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Influence of Lipoteichoic Acid D-Alanylation on Protein Secretion in *Lactococcus lactis* as Revealed by Random Mutagenesis

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***Lactococcus lactis*, a food-grade nonpathogenic lactic acid bacterium, is a good candidate for the production of heterologous proteins of therapeutic interest. We examined host factors that affect secretion of heterologous proteins in *L. lactis*. Random insertional mutagenesis was performed with *L. lactis* strain MG1363 carrying a staphylococcal nuclease (Nuc) reporter cassette in its chromosome. This cassette encodes a fusion protein between the signal peptide of the Usp45 lactococcal protein and the mature moiety of a truncated form of Nuc (NucT). The Nuc secretion efficiency (secreted NucT versus total NucT) from this construct is low in *L. lactis* (~40%). Twenty mutants affected in NucT production and/or in secretion capacity were selected and identified. In these mutants, several independent insertions mapped in the *dltA* gene (involved in D-alanine transfer in lipoteichoic acids) and resulted in a NucT secretion defect. Characterization of the *dltA* mutant phenotype with respect to NucT secretion revealed that it is involved in a late secretion stage by causing mature NucT entrapment at the cell surface.**

Lactococcus lactis is widely used in the food industry and is considered a good candidate for production of heterologous proteins for developing nutraceuticals or new live vaccine strategies. Numerous genetic tools have been developed for gene expression and protein secretion in *L. lactis* (14), and many heterologous proteins have already been produced in *L. lactis*, including bacterial and viral antigens (3, 39, 48) and enzymes and cytokines (2, 43). Recently, efficient food-grade production systems were developed in *L. lactis* (19, 42). However, so far there have been few analyses of lactococcal host factors involved in heterologous protein production and secretion machinery.

Factors that affect secretion efficiency (secreted NucT versus total NucT) include elements of the secretion machinery itself, as well as factors involved in protein stability or degradation and folding. Furthermore, conditions that alter the microenvironment at the cell surface may affect protein folding and release into the culture medium (29, 45). For the *L. lactis* secretion machinery, knowledge of the lactococcal genetic apparatus required for secretion is essentially limited to genome sequence information derived from *L. lactis* strain IL1403 (7). Several *sec* genes were revealed by sequence homology (7). Other putative and/or unidentified lactococcal genes may also be involved in the secretion process. It is notable that SecDF, which is involved in the late steps of translocation, is absent in *L. lactis*. Also, *L. lactis* has only a single signal peptidase for nonlipoproteins, compared to six such enzymes for the gram-positive paradigm organism *Bacillus subtilis* (46). The unique signal peptidase and the absence of SecDF are potential bottlenecks when an *L. lactis* strain is engineered for high-level secretion. *L. lactis* encodes a unique cell surface protease, HtrA. An *htrA* mutant stabilizes production of several heterologous exported proteins or fusion proteins in *L. lactis* (31, 36).

We developed a novel mutagenesis screening procedure based on levels of secretion of the staphylococcal nuclease (Nuc) (41) to identify accessory genes involved in secretion. Nuc has been used as a secretion reporter in *L. lactis* and other lactic acid bacteria (15, 26–28). The strain developed for this mutagenesis procedure carries a single copy of the stable expression cassette integrated in the chromosome. This work is the first random insertional mutagenesis analysis reported in which accessory genes involved in the production and secretion of heterologous proteins in *L. lactis* were identified. In the mutants isolated by this novel screening method, several independent insertions were mapped to the *dltA* gene and resulted in a NucT secretion defect. Characterization of *dltA* inactivation demonstrated that cell wall modifications affect cell viability and late-stage protein secretion.

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MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions. *L. lactis* subsp. *cremoris* MG1363 (17) and *Escherichia coli* TG1 (18) [*supE hsd*-5 *thi* Δ(*lac-proAB*) F′(*traD6 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] and TG1*rep* (containing a chromosomal copy of the pWV01 *repA* gene; kindly provided by K. Leenhouts) were used as bacterial hosts. The plasmids used are listed in Table 1. *E. coli* was grown on Luria-Bertani medium (40) and incubated at 37°C with vigorous shaking. *L. lactis* was grown in M17 medium (44) containing 0.5% glucose (GM17) or on brain heart infusion (Difco) when Nuc detection was required. Most *L. lactis* cultures

