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Molecular Cloning, Sequencing, and Expression of *lytM*, a Unique Autolytic Gene of *Staphylococcus aureus*

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A gene encoding an autolytic activity was identified in an autolysis-deficient mutant (Lyt$^-$) of *Staphylococcus aureus* which produces only a single band in autolytic-activity gels (N. Mani, P. Tobin, and R. K. Jayaswal, J. Bacteriol. 175:1493–1499, 1993). An open reading frame, designated *lytM*, of 948 bp that could encode a polypeptide of 316 amino acid residues was identified. The calculated molecular mass of the *lytM* gene product (34.4 kDa) corresponded to that of the autolytic activity detected (~36 kDa) in the Lyt$^-$ mutant. Results deduced from amino acid sequence analysis and N-terminal amino acid sequencing data suggest that LytM is a secreted protein. The C-terminal region of the putative protein encoded by *lytM* showed 51% identity with the N-terminal region of the mature lysostaphin from *Staphylococcus simulans* and 50% identity with the N-terminal region of ALE-1 from *Staphylococcus capitis* EPK1. Northern blot analysis showed that *lytM* expresses a transcript of ~955 bp, as predicted from the DNA sequence. *Escherichia coli* clones carrying the *lytM* gene exhibited autolytic-activity bands of ~36 kDa as well as of 19 and 22 kDa in activity gels. The *lytM* gene was mapped to the *Sma*I-D fragment on the *S. aureus* chromosome. Mapping data and results of hybridization experiments with primers generated from gene sequences of known autolytic genes of *S. aureus* clearly indicate that the *lytM* gene is distinct from other staphylococcal autolytic genes reported to date.

The peptidoglycan hydrolase enzymes, which are capable of hydrolyzing the peptidoglycan of the bacterial cell envelope, are ubiquitous among bacteria (9). Bacteria elaborate various peptidoglycan hydrolases which include *N*-acetylglucosaminidases, *N*-acetylmuramidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (1, 7, 8, 10, 13, 14, 16, 19, 22, 30, 35, 39, 43). The physiological functions of these enzymes remain largely a matter of speculation. Several roles have been proposed for these enzymes in cell wall growth, cell separation, cell wall turnover, lysis initiated by cell wall-acting antibiotics, competence for genetic transformation, flagellum formation, sporulation, and bacterial pathogenicity (7–10, 13, 16, 19, 22, 30, 34–36, 43). The peptidoglycan hydrolases involved in cell lysis are known as autolysins (39). The involvement of the *Staphylococcus aureus* autolysins in all of the above processes remains a matter of conjecture and has yet to be investigated at the molecular and genetic levels. It was only recently that a role for the *S. aureus* ATL autolysin, encoding glucosaminidase and amidase domains, in cell separation after cell division was delineated (31).

It has proved to be challenging to study autolytic systems of *S. aureus* due to the inherent difficulties in isolating autolysis-deficient mutants. Several *S. aureus* peptidoglycan hydrolase genes have been cloned and/or characterized. Cloning of a gene encoding a glucosaminidase (2) and cloning and characterization of an amidase gene which originated from a prophage in *S. aureus* NCTC 8325 (18, 41, 42), a bifunctional structural autolytic gene encoding the *S. aureus* β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase (6, 25, 26), and a two-component autolytic system (*lytS-lytR*) in *S. aureus* that regulates the transcription of the *lrgA* and *lrgB* genes, presumed to encode a murein hydrolase exporter and a murein hydrolase activity, respectively, have been reported (3, 4). We have previously reported the isolation and preliminary characterization of two *S. aureus* mutants (Lyt$^-$) defective in the production of all major autolysins except one (21). Another mutant (*atl*) exhibiting a single lytic band was reported subsequently by Foster (6), who proposed that most autolysins of *S. aureus* are the products of ATL and that proform processing results in the multiple bands visualized on activity gels. The Lyt$^-$ mutants isolated in our laboratory were normal with respect to growth rate, cell division, cell size, and adaptive responses to environmental changes but exhibited attenuated virulence in an experimental model of rat endocarditis (20, 21). This questioned the validity of the numerous physiological functions attributed to *S. aureus* autolysins and simultaneously raised the following questions. (i) Are these autolysins redundant? (ii) Is the residual autolytic activity still being expressed in the Lyt$^-$ mutant able to compensate for the functions of the missing autolysins? The presence of a single lytic activity of the same molecular mass in our Lyt$^-$ mutant and the *atl* mutant (6) suggested that the residual lytic activity still being expressed by Lyt$^-$ was not a product of ATL or under the control of the putative regulatory gene (17, 21). The present study was initiated to identify the gene encoding the functional autolysin in the Lyt$^-$ mutant in an effort to unravel some of the ambiguity surrounding the role of autolysins in *S. aureus*. Here we report on the cloning, sequencing, expression, and mapping of the *lytM* gene, which probably encodes a secretory protein that is responsible for the functional autolytic activity in the Lyt$^-$ mutant. Sequence analysis, hybridization data, Northern analysis, in vitro transcription-translation, and localization data clearly indicate that *lytM* is distinct from other staphylococcal autolytic genes reported to date (2, 3, 6, 18, 26, 42).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) broth was used for the cultivation of *Escherichia coli* cells, while trypticase soy broth (TSB) (Difco Laboratories) and PYK (24) was used for the cultivation of *S. aureus* strains. Cells were cultured at 37°C with shaking (200 rpm). When required, the antibiotics (Sigma Co.) ampicillin (25 to 50 μg/ml), tetracycline (10 to 20 μg/ml),

