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Cloning and Characterization of a Gene Affecting the Methicillin Resistance Level and the Autolysis Rate in *Staphylococcus aureus*

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Tn*918* mutagenesis of a high-level methicillin-resistant *Staphylococcus aureus* (methicillin MIC, 800 μg/ml) led to the isolation of a low-resistance mutant. The Tn*918* insert was transferred back to the parent to produce strain SRM563 (methicillin MIC, 12.5 μg/ml), which showed heterogeneous resistance. Twenty-two clinical isolates of methicillin-resistant *S. aureus* were transformed with DNA of SRM563. In the transformants of most strains, instances of reduced resistance were observed with concomitant increases of autolysis rate induced by Triton X-100 and were generally more profound in high-resistance strains. Two transformants exhibited a decrease of the autolysis rate and little reduction of resistance. In the transformant of methicillin-susceptible strain RN2677, an increase of the autolysis rate and little reduction of resistance were observed. The production of low-affinity penicillin-binding protein (PBP2′) did not significantly decrease in the mutants. Insertion of Tn*918* occurred within the 3′-terminal region of a novel gene designated *llm*, which was cloned and sequenced. RNA blot analysis demonstrated that the gene was transcribed. The encoded protein was composed of 351 amino acid residues with a molecular weight of 38,512 and was hydrophobic, suggesting its location on the membrane. The gene was detected by PCR in all *S. aureus* strains tested but not in the other 26 staphylococcal species. Comparison of the 3′-terminal sequences of the gene among several *S. aureus* strains showed that, whereas nucleotide substitutions occurred at the third position in seven of eight 3′-terminal codons, only C-terminal amino acid variation of glutamate or aspartate was observed.

Methicillin-resistant strains of *Staphylococcus aureus* produced a low-affinity penicillin-binding protein (PBP), PBP2′ or PBP2a, in addition to the ordinary PBPs (16, 39). PBP2′ was considered to take over the function of peptidoglycan biosynthesis from ordinary PBPs in the presence of otherwise inhibitory concentrations of β-lactam antibiotics such as methicillin because of the low affinity of PBP2′ for β-lactam antibiotics (11, 32). While the production of PBP2′ was known to be prerequisite for methicillin resistance, earlier reports indicated that the amount of PBP2′ produced did not correspond to the resistance level; that is, strains with a high level of resistance did not necessarily produce a larger amount of PBP2′ than strains with a low level of resistance (4, 17, 28). These observations were subsequently confirmed and extended by the finding that *mecA*, the structural gene for PBP2′, was not the single factor determining the resistance level—another genetic determinant(s) was involved in the expression of the high-level methicillin resistance, thus differentiating high-level resistant strains from low-level ones (29). Furthermore, transposon insertional mutagenesis with Tn*551* revealed that, for the expression of both low-level and high-level methicillin resistance, several other genetic determinants such as *fem* genes or auxiliary genes were necessary (1, 20). Inactivation of these determinants by transposon insertion resulted in reduction of the resistance level to a susceptible or low-resistance level without any influence on PBP2′ production. Thus, several additional factors seem to be working together with PBP2′ to make the bacteria methicillin resistant. Among these factors, the products of *femA* and *femB* were revealed to be involved in the biosynthesis of pentaglycine cross bridges of peptidoglycan (9, 18, 26) and that of *femC* was shown to be the glutamine synthetase repressor (14).

As shown with Tn*551*, isolation and characterization of the transposon insertional mutants with reduced phenotypic expression of methicillin resistance seem to be useful for identifying genes participating in peptidoglycan biosynthesis and, furthermore, for elucidating the mechanism of high-level methicillin resistance. In the present study, using a conjugative transposon Tn*918* carrying a tetracycline resistance marker (7), we isolated an *S. aureus* mutant which lost its high-level methicillin resistance to become a low-resistance strain. The transposon insertion occurred within a gene whose nucleotide sequence revealed that it was a novel gene differing from *femA*, *femB*, and *femC*.