TABLE 1. Plasmids used

Plasmid (replicon)	Characteristics	Reference
pBS:UNuc4 (ColE1)	Ap^r, gene expressed from P_{usp} encodes SP_{Usp}:NucTprecursor	28
pBS:*his* (ColE1)	Ap^r, fragment of MG1363 *his* operon	12, 13
pBS:*his*:*uspnucT*:*his* (ColE1)	Ap^r, contains P_{usp}-SP_{Usp}:*nucT* in the *his* operon	This study
pGHost9 (pWV01, rep^{Ts})	Em^r, nonreplicative in *L. lactis* at 37°C	30
pGHost9:IS*SI* (pWV01, rep^{Ts})	Em^r, lactococcal IS*SI*, nonreplicative in *L. lactis* at 37°C	30
pGHost9:*his*:*uspnucT*:*his* (pWV01, rep^{Ts})	Em^r, contains P_{usp}-SP_{Usp}:*nucT* in the *his* operon	This study
pSEC:NucT (pWV01)	Cm^r, contains P_{nis}-SP_{Usp}:*nucT*	28
pIL:*nisRK* (pAMβ1)	Em^r, contains *nisRK* nisin regulatory genes	23

were incubated at 30°C; the only exceptions were cultures used during pGhost9:IS*S1*-mediated mutagenesis (see below). Antibiotics were added at the following concentrations: erythromycin, 150 μg/ml for *E. coli* 5 and 2.5 μg/ml for plasmid and chromosome-encoded resistance in *L. lactis*, respectively; ampicillin, 100 μg/ml for *E. coli*; and chloramphenicol, 5 μg/ml for *L. lactis*. Induction with nisin (Sigma) was performed at a concentration of 1 ng/ml for 1 h with cultures at an optical density at 600 nm (OD_{600}) of ~0.5.

DNA manipulations. The general procedures for DNA manipulation were performed as described previously (40), with the following modifications. Chromosomal DNA extraction from *L. lactis* involved addition of lysozyme (10 mg/ml) to prepare protoplasts and then addition of proteinase K (0.8 mg/ml) to eliminate mature Nuc forms associated with protoplasts prior to cell lysis. For plasmid DNA extraction (4) from *L. lactis*, TES (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl [pH 8]) containing lysozyme (10 mg/ml) was added for 10 min at 37°C to prepare protoplasts. Enzymes were used as recommended by the suppliers. Electroporation of *L. lactis* was performed as described previously (25), and transformants were plated on GM17 agar plates containing the required antibiotic.

Preparation of protein extracts, Western blotting, and immunodetection. Cellular and supernatant protein fractions of *L. lactis* were prepared and concentrated as described previously (27). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotting onto polyvinylidene difluoride membranes (Millipore), and immunoblotting were performed as described previously (40) or according to the suppliers' recommendations. Antibodies against Nuc, raised in rabbits (Eurogentec), were used at a dilution of 1:1,000. Immunodetection was performed with protein G-horseradish peroxidase conjugate (Bio-Rad) and a chemiluminescence kit (Dupont-NEN) used as recommended by suppliers. After enhanced chemiluminescence detection, different nonsaturated film exposures were scanned (Scanjet 7400C; Hewlett-Packard). Amounts of mature NucT and preNucT were estimated with the ImageQuant program. For liberation of cell-associated NucT, 2-ml portions of overnight cultures were pelleted, and the cells were washed with TES and incubated for 15 min on ice with 10% trichloroacetic acid in order to stop cellular metabolism instantaneously. (Note that milder treatments, such as the use of glucose analogs, were not rapid enough to block metabolism and the secretion process.) The cells were then washed with TES, treated or not treated with 10 mg of lysozyme per ml (5 min at 37°C) for partial digestion of the cell wall, and washed with TES. Cell wall-associated Nuc was released by incubation in 1 ml of glycine-NaOH buffer (pH 9 or 10) (wash buffer) for 15 min at room temperature with gentle shaking. The proteins present in the cell and wash buffer were treated as described previously (27). The nisin MIC (i.e., the lowest nisin concentration that inhibited bacterial growth) was determined as follows. An overnight culture was diluted 200-fold in fresh GM17 and incubated for 90 min at 30°C, and different amounts of nisin (ranging from 10 to 100 ng/ml) were added. The OD_{600} of overnight cultures were then determined. Preparation of protein samples and adjustment of the concentration to an OD_{600} of 1 were performed as described previously (27).