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant phenotype or genotype	Source or reference
S. aureus		
ISP2018	RN450 containing pTV32	P. A. Pattee
NCTC 8325-4	Lab strain cured of prophage	Novick et al. (24)
SH108	*atl*	Foster (6)
E. coli		
LE392	el4$^-$ (MrcA$^-$) *hsdR514 supE44 supF58 lacY1* [or Δ(*lacIZY*)*6*] *galK2 galT22 metB1 trpR55*	Promega
JM109	*recA1 endA1 gyrA96 hsdR17* (r_K^- m_K^+) *supE44 thi* Δ(*lac proAB*) *relA1* F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Yanisch-Perron et al. (44)
BLR(DE3)pLysS	F$^-$ *ompT* *hsdS*$_B$(r_B^- m_B^-) *gal dcm* Δ(*srl-recA*)*306*::Tn*10*(DE3)pLysS Cmp^r Tet^r	Novagen
Plasmids		
pCP13	Tet^r	Darzins and Chakrabarty (5)
pTZ18R	Amp^r	Mead et al. (23)
pCRII	Amp^r	Invitrogen
pRSETa	Cam^r Tet^r	X-press systems (Invitrogen)
pLR-01	Tet^r; pCP13 carrying a 23-kb *S. aureus* chromosomal insert with *lytM* gene; Lyt$^+$	This work
pLR-02	Amp^r; pTZ18R carrying a 12-kb *Bam*HI insert of pLR-01; Lyt$^+$	This work
pLR-03	Amp^r; pTZ18R with 3-kb Δ*Eco*RI insert of pLR-02; Lyt$^+$	This work
pLR-03.2 to pLR-03.9	Amp^r; clones of pLR-03 with deletions made by exonuclease III carrying inserts ranging from 0.9 to 1.8 kb in pTZ18R	This work
pLR-04	Amp^r; pCRII carrying *lytM* gene at the *Eco*RV site; Lyt$^+$	This work
pLR-05	Amp^r; pRSETa carrying the *lytM* gene at the *Bam*HI site in the appropriate reading frame; Lyt$^+$	This work

and chloramphenicol (30 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) (Fisher Scientific) were added as indicated.

Preparation of autolysins. (i) Freeze-thawed extracts. Freeze-thawed extracts were prepared essentially as described previously (15, 21). The clarified supernatant was used for the determination of autolytic activity.

(ii) Sonication. Mid-exponential-phase cultures of *E. coli* harboring recombinant plasmids were grown in LB broth containing the appropriate antibiotics, harvested by centrifugation, and resuspended in a minimum volume of 50 mM potassium phosphate buffer (pH 7.2). Cell suspensions were subjected to sonication (7 pulses of 30 s spaced 30 s apart, with settings at an output control of 5 and 50% duty cycle) with a Sonifer Cell Disruptor (Branson Sonic Power Co.). Unbroken cells were removed by centrifugation (10,000 × *g* for 15 min at 4°C). The supernatant was centrifuged several times at 10,000 × *g* for 15 min at 4°C until a clarified supernatant was obtained. This supernatant was used for the determination of lytic activity.

Detection of autolytic activity on SDS-PA gels. The autolytic activity of enzyme extracts was determined on a sodium dodecyl sulfate-polyacrylamide (SDS-PA) (15% polyacrylamide) gel containing 0.2% (wt/vol) lyophilized and autoclaved crude cell walls (CCW) from *S. aureus* and *Micrococcus luteus* as described earlier (21, 28). CCW were prepared from exponential-phase cultures of the strains grown in PYK medium as described by Jayaswal et al. (18). Gels were stained with 1% methylene blue in 0.01% KOH prior to photography (18). Molecular weights were determined by running prestained markers (Bio-Rad). SDS-PA gels without cell walls were stained with Coomassie brilliant blue or with silver stain (Bio-Rad).

Mutant (Lyt$^-$) genomic library construction. A genomic library of the Lyt$^-$ mutant was constructed in cosmid pCP13 (5). Total DNA from the Lyt$^-$ mutant was isolated and partially digested with *Sau*3AI. The digested DNA was fractionated by centrifugation through a 12-ml 10 to 40% (wt/vol) sucrose gradient at 141,000 × *g* (Beckman SW 28 rotor) for 18 h at 25°C. Fractions that yielded fragments of about 23 kbp were pooled and dialyzed against 10 mM Tris-HCl–1 mM EDTA buffer (pH 8.0). The dialyzed DNA was precipitated and ligated to *Bam*HI-digested and dephosphorylated cosmid vector in a ratio of 1:3 (vector/insert). The ligated DNA was packaged into lambda phage heads by using Gigapack II XL packaging extract (Stratagene) and transfected into *E. coli* LE392 to form the genomic library.