MATERIALS AND METHODS

Bacterial strains. Methicillin-resistant *S. aureus* strains used in this study were clinical isolates from Japan. *S. aureus* SRM551 was a penicillinase-negative derivative of a high-level methicillin-resistant strain SR3626 (28) which was also resistant to erythromycin. Methicillin-susceptible *S. aureus* RN2677 (20) was kindly supplied by R. P. Novick (New York Public Health Research Institutes, New York, N.Y.), and *Enterococcus faecalis* JH2SS(pAM378) which harbored plasmid pAM378 carrying Tn*918* with the tetracycline-resistant determinant (7) was supplied by D. B. Clewell (University of Michigan, Ann Arbor, Mich.).

Transposon mutagenesis with Tn*918*. Mutagenesis was carried out by a filter mating method as described previously, with some modifications (7). The donor, *E. faecalis* JH2SS (pAM378), and the recipient, *S. aureus* SRM551, were grown separately with shaking at 37°C overnight in Todd-Hewitt

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broth (Difco Laboratories, Detroit, Mich.) with and without 10 μg of tetracycline per ml, respectively. Five-milliliter cultures of each strain were mixed and collected on a membrane filter (type HA; pore size, 0.45 μm; diameter, 47 mm; Millipore Corp., Bedford, Mass.). The filters were placed on Todd-Hewitt agar (Todd-Hewitt broth plus 1.5% agar) containing 4% horse blood (Toyo Kessei, Tokyo, Japan) and incubated at 37°C for 18 h. The bacteria grown were then suspended in 2 ml of Todd-Hewitt broth and spread on Todd-Hewitt agar plates containing 10 μg of both tetracycline and erythromycin per ml. Since pAM378 could not replicate in *S. aureus* cells, the selection plate allowed the growth of only *S. aureus* cells with the transposon insertion. After overnight incubation at 37°C, colonies grown were replica plated onto plates containing 25 μg of methicillin per ml and 10 μg each of tetracycline and erythromycin per ml and incubated for 18 to 20 h at 37°C. The colonies which did not grow on the methicillin plate were picked from the master plates.

Colony hybridization. Colony hybridization was performed as described previously (33). Briefly, colonies grown on the plate were overlaid with a Hybond-N nylon membrane (Amersham International plc., Buckinghamshire, England). The membranes were removed and placed successively on filter paper impregnated with 10% sodium dodecyl sulfate (SDS), with 0.5 M NaOH plus 1.5 M NaCl, with 0.5 M Tris-HCl (pH 7.4) plus 1.5 M NaCl, and finally with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA adsorbed was cross-linked to the membrane by UV irradiation with UV-Crosslinker (Stratagene, La Jolla, Calif.). The membranes were prehybridized in GMC buffer (0.5 M sodium phosphate buffer [pH 7.2] containing 7% SDS, 1% bovine serum albumin, and 1 mM EDTA) (5) at 60°C for 1 h prior to hybridization at 60°C overnight in the same buffer with a radiolabeled Tn*918* probe. The membranes were washed with 2× SSC plus 0.1% SDS and then with 0.2× SSC plus 0.1% SDS for 30 min each time at room temperature and finally washed twice with 0.2× SSC plus 0.1% SDS at 60°C for 15 min each time. Colonies that gave a signal on the autoradiogram were picked out from the master plate.

Screening of λZAPII gene library. Chromosomal DNA of *S. aureus* SRM551 was prepared from protoplasts (36). The λZAPII (Stratagene) (35) library of SRM551 genomic DNA was constructed by the general method as described previously (13). Briefly, genomic DNA was sheared by sonication. Fragments of 2 to 7 kb were enriched by gel filtration with Sepharose CL-2B (Pharmacia Biotechnology, Uppsala, Sweden), and blunt ends were produced with a DNA Blunting kit (Takara Shuzo, Kyoto, Japan). The DNA fragments were inserted into the *Eco*RI site of λZAPII by using *Eco*RI-*Not*I-*Bam*HI linkers (Takara Shuzo). Recombinant phage DNA was packaged with a λ in vitro packaging kit (Amersham International plc.)