Construction of the *L. lactis* strain used for random insertional mutagenesis. To screen for *L. lactis* mutants affected in Nuc secretion, we constructed an MG1363 derivative strain carrying a *nuc* cassette on the chromosome. It has been reported previously that native Nuc is efficiently secreted via the signal peptide of the lactococcal protein Usp45 (SP_{Usp45}) (28). In contrast, deletion of 17 of the 21 amino acids of the Nuc propeptide (referred to as NucT) results in impaired secretion. For this construct, the majority of the precursor remains cell associated (28). The strategy used to obtain the *L. lactis* strain used for mutagenesis is as follows. The *uspnucT* cassette (containing the *usp45* promoter [P_{usp}] and SP_{Usp45}) was isolated on a *Bam*HI/*Eco*RI fragment from pBS:UNuc4 (28) (Table 1). This cassette was blunted and cloned into *Sna*BI-linearized pBS:*his* (13), resulting in pBS:*his*:*uspnucT*:*his* (with *his* and *uspnucT* in the same orientation). In this plasmid, the *uspnucT* cassette is flanked by a 1.2-kb fragment (5′ end) and a 1.1-kb fragment (3′ end) of the inactive histidine biosynthesis (*his*) operon (12) (Fig. 1A). The *his*:*uspnucT*:*his* cassette was then isolated on an *Eco*RI fragment, cloned into *Eco*RI-digested pGhost9 (30), and established in *E. coli* TG1*rep*. The resulting plasmid, pGhost9:*his*:*uspnucT*:*his* (Nuc^+ Em^r), was then established in wild-type *L. lactis* subsp. *cremoris* MG1363. In spite of the lack of a complete genome sequence for MG1363, we used this strain instead of *L. lactis* subsp. *lactis* IL1403, which contains six prophages inducible by various stresses (9a). Furthermore, A. Bolotin confirmed that the genes which we studied were quite similar in these two *L. lactis* model strains (unpublished data). Stable integration of the *uspnucT* expression cassette by a double-crossover recombination event into the *L. lactis* chromosome was performed as described previously (5). The resulting strain, MG[*uspnucT*], was verified by Southern blotting by using a ^{32}P radiolabel (Ready-to-Go kit; Pharmacia Biochemicals) and a DNA fragment encoding *uspnucT* as the probe. Growth was not affected by the *uspnucT* integration (data not shown). NucT expressed from the chromosome had a secretion efficiency (as evaluated by Western blot analysis) comparable to the secretion efficiency when NucT was expressed from a plasmid (28). NucT was poorly secreted from MG[*uspnucT*]; the majority of SP_{Usp45}:NucT was found in the precursor form in the cell fraction (Fig. 1B).

Mutagenesis and screening conditions. Plasmid pGhost9:IS*S1* was introduced into MG1363[*uspnucT*]. Mutagenesis with pGhost9:IS*S1* was performed essentially as described previously (30). Cells were plated in order to obtain about 300 CFU/petri plate. The plates were overlaid with TBD agar to reveal halos caused by Nuc enzymatic activity (26). From the colonies screened, mutants having significantly larger or smaller halos were selected for further examination.

Determination of the pGhost9:IS*S1* integration loci in MG[*uspnucT*] and pGhost9:IS*S1* excision procedure. Plasmid pGhost9:IS*S1* contains unique sites adjacent to the IS*S1* element (*Eco*RI and *Hin*dIII) that were used after transposition to clone chromosomal DNA flanking the pGhost9:IS*S1* insertion site (referred to below as junctions) (30). Chromosomal junctions were sequenced

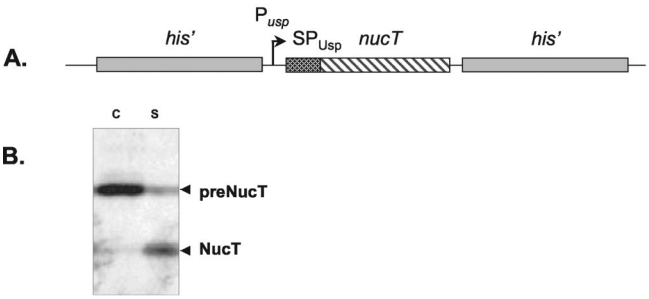

FIG. 1. Structure and secretion phenotype of the *L. lactis* strain carrying the *uspnucT* expression cassette. (A) *nucT* was fused to the Usp45 promoter (P_{usp}) and the signal peptide (SP_{Usp}). The *uspnucT* expression cassette was integrated by double crossover in the middle of the *his* operon in MG1363. (B) Secretion profile for NucT in MG[*uspnucT*] as analyzed by Western blotting with Nuc-specific antibodies. Protein samples in cell (lane C) and supernatant (lane S) fractions were prepared from an overnight culture of MG[*uspnucT*]. The majority of NucT remained in the cell fraction in the precursor form (preNucT), whereas only about 40% was processed to the mature form (NucT) and released into the culture medium.

TABLE 2. Genes identified by sequence similarity and secretion phenotype

Mutant	Gene[a]	Gene function[b]	preNucT[c]	NucT[c]	Secretion efficiency[f]	Growth rate (h^{-1})
VEL11461	*uspnucT*	Staphylococcal nuclease reporter gene	1	1	30	2.5
2[d]	*dltA*	LTA D-alanylation	1	0.6	26	2.4
5[e]	*dltA*[e]	LTA D-alanylation	1	0.7	30	2
7	*dltA*	LTA D-alanylation	1	0.7	25	1.8
12	*dltA*	LTA D-alanylation	1	0.6	18	1.8
17[d]	*dltA*	LTA D-alanylation	1	1.1	34	2.3
15	*celB*	Cellobiose-specific PTS system component IIC	0.4	1.2	58	2
19	*ptnC*	Mannose-specific PTS system component IIC	0.6	1.7	54	2.1
18	*ptnD*	Mannose-specific PTS system component IID	0.6	1.3	54	2.1
25	*ptnD*	Mannose-specific PTS system component IID	0.9	1.8	44	1.7
35[d]	*rgrA*	Trehalose operon repressor	1.4	1.6	36	2.2
3	*ssbA*	DNA recombination and repair	1.2	0.7	24	1.8
26	*vacB2*	RNase	0.7	2.5	58	1.5
8	*ybdD*	Unknown	1.3	2.7	44	2.7
27	*ybdD*	Unknown	1.2	2.6	47	2.3
32	*ybdD*	Unknown	1.2	2.4	46	2.5
33	*yccF*	Unknown	1.3	0.4	43	2.5
16	*ypcH*	Unknown	0.5	1.5	61	2.4
11	*yqjB*	Unknown	1	1.6	44	2.1
24	*ythA*	Unknown	1.2	1.3	29	2.2
31	*yuaE*	Unknown	0.4	1.1	61	1.7

