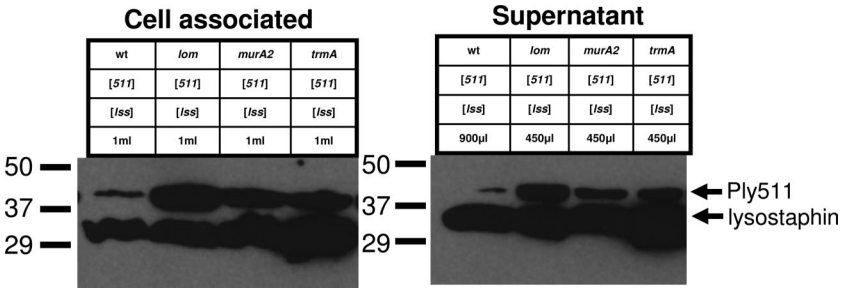

FIG. 3. Western blot detection of MG1363 strains secreting lysostaphin and/or Ply511 in the cell-associated and supernatant fractions. The different strains and the quantities loaded on the Western blots are given in the tables above the blots. Molecular mass standards are expressed in kilodaltons. The amount of cell-associated protein is equivalent to 1 ml of late-exponential-phase culture. The amount of supernatant protein loaded in each lane is equivalent to 900 μl of late-exponential-phase culture for the control strain (MG1363[*lss*][*511*]) and 450 μl for mutant strains. The Ply511 protein resolved at approximately 40 kDa. The lysostaphin protein resolved at approximately 31 kDa. The Ply511 protein can be observed in the cell-associated fraction and supernatant of the control strain after longer exposures. wt, wild type.

cell-associated and supernatant fractions by the *lom*[*lss*][*511*], *murA2*[*lss*][*511*], and *trmA*[*lss*][*511*] mutant strains were significantly greater than those for the control strain, MG1363 [*lss*][*511*] (Fig. 3). The lack of correlation between Western blot analysis and clearing zone size for Ply511 may be because the zone size for wild-type *Lactococcus* expressing Ply511 (MG1363[*lss*][*511*]) is already very large (compared to clearing zones from lysostaphin activity) and therefore may have saturated the assay, despite the fact that only very low levels of enzyme were produced. The large clearing zones generated by Ply511 could be due to its ability to diffuse through the bacterial-cell-containing agar more efficiently than lysostaphin.

***murA2* and *trmA* mutants were more resistant to lysozyme hydrolysis.** A recent study has shown that a mutation in *trmA* results in lysozyme resistance in MG1363 (40). In this study, we found that a *trmA* mutant that expresses lysostaphin (*trmA*[*lss*]) is also more resistant to lysozyme than the wild-type lysostaphin-expressing strain (MG1363[*lss*]) (Fig. 4). Interestingly, we observed that MG1363[*lss*] was much more sensitive to lysozyme than the non-lysostaphin-secreting wild-type strain, MG1363 (Fig. 4B). This result suggests that lysostaphin may be cleaving lactococcal peptidoglycan during its passage through the cell wall, thereby heightening the sensitivity to lysozyme. According to Veiga et al. (40), the resultant interactions within a *trmA* mutant give rise to changes in the acetylation and thickness of peptidoglycan, thereby making it more resistant to lysozyme hydrolysis. Therefore, the *trmA* mutant may also be more resistant to the nonspecific degradation caused during the secretion of lysostaphin and, as a result, may be able to tolerate higher levels of protein secretion. We also observed that the *murA2*[*lss*] mutant strain was moderately more resistant to lysozyme than the control strain (MG1363[*lss*]) and that the lysozyme resistance of wild-type MG1363 was greater at 30°C than at 37°C (Fig. 4B and C). Therefore, protection from cell wall-lytic enzymes (lysozyme and lysostaphin) is likely to be attributable to modifications in the peptidoglycan structure resulting from mutations in *trmA* and *murA2*; however, this has yet to be confirmed.

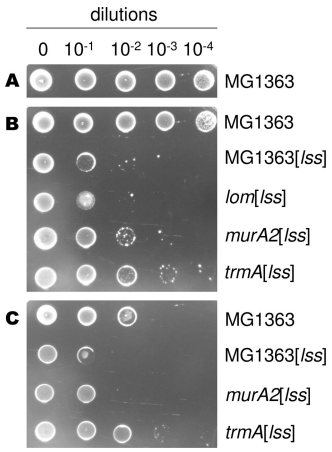

FIG. 4. The *murA2*[*lss*] and *trmA*[*lss*] mutant strains are more resistant to lysozyme hydrolysis than the control strain, MG1363[*lss*]. The wild-type strain, MG1363, is more resistant than the control strain, which expresses lysostaphin. All strains showed identical growth on GM17 agar plates without lysozyme at 30°C and 37°C. Therefore, only the growth of MG1363 is shown. Strains were grown at 30°C without lysozyme (A) or with 0.25 mg/ml lysozyme (B) or at 37°C with 0.25 mg/ml lysozyme (C).

In conclusion, we have identified four genes involved in the secretion of lysostaphin in MG1363. These genes are different from those previously identified in the NucT study (25), suggesting that lactococcal host factors that affect the secretion of heterologous proteins differ depending on the protein of interest. While the mechanisms remain unknown, the inactivation of these four genes significantly increased the amount of lysostaphin secreted without any detrimental effects to the host cell and the growth rate. We also describe the construction of novel MG1363 strains that are able to secrete two kinds of peptidoglycan hydrolases, lysostaphin and Ply511. The results of this study clearly provide new insights into lactococcal host factors important for the secretion efficiency of heterologous proteins, which may have applications in the food and pharmaceutical industries.

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