The λZAPII library was screened by the plaque hybridization method (33). The phages of 10^4 PFU were grown on *Escherichia coli* XL1-Blue for 8 h at 37°C. Hybond-N nylon membranes were overlaid for 2 to 4 min. The membranes were transferred to the filter paper impregnated with 0.5 M NaOH plus 1.5 M NaCl to denature the DNA. Neutralization and hybridization with radiolabeled oligonucleotide probe were performed as described in the colony hybridization section. Plaques giving a signal on the autoradiogram were picked out and purified by repeated plaque hybridization. The pBluescript phagemid containing the insert was excised in vivo from the corresponding recombinant λZAPII phages with helper phage R404 and introduced into *E. coli* XL1-Blue by infection according to the instructions of the manufacturer.

PCR. Preparation of template DNA from bacterial colonies and PCR amplification were carried out as described previously (27) except that the addition of SDS for DNA preparation was omitted and the denaturation of the first cycle of the amplification reaction was done at 94°C for 2 min. The primer concentration employed was 0.25 μM unless otherwise noted herein.

DNA sequencing and homology analysis. DNA sequences of both strands were determined by the dideoxy chain termination method (34) with the Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) with [^{35}S]dCTPαS (222 TBq/mmol; Amersham) and the synthesized primers. Recombinant pBluescript plasmids which had the insert to be sequenced were usually used as templates. For direct sequencing of the genomic DNA, DNA fragment containing the sequence of interest was amplified by two sequential PCRs. In the first PCR, a target fragment was amplified with genomic DNA as a template and 0.30 μM (each) primers. The second asymmetrical PCR was performed in 100 μl of reaction mixture, with 5 μl of the first PCR product as a template and the same primers as those used in the first PCR at 0.05 μM for one primer and 0.5 μM for the other. The second PCR product was purified with SUPREC-02 (Takara Shuzo) and used as the template for sequencing with the primer used at 0.05 μM in the second PCR.

The nucleotide and amino acid sequence analysis and sequence comparison with DNAs registered in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries were performed with MacDNASIS software (Hitachi Software Engineering Co., Ltd. Tokyo, Japan) and the FASTA program (31), respectively.

Blot analysis of nucleic acids. The digests of chromosomal DNA by restriction enzyme were separated by 0.4 or 0.7% agarose gel electrophoresis. After the denaturation of DNA under alkaline conditions, capillary transfer of the DNA to FLASH nylon membranes (Stratagene) was performed overnight (33). The membranes were hybridized with the biotin-labeled probe before the chemiluminescent detection of the probe on the membrane with the FLASH Prime-It detection kit (Stratagene) according to the instructions of the manufacturer.

For the analysis of mRNA, total RNA was extracted from exponentially growing *S. aureus* cells as described previously (21). RNA in the lysate was further purified by a CsCl purification procedure (10). Twenty micrograms of total RNA was electrophoresed on formaldehyde agarose gels and transferred to FLASH nylon membranes (Stratagene). Membranes were prehybridized for 1 h at 60°C and hybridized at 60°C overnight with radiolabeled probe under essentially the same conditions as those for colony hybridization.

Transformation of *S. aureus*. DNA of *S. aureus* was prepared from protoplasts (36). Competent recipient cells were prepared by treating the bacteria with helper phage 55 and 0.1 M $CaCl_2$ (37). Tetracycline-resistant or methicillin-resistant transformants were selected by plating on tryptic soy agar (Difco) containing 10 μg of tetracycline or 2.5 μg of methicillin per ml, respectively, and incubating at 37°C overnight.

Susceptibility test. MICs were determined by using serial twofold dilutions of antibiotic in Mueller-Hinton agar (Difco) supplemented with 0.5% NaCl. The overnight culture of bacteria in tryptic soy broth was diluted to about 10^6 CFU/ml. A bacterial suspension of 1 μl was inoculated onto the agar and incubated at 37°C for 18 to 20 h before the MIC was scored.