[a] Gene in which pGhost9:IS*S1* integration occurred.
[b] Gene function as determined by sequence similarity (7).
[c] The phenotype of each mutant was confirmed by Western blot analysis by determining the amount of the precursor form detected inside the cells or the amount of the mature NucT form secreted into the culture medium in comparison with the MG [*uspnucT*] secretion profile. The index 1 was assigned to preNucT and NucT productions in VEL11461.
[d] Two pGhost9:IS*S1* plasmids were integrated into the chromosome.
[e] pGhost9:IS*S1* integration occurred in the *thiE-dltA* intergenic region.
[f] Secretion efficiency, secreted NucT versus total NucT detected.

(with an ABI Prism 310 genetic analyzer) by using primers present on the IS*S1* element and directed towards the cloned chromosomal junction (pEco for the *Eco*RI junction [5′-ATAGTTCATTGATATATCCTCGCT]; pHind for the *Hin*dIII junction [5′-TCGGTATCTACTGAGATTAAGGTC]). Genes that were mutated in *L. lactis* subsp. *cremoris* MG1363 were identified by comparison of the sequenced junction with the closely related *L. lactis* subsp. *lactis* IL-1403 genome sequence (7; http://spock.jouy.inra.fr). The number of pGhost9:IS*S1* integrations into the chromosome was determined by Southern blotting by using a ^{32}P-radiolabeled IS*S1* fragment as a probe.

Excision of pGhost9:IS*S1* from the initial integration locus resulted in a stable IS*S1* insertional mutant that grew at 30°C without selective pressure (30). Stable erythromycin-sensitive excision derivatives of the candidate secretion mutants were first tested to determine their Nuc phenotypes by depositing 5-μl portions of culture supernatants on TBD agar medium to detect Nuc activity. They were confirmed by Western blot analysis and Nuc immunodetection.

RESULTS

Mutagenesis and identification of transposition loci. Random mutagenesis was performed on the MG[*uspnucT*] strain by using plasmid pGhost9:IS*S1*. Colonies were screened by the Nuc plate assay for increased or decreased amounts of secreted NucT. Of 35,000 mutant clones, 200 were selected after the first screening step. As the halo size for Nuc activity may vary widely with the colony size, mutant colonies were patched onto brain heart infusion agar medium and subjected to a second screening step. After this step, 34 of the 200 initial mutants exhibited a halo size significantly different from that of the parent, strain MG[*uspnucT*]. These 34 mutants were analyzed further by Western blotting and Nuc immunodetection by using overnight cultures, and the secretion profiles were compared to that of MG[*uspnucT*]; 20 mutants exhibited significantly different NucT secretion patterns. Junctions of these mutants were cloned, and sequences of the interrupted genes were determined by sequence comparison with the IL1403 genome sequence (7). The secretion phenotypes (as determined by Western analysis) and the genes identified by sequence similarity are listed in Table 2. For the parental strain used as a control, an index value of 1 was assigned for the amounts of preNucT and mature NucT. For each mutant, the amounts of preNucT and mature NucT were estimated as described in Materials and Methods and compared to the amounts obtained for the parental strain (Table 2). Furthermore, the secretion efficiency of NucT (secreted NucT/total Nuc forms detected) was determined. In addition, the growth rates of all 20 mutants were determined (Table 2). For six mutants, no sequence similarity with the reference IL1403 genome sequence or with the public databases was found. Eight integrations occurred in putative open reading frames having unknown functions, and three independent integrations occurred in *ybdD*; the *ybdD* mutants contained increased amounts of the precursor preNucT and of NucT (S. Nouaille, E. Morello, Y. Le Loir, J. Commissaire, A. Bolotin, A. Gruss, and P. Langella, unpublished data). Four mutations occurred in genes encoding putative components of the phosphotransferase systems in *E. coli*; three of the mutations were in the mannose-specific phosphotransferase system, and one was in the cellobiose-specific phosphotransferase system. These four mutations led to slightly improved secretion efficiency, which may have been due to changes in metabolism rather than to direct involvement of the genes in the secretion process. Mutant 35 produced increased amounts of both preNucT and NucT and resulted from double integration of pGhost9:IS*S1*;