Screening of library for lytic clones. Three thousand clones were patched in triplicate on LB agar containing tetracycline (20 μg/ml) and grown at 37°C for 16 h. One hundred clones from each plate were pooled together in 10 ml of LB (with 20 μg of tetracycline per ml) and incubated at 37°C for 3 to 4 h. Cells were harvested by centrifugation and resuspended in 50 mM potassium phosphate buffer (pH 7.2). Cell extracts were prepared by sonicating the cell suspension on ice for 2 min (30-s pulses). Extracts were centrifuged, and the clarified extracts were assayed for lytic activity by using SDS-PA gels containing 0.2% CCW from *S. aureus* as described earlier (18). This process was repeated with progressively smaller number of clones from lysis-positive pools until the clone expressing the lytic activity was obtained (pLR-01).

Construction of clones overexpressing recombinant His$_6$-LytM fusion protein. The *lytM* gene was amplified by PCR with insertion of *Bam*HI and *Hin*dIII sites at the 5′ and 3′ ends, respectively. Primers used were L1 (5′ GGATCCATGG AGGATGTTTTATACATGAAA 3′) and L2 (5′ AAGCTTCAACTTGGGAT TTTCTGTATTATC 3′). The fragment was purified by the gene clean method and ligated into pCRII (Invitrogen) at the *Eco*RV site. *lytM* was subcloned under the *tac* promoter from the resultant plasmid, pLR-04, into the expression vector pRSETa (Invitrogen) to generate plasmid pLR-05, which was inducible by IPTG. This plasmid was transferred into *E. coli* BLR(DE3)pLysS (Novagen) by electroporation with a Bio-Rad Gene Pulser. The transformants were grown in LB broth (containing 25 μg of ampicillin per ml, 10 μg of tetracycline per ml, and 30 μg of chloramphenicol per ml) to an optical density at 580 nm (OD_{580}) of 0.3 and then induced by the addition of IPTG (to a final concentration of 1 mM) and further incubated for 4 h. Cells were harvested by centrifugation (10,000 × *g* for 10 min at 4°C). Pellets were resuspended in 50 mM potassium phosphate buffer, pH 7.2, and sonicated as described above. Cell debris was removed by centrifugation, and the supernatant was analyzed by SDS-PA gel electrophoresis (SDS-PAGE).

Northern blot analysis. Total RNA was isolated from various strains of *S. aureus* by using the Promega RNA isolation kit with minor modifications. Ten micrograms of total RNA electrophoresed under denaturing conditions in a 1.4% agarose gel was transferred to a nitrocellulose membrane as described by Sambrook et al. (32). Hybridization was carried out at 42°C for 20 h in hybridization buffer (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1× Denhardt's solution, 50 mM Na_2HPO_4 [pH 6.5], 250 μg of denatured herring sperm DNA per ml). The membrane was hybridized with an [α-^{32}P]dCTP (ICN Biochemicals)-labeled *lytM* probe and washed with 2× SSC buffer at room temperature for 5 min and washed twice in 1× SSC–0.1% SDS–1 mM EDTA buffer at 50°C for 10 min until background levels of radioactivity were undetectable. The bands hybridizing to *lytM* were visualized by autoradiography. Transcript sizes were determined by running standard RNA markers (Promega). To determine the levels of *lytM* gene expression during the growth cycle, total RNA was isolated from cells at different stages of growth (at OD_{580} of 0.2, 0.4, 0.8, 1.2, and 2.0) and probed with *lytM* by the procedure described above.

Mapping of *lytM* on the *S. aureus* chromosome. The location of *lytM* on the *S. aureus* chromosome was determined by pulsed-field gel electrophoresis (27, 37). The *Sma*I-digested DNA from the parent strain was electrophoresed and probed with the biotinylated plasmid pLR-03 carrying the *lytM* gene. This work was performed in John Iandolo's laboratory at Kansas State University.

In vitro transcription-translation of *lytM*. The in vitro transcription-translation reactions were carried out by using *E. coli* S30 extract for circular DNA (Promega in vitro transcription translation system) per the manufacturer's in-