For population analysis of methicillin-resistant *S. aureus*, overnight cultures of bacteria were plated at different cell concentrations onto L agar (5 g of NaCl, 5 g of yeast extract [Difco], and 10 g of tryptone [Difco] per liter [pH 7.2] solidified

with 1.5% agar) containing serial twofold dilutions of methicillin and, when the transposon mutant was analyzed, 10 μg of tetracycline per ml and incubated at 37°C for 72 h.

Other procedures. Analysis of PBP was carried out as described previously (28). Triton X-100-induced autolysis was spectrophotometrically measured as described elsewhere (8).

Primers and probes. Oligonucleotides were synthesized with a Gene Assembler Plus DNA synthesizer (Pharmacia Biotechnology). Primers TR1 (5'TGATAAAGTGTGATAAGTCC) and TL1 (5'AAATACTCGAAAGCACATAG) were synthesized according to the nucleotide sequences of the right and the left ends, respectively, of Tn*916*, which was reported to have the same size and the same restriction maps as Tn*918* (6, 7). Oligonucleotides were labeled with the Megalabel kit (Takara Shuzo) with [γ-^{32}P]ATP (222 TBq/mmol; Amersham). The pAM378 (used as Tn*918* probe) and PCR products were labeled with the Prime It Random Primer labeling kit (Stratagene) with [α-^{32}P]dCTP (111 TBq/mmol; Amersham). The biotin-labeled Tn*918* probe for chemiluminescent detection was prepared with the FLASH Prime-It labeling kit (Stratagene) with pAM378 as a template.

Nucleotide sequence accession number. The nucleotide sequence data for the *llm* gene will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number D21131.

RESULTS

Isolation of a transposon insertional mutant with reduced methicillin resistance. Tn*918* was introduced into the chromosome of high-level methicillin-resistant *S. aureus* SRM551 (methicillin MIC, 800 μg/ml) by filter mating. About 30,000 tetracycline-resistant transconjugants were examined for the methicillin resistance level by replica plating. Four colonies which appeared to have become more susceptible to methicillin were picked out. Association of Tn*918* insertion with the reduction of methicillin resistance was examined by transforming the parent strain, SRM551, with the chromosomal DNA of each transconjugant. Clear association was confirmed for only one transconjugant, and the transformant thus obtained was named SRM563 (methicillin MIC, 12.5 μg/ml) and used for further studies.

Strain SRM563 was proved to have a single copy of Tn*918* in the chromosome by DNA blot analysis with Tn*918* probe. When the chromosomal DNA of the mutant SRM563 was digested with *Eco*RI, whose recognition site was absent from Tn*918* (7), a single 30-kb fragment became hybridized with the probe (Fig. 1A). On the other hand, digestion with *Hin*dIII, which had a single recognition site within Tn*918*, produced 12- and 8-kb fragments hybridizing with the probe (Fig. 1B). Digests of chromosomal DNA of the parent gave no signal. These results showed that *S. aureus* SRM563 had a single copy of Tn*918* in the chromosome.

Genetic linkage between *mecA* and the insertion site of Tn*918* was examined by the transformation of *S. aureus* RN2677, which was methicillin susceptible and novobiocin resistant, with chromosomal DNA of the mutant SRM563. None of the 105 tetracycline-resistant transformants exhibited methicillin resistance, nor did any of the 87 methicillin-resistant transformants show tetracycline resistance, thus revealing no linkage between *mecA* and Tn*918*, whereas *mecA* and novobiocin marker were cotransferred as reported previously (23).

Cloning and sequencing of Tn*918* insertion site. The 12-kb fragment of the *Hin*dIII digests of SRM563 chromosomal DNA detected with the Tn*918* probe (Fig. 1B) was excised from agarose gel and ligated into the *Hin*dIII site of ΔpBR322 (Δ*Sal*I-*Pvu*II; ampicillin resistant and tetracycline susceptible) prepared by deletion of the *Sal*I-*Pvu*II region containing a part of the tetracycline determinant from pBR322 to produce pSR931. *E. coli* HB101 transformants harboring pSR931 were successfully isolated by colony hybridization using the Tn*918* probe. However, cloning of the 8-kb fragment of *Hin*dIII digests or the 30-kb fragment of *Eco*RI digests into the same vector was not successful.