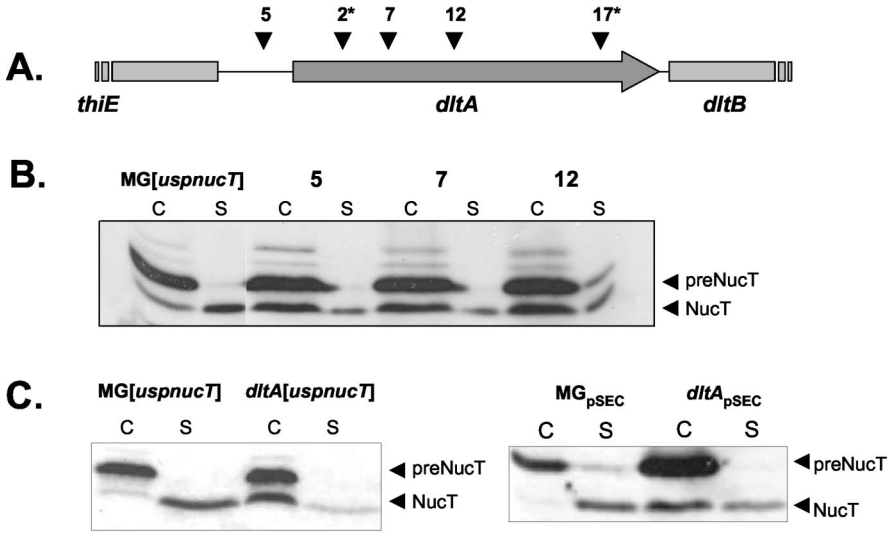

FIG. 2. *dltA* mutations and phenotypes of the *L. lactis* mutants. (A) Positions of IS*S1* insertions in the five *L. lactis dltA* mutants. Four independent pGhost9:IS*S1* insertions occurred in the coding sequence of *dltA* at nucleotide positions 369 (mutant 2), 450 (mutant 7), 649 (mutant 12), and 1215 (mutant 17) with respect to the ATG start codon. One integration event occurred 141 nucleotides upstream of the *dltA* ATG start codon (mutant 5). An asterisk indicates a mutant in which another pGhost9:IS*S1* integration occurred. (B) Comparison of Nuc secretion profiles in *dltA* and MG[*uspnucT*] strains. At 37.5°C, the three single *dltA* mutants exhibited the same Nuc phenotype. There was no difference in the amounts of preNucT observed in the mutants and the parental strain. A reduced amount of NucT was detected in the culture medium of all mutants, whereas the majority of NucT was detected in the cell fraction. (C, left panel) NucT secretion profile of *dltA*[*uspnucT*] was conserved when the organism was grown at 30°C without erythromycin. (C, right panel) Secretion phenotype of *L. lactis dltA*[*uspnucT*] overproducing NucT. NucT secretion was analyzed by Western blotting by using Nuc-specific antibodies after 1 h of nisin induction of MG[*uspnucT*](pSEC:NucT/pIL:*nisRK*) and *dltA*[*uspnucT*](pSEC:NucT/pIL:*nisRK*). Overexpression of NucT did not alter the secretion profile in the parental strain or in the *dltA* mutant. C, cell fraction; S, supernatant fraction; MG_{pSEC}, MG[*uspnucT*](pSEC:NucT/pIL:*nisRK*); $dltA_{pSEC}$, *dltA*[*uspnucT*](pSEC:NucT/pIL:*nisRK*).

one of the insertions was in a gene similar to *rgrA* encoding the repressor of the trehalose operon in *B. subtilis*. The other integration locus was not identified. Two mutants (mutants 3 and 26) were affected in genes involved in nucleic acid metabolism. The effect of an *ssbA* mutation (mutant 3) on NucT secretion might have been indirect and may have resulted from a general defect in DNA metabolism. The effect of a *vacB2* mutation (mutant 26) on NucT may have been linked to an increased half-life of mRNAs (for *nucT* or other genes involved in the secretion process) in the cell. Five independent pGhost9:IS*S1* integrations mapped in the *dltA* gene, the first gene of the *dlt* operon, which comprises four genes (*dltA*, *dltB*, *dltC*, and *dltD*) that catalyze the incorporation of D-alanine residues into lipoteichoic acids (LTA) (see below) involved in the net global charge of the bacterial surface. In this work, we focused on analysis of the influence of the *dlt* operon on protein secretion in *L. lactis*. The growth curves, generation times, and final pH and biomass values for the three *dltA* single-insertion mutants were the same as those for the parental strain (data not shown).

These results show that the screening procedure in which the NucT reporter was used was effective for identification of 20 putative secretion mutants in *L. lactis*.