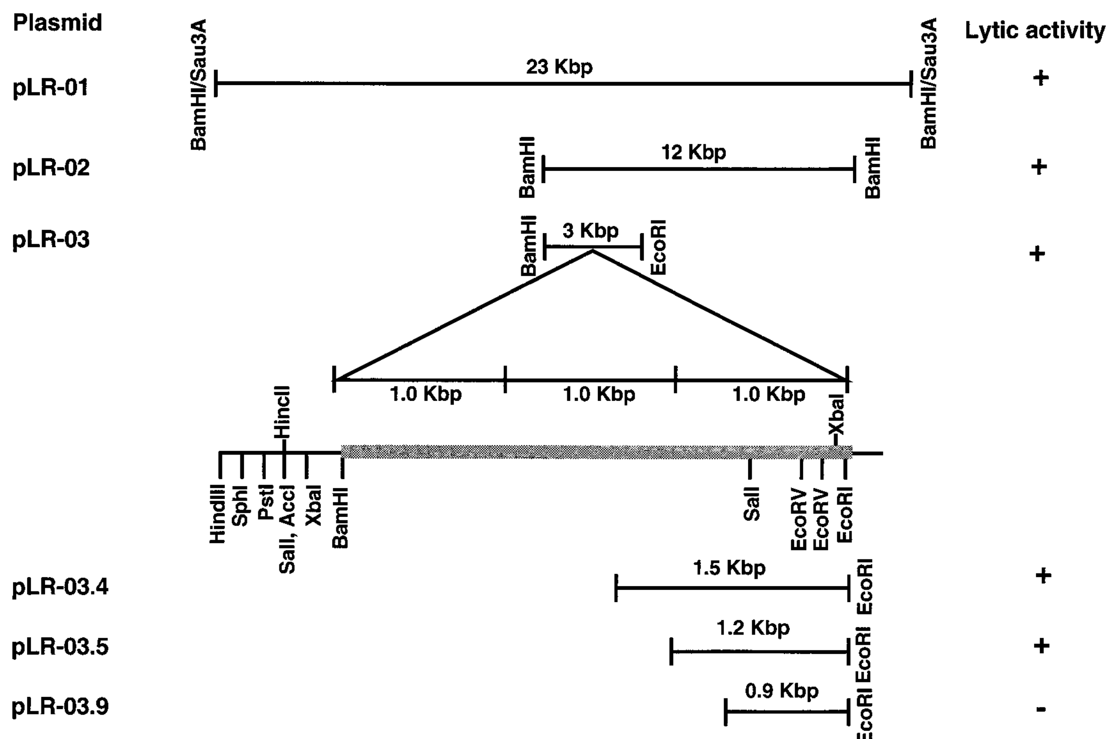

FIG. 1. Physical map of the chromosomal fragment of *S. aureus* DNA containing the *lytM* gene. The subclones were constructed into various plasmids as described in Materials and Methods. Each clone was tested for its ability to hydrolyze whole cells of *S. aureus* and *M. luteus*. The chromosomal fragment of *S. aureus* containing the *lytM* gene (stippled bar) is shown. Symbols: +, lysis; −, no lysis.

structions. A trans^{35}S-label mixture of cysteine and methionine (ICN Biochemicals) with a specific activity of 40.59 TBq/mmol was used to label the newly synthesized proteins. Samples (3 μg) of plasmid DNA from pLR-03, pLR-03.2 to pLR-03.9, and the pTZ18R vector were used for the in vitro reaction. Labeled protein products were analyzed by SDS-PAGE (15% polyacrylamide gels) followed by Coomassie brilliant blue staining and autoradiography.

General DNA techniques. Subclones were created through use of various unique restriction sites within the lysis-positive cosmid clone (pLR-01). Unidirectional exonuclease III-ordered deletion was performed essentially as described by Henikoff (12). The nucleotide sequence of *lytM* was determined by the dideoxy-chain termination method of Sanger et al. (33) with modified T7 polymerase (Sequenase DNA sequencing kit; United States Biochemicals) and automated sequencing (ABI Prism 310 Genetic Analyzer; Perkin Elmer). Sequencing was completed with synthetic DNA primers (DNAgency, Malvern, Pa.). Nucleotide sequences were analyzed by McVector (version 5.0) and Assembly Lign programs. Nucleotide and amino acid sequence homology searches were performed on NCBI Entrez databases by Blast searches. The N-terminus amino acid sequence of the *lytM* product overexpressed in *E. coli* was determined by microsequencing technique (Applied Biosystems Automatic 477 Amino Acid Sequencer). DNA restriction, digestion, ligation, cloning, transformation, and Southern blot procedures were performed as described by Sambrook et al. (32).

Nucleotide sequence accession number. The sequence reported in this study has been submitted to GenBank and assigned accession no. L77194.

RESULTS AND DISCUSSION

Molecular cloning and nucleotide sequence analysis of the gene encoding the lytic activity. Three thousand clones from the Lyt$^-$ mutant library were screened by zymography to isolate pLR-01, the cosmid clone carrying the *S. aureus* DNA fragment exhibiting the lytic activity. The lytic activity was mobilized with pLR-01 into another strain of *E. coli*, indicating that we had cloned the correct DNA fragment. A 3-kb fragment from pLR-01 was further subcloned into pTZ18R in both orientations to generate pLR-03 and pLR-03.1. A partial restriction map of the cloned fragment in pLR-03 is shown in Fig. 1. Analysis of several overlapping deletion clones for lytic activity suggested that the gene encoding the lytic activity is located within a 1.2-kb fragment (pLR-03.5).