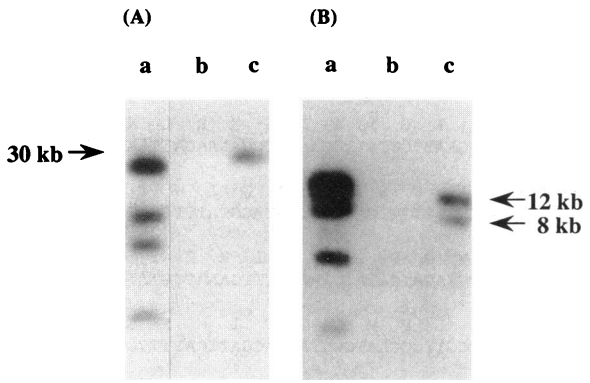

FIG. 1. Southern blot analysis of Tn*918* insertion in *S. aureus* chromosome. Total DNAs from strains SRM551 and SRM563 were digested with *Eco*RI (A) or *Hin*dIII (B) and hybridized with the Tn*918* probe after separation on a 0.4 or 0.7% agarose gel, respectively. Lanes: a, *Eco*RI digest of pAM378 as marker; b, SRM551; c, SRM563. Fragments which hybridized with the probe are indicated (arrows).

Using pSR931 as a template, we sequenced the region adjacent to the Tn*918* insert with primer TR1 which corresponded to the right end of the transposon. The region sequenced was the 3' end of the open reading frame. On the basis of the sequence determined, a 52-mer probe, corresponding to nucleotides 1106 to 1157 in Fig. 2, was synthesized, and the λZAPII genomic library of *S. aureus* SRM551 was screened with this probe. Five positive phage clones (B1, B2, B3, B4, and B5) were obtained by plaque hybridization, and the pBluescript phagemids with the inserts were excised from the recombinant phages and introduced into *E. coli* XL1-Blue to produce the corresponding plasmids B1, B2, B3, B4, and B5. Plasmid B5 contained the full length of the open reading frame, and *E. coli* carrying this plasmid grew very slowly. All the other recombinant plasmids had the open reading frames truncated in the 5'-terminal region, and their host *E. coli* grew normally. Thus, the product of the cloned gene showed a growth-inhibitory effect on *E. coli*.

The nucleotide sequence of the 1,436-bp region around the Tn*918* insertion site was determined with these recombinant plasmids as template DNA (Fig. 2). The plasmid B5 was used for sequencing of the 5' region, which the other recombinant plasmids did not have. However, there was the possibility that, because of the growth-inhibitory effect of the gene cloned, the plasmid B5 had mutations in the 5' region which allowed the host to grow. Thus, the sequence of the 5' region was confirmed by direct sequencing of the genomic DNA of strain SRM551. The open reading frame extended between nucleotides 148 and 1200, which corresponded to a protein of 351 amino acid residues with a molecular weight of 38,512. The protein encoded was hydrophobic (Fig. 3) and was considered a membrane protein. An amino acid sequence homology search using EMBL or GenBank nucleotide sequence databases revealed no significant homologies of the protein with