Effects of *dltA* disruption on NucT secretion. The *dlt* operon is required for D-alanylation of LTA in various gram-positive bacteria (1, 8, 10, 32, 34, 35). One of the five *dltA* insertions (Fig. 2A) occurred just upstream of the *dltA* start codon, and four occurred in the *dltA* coding sequence. In the latter cases, two mutants experienced double integration events; the second integration loci were not identified. However, the Nuc phenotype of these mutants was similar to that of the *dltA* single mutants, suggesting that a *dltA* mutation is responsible for the secretion phenotype. Only the three mutants with a single integration event in *dltA* (mutants 2, 7, and 12) were analyzed further. All of these mutants were impaired for NucT secretion, as determined by the Nuc plate assay, suggesting that the *dlt* operon is relevant in secretion. NucT secretion in these mutants was analyzed by Western blotting by using anti-Nuc antibodies and was compared to NucT secretion in the MG[*uspnucT*] parental strain (Fig. 2B). All three mutants displayed similar NucT secretion profiles: (i) the amounts of cell-associated preNucT in the cell fraction were the same as the amount in MG[*uspnucT*]; (ii) the amounts of the secreted mature form in the supernatant were reduced, consistent with observations made with the Nuc plate assay; and (iii) strikingly, the major part of mature NucT was cell associated (Fig. 2B). As the *dltA* mutant phenotypes were similar, we selected *dltA* mutant 7, containing the most 5′-proximal pGhost9:IS*S1* insertion, for further characterization. The pGhost9:IS*S1* excision mutant, *dltA*[*uspnucT*], exhibited the same phenotype as the initial mutant 7 (data not shown). Comparison of Nuc secretion in *dltA*[*uspnucT*] and MG[*uspnucT*] at 30°C without erythromycin showed that the initial Nuc phenotype was due neither to high temperature (37.5°C) nor to erythromycin selection (Fig. 2C, left panel).

In *dltA*[*uspnucT*], the majority of mature NucT was cell associated. This phenotype differs from the phenotype of the parent, MG[*uspnucT*], in which all of the mature NucT was

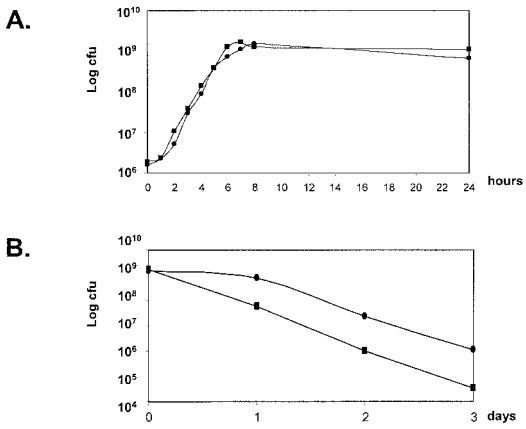

FIG. 3. (A) Growth of *L. lactis* MG[*uspnucT*] and *dltA*[*uspnucT*]. MG[*uspnucT*] (●) and *dltA*[*uspnucT*] (■) were grown in GM17. Viable cell counts (CFU per milliliter) were determined by plating on GM17 agar. (B) Long-term survival of *L. lactis* MG[*uspnucT*] and *dltA*[*uspnucT*]. On days 1, 2, and 3 after cultures reached the stationary growth phase, viable cell counts (CFU per milliliter) were determined by plating on GM17 agar.

efficiently released into the supernatant (Fig. 2C). These results show that IS*1* integration in *dltA* led to modification of the distribution of mature NucT between the cell and supernatant fractions. In the case of *dltA*, the maturation efficiency was identical to that of the parental strain, but the sensu stricto secretion efficiency was lower because mature NucT was retained at the cell surface instead of being secreted into the culture medium.

Effects of the *dltA* mutation on growth, survival, and nisin sensitivity. *dltA* inactivation affects the charge balance of LTA at the cell surface. It reportedly influences viability, cell shape, autolytic enzyme concentration within the cell envelope, and sensitivity to cationic antimicrobial peptides, as previously reported for other gram-positive species, such as *B. subtilis*, *Streptococcus gordonii*, *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Listeria monocytogenes* (1, 10, 35, 38, 47). Light microscopy of the MG[*uspnucT*] and *dltA*[*uspnucT*] strains did not reveal differences in morphology or chain length in exponential- or stationary-phase cells (data not shown). A zymogram analysis performed with supernatant and cell fractions did not reveal any differences in quantity, localization, or processing of the major autolysin AcmA (9) between the two strains, in accordance with the similar chain lengths observed (data not shown).