Overlapping clones with deletions made by exonuclease III were used for nucleotide sequencing which revealed two overlapping open reading frames of 966 and 948 bp with start codons at positions 314 and 332 (Fig. 2). However, the second start codon was considered to be the start site for translation of the gene, as an appropriate ribosome binding site was found upstream of this start codon. This open reading frame, designated *lytM*, starts with ATG at position 332 and extends to position 1279 (TAA as stop codon at 1280) and is 948 bp long. The stop codon (TAA) at position 1280 is followed by a sequence with dyad symmetry. The sequence beginning 5 bp downstream of the TAA stop codon at position 1280 (indicated by horizontal arrows under the sequence) could form a stem-loop structure and may be a transcription terminator. The G+C content of *lytM* is 36.7%, which is similar to the overall G+C (32 to 36%) content of *S. aureus*.

Deduced amino acid sequence of LytM. The primary amino acid sequence deduced from the nucleotide sequence of the *lytM* gene revealed a putative protein of 316 residues with an apparent molecular mass of 34.4 kDa and a pI of 6.3. The hydropathy profile of the amino acid sequence showed the pronounced hydrophilicity of the protein with a short hydrophobic domain at the N terminus. A putative signal sequence, as well as a putative signal peptide cleavage site, is present within the first 26 amino acids (Fig. 2). This latter site was identified as described by Von Heijne (40). The signal sequence is the only strongly hydrophobic region of LytM, a feature which is highly characteristic of secreted proteins and consistent with LytM being a secreted protein. The processed

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CAATAGCGATTGATAATGGAATTTGGTTGATATGATTTGATGGATGATTACTATCATGCATTAGCGTTGAATCGCGAACATGGACGAACGATTAGTTGTT  100

AATTTTGAAAATTTATAAAAGGTTAAACGAATGCAGTGAGAGATGTCATTTTAATATCAATGTATGGCTATTTTGTAATGACAATGTAATGAGTTTAGTA  200

AAACATTTCGGGAATATTAAATAGTTGGAAAATGAGAATTAAATCCTTTACTCAGTTGTCTAATTCTTTTAGTATGTGCAGTACAGTTTAATTGAAAACT  300

AACTTTAACTTTAATggaggaTGTTTTATACATGAAAAAATTAACAGCAGCAGCGATTGCAACGATGGGCTTCGCTACATTTACAATGGCGCATCAAGCA  400
               rbs              M  K  K  L  T  A  A  A  I  A  T  M  G  F  A  T  F  T  M  A  H  Q  A   a23

GATTCAGCAGAAACGACAAACACCCAACAAGCACATACACAAATGTCAACACAATCACAAGACGTATCTTATGGTACTTATTATACAATTGATTCTAATG  500
 D  S↑A  E  T  T  N  T  Q  Q  A  H  T  Q  M  S  T  Q  S  Q  D  V  S  Y  G  T  Y  Y  T  I  D  S  N   a57

GGGATTATCATCACACACCTGATGGTAACTGGAATCAAGCAATGTTTGATAATAAAGAATATAGCTATACATTCGTAGATGCTCAAGGACATACGCATTA  600
G  D  Y  H  H  T  P  D  G  N  W  N  Q  A  M  F  D  N  K  E  Y  S  Y  T  F  V  D  A  Q  G  H  T  H  Y   a90

TTTTTATAACTGTTATCCAAAAAATGCAAATGCCAATGGAAGCGGCCAAACATATGTGAATCCAGCAACAGCAGGAGATAACAATGACTACACAGCGAGT  700
  F  Y  N  C  Y  P  K  N  A  N  A  N  G  S  G  Q  T  Y  V  N  P  A  T  A  G  D  N  N  D  Y  T  A  S  a123

CAAAGCCAACAGCATATTAATCAATATGGTTATCAATCAAATGTAGGTCCAGACGCGAGCTATTATTCACATAGTAACAACAACCAAGCGTATAACAGCC  800
 Q  S  Q  Q  H  I  N  Q  Y  G  Y  Q  S  N  V  G  P  D  A  S  Y  Y  S  H  S  N  N  N  Q  A  Y  N  S   a157

ATGATGGTAATGGAAAGGTCAATTATCCTAATGGCACATCTAATCAAAATGGTGGATCAGCAAGTAAAGCGACACGTAGTGGTCATGCGAAAGACGCAAG  900
H  D  G  N  G  K  V  N  Y  P  N  G  T  S  N  Q  N  G  G  S  A  S  K  A  T  R  S  G  H  A  K  D  A  S  a190

CTGGTTAACAAGTCGTAAACAACTACAACCATATGGACAATATCACGGTGGTGGTGCGCATTACGGTGTCGACTATGCAATGCCTGAAAATTCACCAGTT 1000
  W  L  T  S  R  K  Q  L  Q  P  Y  G  Q  Y  H  G  G  G  A  H  Y  G  V  D  Y  A  M  P  E  N  S  P  V  a223