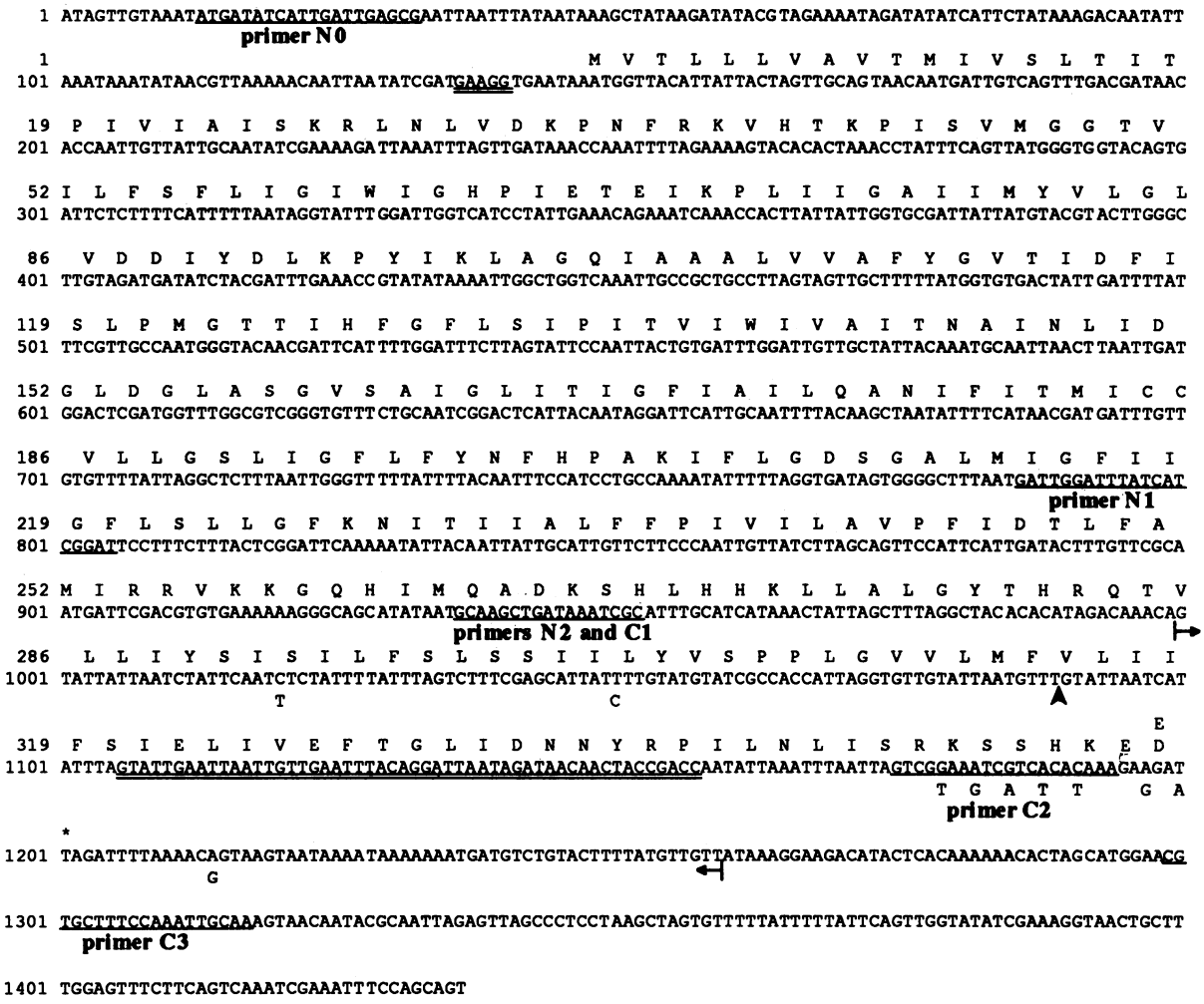

FIG. 2. Nucleotide sequence of *llm* gene and predicted amino acid sequence of *S. aureus* SRM551. Nucleotides and amino acid residues of *S. aureus* SRM401 gene which differ from those of SRM551 gene are shown under and above the SRM551 sequence, respectively. The region of sequence determined for the SRM401 gene is indicated by arrows. The arrowhead indicates the Tn*918* insertion site. The putative ribosome binding site and the sequence for the 52-mer probe used in plaque hybridization are indicated by double underlines. The primers used in PCR are noted under the corresponding underlined sequences. "C" in the primer name means that the sequence is complementary to that of corresponding underlined region.

those reported thus far. We temporarily designated the gene *llm* (for lipophilic protein which affects bacterial lysis rate and methicillin resistance level).