The growth rates and long-term survival of the MG[*uspnucT*] and *dltA*[*uspnucT*] strains were determined in GM17 at 30°C by plate counting. No significant difference was observed between MG[*uspnucT*] growth and *dltA*[*uspnucT*] growth (Fig. 3A). Long-term survival was estimated on days 1, 2, and 3 after cultures reached the stationary growth phase (Fig. 3B). The *dltA*[*uspnucT*] viability in stationary-phase cultures was reduced compared to that of MG[*uspnucT*]. After 3 days, there was a 35-fold difference in the survival of the two strains (1.15×10^6 CFU $ml^{-1}$for MG[*uspnucT*] versus 3.3×10^4 CFU $ml^{-1}$for *dltA*[*uspnucT*]). This loss of viability is less drastic than that reported for other gram-positive *dltA* mutants (47). As LTA is more negatively charged in a *dltA* mutant than in a wild-type strain, *dltA* mutants are expected to be more sensitive to cationic antimicrobial peptides (such as nisin) present in the medium. To test this, the MIC of nisin was determined for both strains and was found to be 60 ng/ml for MG[*uspnucT*] and 35 ng/ml for *dltA*[*uspnucT*]. The difference between the nisin MIC for the *dltA* mutant of *L. lactis* and the wild type is similar to the difference that was recently observed for a *dltA* mutant of *S. agalactiae* and the wild type (38). The nisin MIC were evaluated for *dltA* mutants of several other gram-positive species, and the values were 2- to 4,000-fold less than the values for the wild-type strains (1, 35, 38). These results may indicate that other charged moieties are present on the cell envelope, which compensate for the negative charges associated with the *dltA* mutation.

Altogether, these results show that the *L. lactis dltA* mutant is not affected in growth but is slightly affected in long-term survival and in sensitivity to nisin compared to the wild-type strain.

Effect of the *dltA* mutation when Nuc is overproduced. Nuc secretion phenotypes described above were obtained for a strain expressing a single chromosomal copy of *uspnucT*. Overexpression of NucT was achieved by introducing pSEC:NucT into *dltA*[*uspnucT*] (28); in this construct, *uspnucT* expression is under control of the nisin-inducible P_{nisA} promoter and is regulated by *nisRK* expressed from plasmid pIL:*nisRK* (23). MG[*uspnucT*] containing the same two plasmids was used as a control. The NucT distribution in the parental MG[*uspnucT*] strain was similar regardless of overproduction of *uspnucT*. Similarly, when there was high-level NucT expression in the *dltA* background, the NucT mature form remained mainly associated with the cell fraction (Fig. 2C, right panel). This result showed that high-level NucT production did not alter the distribution of mature NucT in *dltA*[*uspnucT*], and the majority of the mature form was cell associated.

NucT is trapped in the peptidoglycan of the *dltA* mutant by electrostatic interactions and a peptidoglycan network. Previous work with gram-positive bacteria demonstrated that the absence of D-alanylation of LTA leads to a negatively charged cell surface (11, 35). The NucT isoelectric point is 9.48, while the pH of MG[*uspnucT*] overnight GM17 culture medium is around 5.7. Since at this pH NucT has a global positive net charge, there were electrostatic interactions between the positively charged NucT and the negatively charged LTA of *dltA*[*uspnucT*], as expected. This could explain the accumulation of the NucT mature form in the *dltA*[*uspnucT*] cell fraction. We tried to release NucT from the cell surface by washing the cell pellets with buffers containing high Ca^{2+} concentrations in order to displace the charge equilibrium by charge competition between Ca^{2+} ions and the positively charged NucT interacting with the modified cell wall. This treatment did not result in any release of mature NucT from the surface of the mutant, suggesting that it was not able to disrupt established interactions between NucT and the cell surface (data not shown). We then modified the net charge of NucT itself. Cells of MG[*uspnucT*] and *dltA*[*uspnucT*] were harvested after overnight growth and washed with buffers at pH 9 (less than the isoelectric point of NucT) and pH 10 (more than the isoelectric point of NucT). In the pH 10 buffer, NucT was negatively charged and was expected to be associated with the cell surface

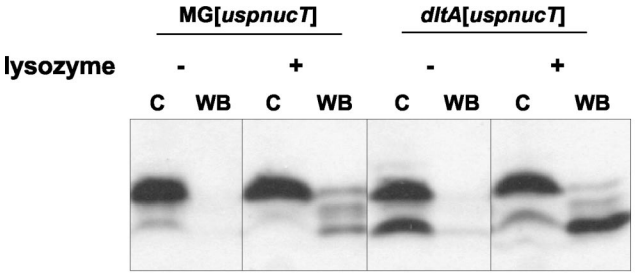

FIG. 4. Release of cell-associated mature NucT from *L. lactis*. Cells of MG[*uspnucT*] and *dltA*[*uspnucT*] overnight cultures were harvested, treated with lysozyme (+) or not treated (−), and washed in pH 10 wash buffer. Protein samples prepared from washed cells (lanes C) and wash buffer (lanes WB) were analyzed by Western blotting by using Nuc-specific antibodies.

in MG[*uspnucT*], whereas it was released from the cell surface of *dltA*[*uspnucT*]. In the pH 9 buffer, NucT was expected to remain associated. Neither pH 9 treatment nor pH 10 treatment resulted in release of mature NucT from the cell fraction (data not shown). This suggests that electrostatic interactions are not solely responsible for the *dltA* phenotype and/or that the peptidoglycan network is somehow involved in NucT association with the cell. To examine these possibilities, cells were treated with lysozyme (10 mg/ml, 5 min, 37°C) to disrupt the peptidoglycan network prior to washes at pH 9 or 10. The combination of lysozyme treatment and washing at pH 9 did not lead to release of NucT from the cell fraction (data not shown). In contrast, the combination of lysozyme treatment and washing at pH 10 resulted in clear and efficient release of NucT into the wash buffer (Fig. 4). These results show that (i) NucT found in the cell fraction matures correctly but remains in the peptidoglycan and (ii) NucT-cell association involves both electrostatic interactions and physical entrapment in the peptidoglycan network.