TACTCATTAACTGATGGTACAGTAGTACAAGCAGGTTGGAGTAACTATGGTGGCGGCAATCAAGTAACGATTAAAGAAGCGAACAGTAATAACTACCAAT 1100
 Y  S  L  T  D  G  T  V  V  Q  A  G  W  S  N  Y  G  G  G  N  Q  V  T  I  K  E  A  N  S  N  N  Y  Q   a257

GGTATATGCATAATAATCGTTTAACTGTTTCAGCTGGTGATAAAGTCAAAGCTGGTGACCAAATTGCATATTCAGGTAGTACGGGTAATTCAACAGCGCC 1200
W  Y  M  H  N  N  R  L  T  V  S  A  G  D  K  V  K  A  G  D  Q  I  A  Y  S  G  S  T  G  N  S  T  A  P  a290

TCACGTACACTTCCAACGTATGTCTGGTGGCATCGGTAATCAATATGCAGTAGACCCAACGTCATACTTGCAAAGTAGATAATACAGAAAATCCCAAGTT 1300
  H  V  H  F  Q  R  M  S  G  G  I  G  N  Q  Y  A  V  D  P  T  S  Y  L  Q  S  R  *          ___________

GCGATATCATACGCAGCTTGGGATTTTTTCGTTTTAATAATAGGTATAAGTCCATTGTTTGTCCTCTAAAACATGTTGATAAAAAGGATCTTGGCCAATT 1400
--->   <----------

AACTTGATATCATCTGCAAGTGCTTCAACTTCATCTAAATGATGGGTAGTTAATATAATTAAACATTTAGATTTCATGATGTTAAGTAGTTGGTGGATGT 1500

CATGTCTAGATTTTAAATCAATACCAACTGTCGGTTCATCTAAAATGAGAATTCCCATAT
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FIG. 2. Nucleotide sequence and deduced amino acid sequences of the *lytM* gene. A putative ribosome binding site (rbs; sequence in lowercase letters) is indicated. A possible transcription terminator downstream of *lytM* is also indicated by horizontal arrows under the sequence. The possible signal peptide sequence cleavage site is indicated by a vertical arrow. a, amino acid.

protein has a calculated molecular mass of 32 kDa and a pI of 6.02.

The deduced amino acid sequence of LytM was compared to the NCBI Entrez protein databases by Blast searches. A significant amino acid sequence homology was found with the plasmid-encoded lysostaphin of *Staphylococcus simulans* and the glycylglycine endopeptidase, ALE-1, encoded by *Staphylococcus capitis* EPK1 (11, 29, 38). The C-terminal region of LytM showed significant (51%) homology with the N-terminal region of the mature lysostaphin and with that of ALE-1 (50%), with some highly conserved regions (Fig. 3). However, the significance of this sequence homology remains to be determined.

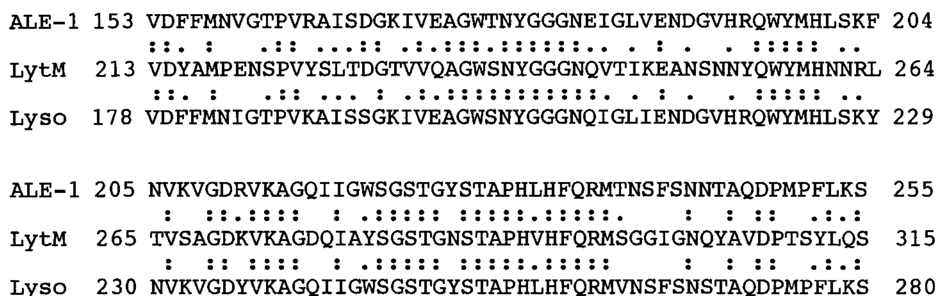

FIG. 3. Alignment of deduced amino acid sequences of LytM, lysostaphin of *S. simulans*, and ALE-1 of *S. capitis* EPK1. Identical (colons) and similar (periods) amino acids in the two proteins are indicated. lyso, lysostaphin.

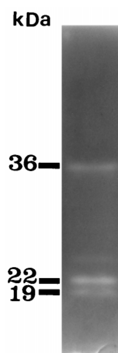

FIG. 4. Lytic activity of sonicated extract of induced *E. coli* BLR(DE3)pLysS harboring pLR-05. The SDS-PA gel contained 0.2% (wt/vol) lyophilized and autoclaved cell wall from *S. aureus*. Molecular masses (in kilodaltons) are shown on the left.