To confirm the Tn*918* insertion site and to deduce the amino acid sequence of the mutant protein, the junction region between Tn*918* and *llm* was amplified by PCR with primers TL1 corresponding to the left end of the transposon and N1 (Fig. 2), and the sequence of the PCR product was directly determined. The Tn*918* insertion site was localized between nucleotides 1089 and 1090, which agreed with the site determined by sequencing of pSR931. The mutant protein lost the C-terminal hydrophilic region of 37 amino acid residues and gained isoleucyl asparagine instead.

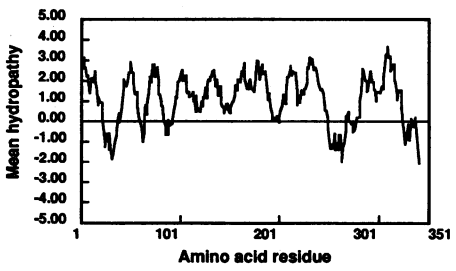

FIG. 3. Hydrophobicity profile of the *llm* product. Hydropathy was calculated by the method of Kyte and Doolittle (24) with a span of 10 amino acid residues.

Existence of *llm* and nucleotide sequence variation of the 3′ end among *S. aureus* strains. PCR with the primers N1 and C1 (Fig. 2) amplified a 167-bp fragment in all 115 *S. aureus* strains tested, including methicillin-resistant and -susceptible strains. In contrast, the fragment was not amplified in the strains of the other 26 staphylococcal species.

Further PCR analysis using primers N1 and C3 revealed that a 532-bp fragment was amplified in 86 of 90 methicillin-resistant strains and in 13 of 25 susceptible strains of *S. aureus* showing sequence variations around the region corresponding

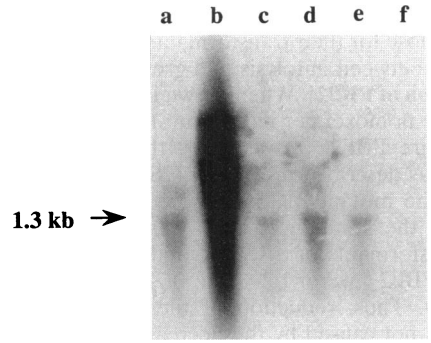

FIG. 4. Blot analysis of mRNA of *llm*. Total RNAs were electrophoresed and hybridized with the *llm* probe. Lanes: a, *S. aureus* SRM551; b, *S. aureus* SRM563; c, *S. aureus* SRM401; d, *S. aureus* RN2677; e, *E. coli* XL1-Blue harboring plasmid B5; f, *E. coli* XL1-Blue harboring a pBluescript without insert. The hybridizing fragment is indicated (arrow).

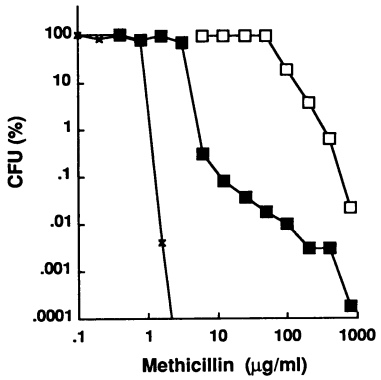

FIG. 5. Population analysis of *S. aureus*. Overnight cultures of bacteria were plated in L agar containing methicillin and incubated at 37°C for 72 h. Symbols: □, *S. aureus* SRM551; ■, *S. aureus* SRM563; ×, *S. aureus* NCTC8325.