DISCUSSION

In the present study, we developed a system to identify new host factors involved in production and secretion of heterologous proteins in *L. lactis*. To our knowledge, previous use of random mutagenesis to examine secretion in gram-positive bacteria was limited to two studies, both with *B. subtilis* (22, 24). In those studies, mutagenesis was performed with chemical mutagenic agents and a plasmid-borne reporter protein. This strategy allowed isolation of secretory mutants, but identification of the mutation was difficult, as the secretion phenotypes might have resulted from combinations of mutations affecting either the secretion machinery or the plasmid. To circumvent these problems, in our system we combined the pGhost9:IS*S1* plasmid designed for random insertional mutagenesis (30) and the MG[*uspnucT*] strain, in which the NucT secretion reporter is expressed chromosomally. The use of a stably integrated expression cassette avoided selection of false positives related to plasmid maintenance. In contrast to chemically induced mutations, the pGhost9:IS*S1* insertion locus can be directly identified.

Screening of 35,000 clones revealed 20 confirmed integration mutants that were affected in NucT secretion. As IS*S1* insertion is reportedly random (30), the number of mutants screened theoretically corresponds to complete coverage of the *L. lactis* genome (~2.37 Mbp [7]). In theory, pGhost9:IS*S1* integration results in gene inactivation. Consequently, interruption of secretion genes, such as *secY*, *secE*, or *secA*, which are essential for cell viability in *E. coli* and *B. subtilis*, was not expected. Similarly, mutations that severely affect cell growth or that produce a growth phase-dependent phenotype might not be selected. However, screening did not reveal any Nuc$^-$ mutant resulting from inactivation of the *uspnucT* cassette, suggesting that pGhost9:IS*S1* insertional transposition is not totally random. A similar mutagenesis coupled to the use of a robot to screen a higher number of secretion mutants will be performed. Among the 20 mutants identified, we focused on characterization of *dltA* and *ybdD* mutants (Nouaille et al., unpublished), which were inactivated several times independently. Among the other mutants, hypersecreting mutant 26, inactivated in *vacB2*, which encodes one of the five putative RNases detected in the *L. lactis* genome (7), is a promising candidate, and further characterization is in progress. Targeted RNase inactivation could be a way to increase heterologous protein production and secretion in this organism. Six of the remaining mutated genes lack homology with known genes.

Five independent insertions occurred in *dltA*, the first gene of the *dltABCD* operon, which is required for D-alanylation of LTA, showing its involvement in the NucT secretion process. Mutations in the *dlt* operon have been studied in other gram-positive bacteria, including *S. aureus* (20, 35), *B. subtilis* (21, 47), *L. monocytogenes* (1), *Lactobacillus casei* (33), *S. gordonii* (10), *S. agalactiae* (37, 38), and *Streptococcus mutans* (8). In all cases, *dlt* inactivation totally eliminates D-alanylation of LTA and the cell surface acquires negative charges which might affect the release of proteins with surface-exposed positively charges residues into the growth medium (6). Other reported phenotypes associated with *dlt* mutations are different in different species. In general, changes in cell morphology and septation and increased sensitivity to cationic antimicrobial peptides were observed. However, in *L. lactis*, the *dltA* mutation had no detectable effect on chain length and cell morphology. In previous work the researchers reported a modification in chain length and a lower growth rate in *L. lactis dltD* mutants (16). As DltD is involved in the last step of D-alanylation, the accumulation of intermediate products could be more deleterious than the complete operon impairment that results from *dltA* inactivation. The less pronounced phenotypes resulting from the *dltA* mutation in *L. lactis* than from the mutations observed in other gram-positive bacteria could reflect differences in cell wall composition.

NucT produced in the *dltA* mutant was found to be predominantly associated with the cell fraction. We believe that the altered distribution of NucT (which has a net positive charge) in the *dltA* context is likely due to electrostatic interactions with negatively charged LTA, leading to entrapment in the peptidoglycan. In *B. subtilis*, mutations in *dlt* genes result in stabilization of recombinant anthrax protective antigen rPA, a highly labile protein (45). Thwaite et al. suggested that anionic polymers in a *dlt* mutant provide a microenvironment that favors rPA folding. Our results for *L. lactis* show that the anionic LTA present in the *dltA* mutant interact with and

accumulate positively charged secreted NucT. The *L. lactis dltA* mutant is now being tested to determine its ability to produce heterologous proteins prone to misfolding and degradation in a wild-type context. Thus, despite its isolation as a down-secretion mutant, the *L. lactis dltA* mutant could be a useful host for secretion of labile heterologous proteins that are concentrated in the peptidoglycan fraction.

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