Analysis of LytM activity profile. The lytic activities of various strains were examined on zymograms containing lyophilized and autoclaved *S. aureus* CCW as described in Materials and Methods. We had earlier reported the presence of several lytic-activity bands (19 to 167 kDa) in the extracts of the parent strain RN450 but only one such band of about 36 kDa in the Lyt$^-$ mutant (21). However, we had also observed earlier that the Lyt$^-$ mutant occasionally exhibited two additional lytic-activity bands at positions corresponding to 19 and 22 kDa which we believed to be degraded products of the 36-kDa band. Analysis of lytic activity of a sonicated extract of *E. coli* containing pLR-05 showed three bands at positions 36, 22, and 19 kDa (Fig. 4). A lytic-activity band at 27 kDa appeared after prolonged incubation (several days) in renaturation buffer at room temperature. Insertional inactivation of *lytM* in pLR-03.5 with a chloramphenicol resistance marker (at the 906-bp position in Fig. 2) resulted in the loss of all these lytic-activity bands. These results clearly suggest that the lytic activity from pLR-03.5 is encoded by *lytM*. These data in conjunction with the nucleotide sequence data (*lytM* encoding a putative protein of 34.4 kDa) strongly indicate that the lower-molecular-mass lytic-activity bands are degraded products of LytM. Uninduced *E. coli* carrying pLR-05 and a control without pLR-03.5 did not show any activity on zymograms, indicating that the autolytic bands observed in the *E. coli* clones are indeed a product of the *lytM* gene (data not shown). This conclusion is further substantiated by the results of experiments on the induction of *lytM* gene fusion product.

In vitro transcription-translation of the *lytM* gene product. The plasmid constructs pLR-03, pLR-03.2 to pLR-03.9 and pTZ18R were used in an in vitro transcription-translation-coupled system from *E. coli* to analyze the *lytM* gene product. As shown in Fig. 5, a polypeptide with an apparent molecular mass of ~36 kDa was synthesized by the pLR-03 and pLR-03.5 plasmids carrying *lytM*. Two higher-molecular-mass bands of ~60 and 66 kDa were also observed. These peptides could originate from readthrough from the vector. Plasmid pLR-03 carried a 3.0-kb DNA fragment, and pLR-03.5 carried a 1.2-kb DNA fragment of *S. aureus* containing intact *lytM* (Fig. 5, lanes 1 and 2). However, plasmid pLR-03.9, which carried a truncated *lytM* (0.9-kb insert), did not synthesize the 36-kDa polypeptide (lane 3) or the high-molecular-mass bands. This 36-kDa polypeptide was also absent in the reaction where the pTZ18R vector was used as template for *in vitro* transcription-translation (data not shown). This result concurs with the predicted molecular weight of the *lytM* gene product calculated from the nucleotide sequence data.

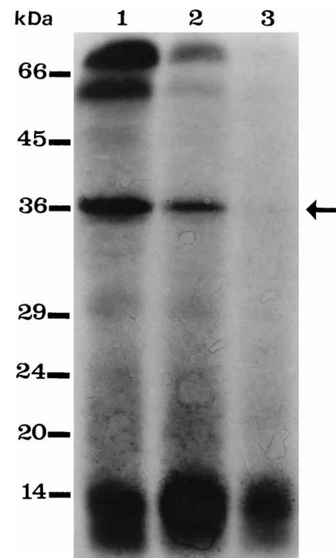

FIG. 5. *In vitro* transcription-translation of the *lytM* gene product. Three micrograms of plasmid DNA from pLR-03, pLR-03.5, and pLR-03.9 was used for in vitro reactions. Products were labeled with trans^{35}S-label mixture of cysteine and methionine, and the products were analyzed by SDS-PAGE (15% polyacrylamide gel). Lane 1, pLR-03; lane 2, pLR-03.5; lane 3, pLR-03.9. The positions (in kilodaltons) molecular size markers are indicated on the left.

RNA analysis of *lytM* during the growth cycle of *S. aureus*. Northern blot analysis was performed to measure the size of the *lytM* transcript. As shown in Fig. 6, the PCR-amplified [α-^{32}P]dCTP-labeled *lytM* probe hybridized with a single transcript of approximately 955 bp in all the strains. This size closely corresponds with the *lytM* size predicted from the nucleotide sequence. Two other transcripts were also observed. However, the origin or significance of these transcripts remains unknown. To determine whether the level of expression of *lytM* is modulated during the growth cycle, total RNA from *S. aureus* RN450, Lyt$^-$, and *atl* (SH108) mutant was isolated at

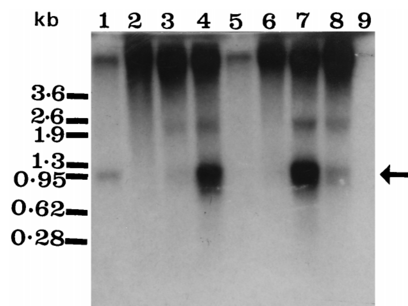

FIG. 6. Transcript analysis of *lytM* RNA. Total RNA was isolated from *S. aureus* RN450 and Lyt$^-$ and *atl* mutants at various stages in the growth cycle as described in Materials and Methods. Ten micrograms of total RNA from each sample was electrophoresed on a 1.4% formaldehyde gel under denaturing conditions, transferred to a nitrocellulose membrane, and probed with [α-^{32}P]dCTP-labeled PCR-amplified *lytM*. Lanes 1, 2, and 3, total RNA from *S. aureus* RN450 isolated at OD_{580}s of 0.4, 0.8, and 1.2, respectively; lanes 4, 5, and 6, total RNA from a Lyt$^-$s mutant isolated at OD_{580}s of 0.4, 0.8, and 1.2, respectively; lanes 7, 8, and 9, total RNA from an *atl* mutant isolated at OD_{580}s of 0.4, 0.8, and 1.2, respectively. The probe hybridized with a single transcript of approximately 955 bp (lanes 1, 4, and 7), as calculated from the positions of the RNA molecular size standards shown on the left.

different time points and subjected to Northern blot analysis. As shown in Fig. 6 (lanes 1, 4, and 7), in all three strains the expression of *lytM* was highest in early exponential growth, declined during late exponential growth, and was almost undetectable in stationary phase. In addition, *lytM* was found to be highly overexpressed (at least 10-fold) in early log phase in the Lyt^{-} (lane 4) and *atl* (SH108) (lane 7) mutants compared to the parent strain (lane 1). This suggests that expression of *lytM* is regulated by an internal or external environmental signal. A two-component regulatory system of *S. aureus* (*lytS-lytR*) bearing strong amino acid sequence similarities to two-component regulatory systems in other organisms has been reported (3). Whether the expression of *lytM* is regulated through *lytS-lytR* remains to be determined. Environmental modulation of our putative regulatory gene (17, 21) and the *atl* gene (6) by various environmental factors has been documented by gene fusion experiments. We are currently investigating the effects of various environmental factors responsible for modulating the expression of *lytM* during the growth cycle of *S. aureus.*

Overexpression and N-terminal sequencing of the *lytM* gene fusion product. The *lytM* gene was cloned into an overexpression vector, pRSETa, under the control of the T7 promoter as described in Materials and Methods. This construct was stable in *E. coli* BLR(DE3)pLysS and overexpressed in an active form. The fusion product had an apparent molecular mass of ~40 kDa (data not shown). However, it appeared that LytM is toxic to the cells, as the growth rate of *E. coli* BLR(DE3)pLysS harboring *lytM* was significantly lower than that of a strain carrying the vector alone. Amino acid sequencing of the N terminus of the overexpressed polypeptide showed that the sequence AETTNTQQAHTQMST matched perfectly with our deduced amino acid sequence in Fig. 2 (amino acids 26 to 40). This result strongly suggests that the LytM fusion protein expressed in *E. coli* is cleaved after the signal peptide sequence as shown in Fig. 2. We are currently in the process of biochemically characterizing the LytM enzyme.

Location of the *lytM* gene on the *S. aureus* chromosome. To localize the *lytM* gene on the *S. aureus* chromosome, a physical mapping technique using pulsed-field gel electrophoresis was performed. The *lytM* gene was localized to the *Sma*I-D fragment on the *S. aureus* chromosome distinct from other autolytic genes of *S. aureus* reported to date.

Conservation and relatedness of *lytM* to other staphylococcal autolytic genes. To determine whether *lytM* was conserved in other staphylococcal species, DNA from *S. aureus* (four different isolates), *S. capitis*, *S. hemolyticus*, *S. hominis*, *S. hyicus*, *S. intermedius*, *S. sciuri*, *S. simulans*, and *S. xylosus* was isolated and probed with [α-^{32}P]dCTP-labeled *lytM*, under conditions of low (1× SSC, 0.1% SDS, 50°C) and high stringency (0.3× SSC, 0.1% SDS, 68°C) by dot blot hybridization. Hybridization was observed only with *S. aureus* strains and not with other staphylococcal species, suggesting that *lytM* is highly conserved only within *S. aureus.*

The nucleotide sequence of *lytM* did not show any significant homology to any known autolytic gene sequences of *S. aureus*. Also, mapping data indicated that *lytM* was at a different position on the *S. aureus* chromosome than other staphylococcal autolytic genes that have been localized thus far. A dot blot analysis was carried out to further rule out or detect any cross-hybridization with other known autolytic genes in *S. aureus*. The DNA carrying the *lytM* gene was probed (under low- and high-stringency conditions) with primers generated from the published sequence of the *atl* gene (26), (primers used were 5′ TTCTGGCACTCAAGTATA 3′, 5′ CACCCCAAGATAAG TAATCC 3′, 5′ CGACAAAACTGGTAAA 3′, and 5′ GTTT GCATTA CATTTA 3′), the gene sequence of *lytI* (a putative regulatory gene of *S. aureus* autolytic system) (17, 21) and a plasmid bearing a partial gene sequence of *lytS-lytR* (3). Lack of hybridization with any of the above probes further confirmed that *lytM* was a distinct autolytic gene in *S. aureus* and bears no sequence homology to any known autolytic genes of *S. aureus.*

Our results indicate that we have cloned the gene encoding the lytic-activity band in the Lyt^{-} mutant reported earlier from our laboratory (21) and that this enzyme is distinct from other *S. aureus* autolytic genes reported so far. We had previously hypothesized that the lytic activity present in the Lyt^{-} mutant was important to *S. aureus*, as the mutant exhibits normal growth and division despite the fact it lacks most autolysins (21). We are currently determining the functional importance of LytM in *S. aureus* cell growth and other vital functions.

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