to the primer C3. In addition, the sequence between nucleotides 1000 and 1259 of the low-level methicillin-resistant strain SRM401 determined by using the PCR product with primers N2 and C3 as a template revealed nine nucleotide substitutions inside the gene, with seven of them at the third position of the 3′-terminal 8 codons (Fig. 2). In contrast to the nucleotide variability, only a C-terminal aspartate in strain SRM551 was replaced by a glutamate in strain SRM401 in the 67 C-terminal amino acids examined. PCR with primers N1 and C2, which distinguish the sequence of SRM551 from that of SRM401, amplified the 409-bp fragment in 66 of 90 resistant strains and 18 of 25 susceptible strains. Further sequence determination with seven other strains, including three susceptible strains, in which the 532-bp fragment was amplified by PCR with primers N1 and C3 revealed three strains which gave the 409-bp amplified fragment and four strains which gave no fragment to have a 3′-terminal sequence identical to that of SRM551 and SRM401, respectively, irrespective of their resistance level. Thus, there were at least two types of *llm*, the SRM401 and the SRM551 types, among the *S. aureus* strains with respect to the 3′-terminal sequence.

Substitution of SRM401-type *llm* for SRM551-type *llm* by precise excision of Tn*918*. *S. aureus* SR3712 (methicillin MIC, 12.5 μg/ml) which had SRM401-type *llm* was transformed with DNA of the mutant SRM563. A representative tetracycline-resistant transformant was spread on an agar plate containing no drug. After incubation for 18 h at 37°C, the plate was stored at 4°C for a week. While most of the colonies became transparent, leaving the central part opaque, some remained opaque and were shown to lose tetracycline resistance. Sequence analysis revealed that Tn*918* was precisely excised and the SRM401-type gene was consequently replaced by an SRM551-type gene in these transformants. However, the methicillin MICs for these strains were identical to that for the parent strain SR3712. Similar results were obtained with the other low-level methicillin-resistant strains, SRM106 derived from susceptible strain RN2677 by introducing *mecA* and SRM401. Accordingly, the SRM551-type gene was not associated with high-level resistance.

Analysis of mRNA. Transcription of *llm* was demonstrated to occur in strains SRM551 (high resistance), SRM401 (low resistance), and RN2677 (susceptible) by RNA blotting analysis with a radiolabeled *llm* probe prepared from a PCR product with primers N0 and C1 (Fig. 2 and 4). In these strains, a 1.3-kb fragment was observed to hybridize with the *llm* probe. The hybridizing fragment of the same size was observed in *E. coli* XL1-Blue harboring plasmid B5 which contained the full-length *llm*. Thus, *llm* was transcribed irrespective of the methicillin resistance level. The reason for the smear seen in the SRM563 blot was unclear. Transcription from Tn*918* with random termination appeared to occur.

Susceptibility and autolytic activity of the transposon mutants. Reduction of the methicillin resistance level of the mutant SRM563 was shown by population analysis (Fig. 5). The mutant showed heterogeneous expression of methicillin resistance characteristic of low-resistance strains.

Next, we examined the influence of transposon insertion into *llm* on resistance level and the autolysis rate in various other strains of methicillin-resistant *S. aureus*. The strains listed in Table 1 were transformed with SRM563 DNA to select tetracycline-resistant transformants for which MICs of methicillin and imipenem were determined. Acquisition of mutant *llm* resulted in the reduction of the resistance level, which was generally more profound in most of the high-level- than in the low-level-resistance strains, with the exception of a transformant of strain SR17164 which showed no resistance reduction. This transformant grew very slowly. Transformants of SRM401 and methicillin-susceptible strain RN2677 also showed little if any resistance reduction.

Heterogeneities of resistance of the transformants derived from four strains with high resistance and five strains with low resistance were examined by spreading the bacteria on agar plates containing 100 μg of methicillin per ml. All transformants tested gave rise to colonies at frequencies of more than 10^{-5}, thus showing heterogeneous resistance.

Autolysis rates were determined for five pairs of the parent and transformant from high-level-resistance strains, six pairs from low-level-resistance strains, and a pair from the susceptible strain, which are listed in Table 1. The transformants lysed more rapidly than their parents irrespective of the resistance level in 8 of 10 pairs from the clinical isolates examined. The transformant of RN2677 also lysed more rapidly. (Fig. 6A shows representative results.) The possibility that Tn*918* itself, and not the insertional mutation of *llm*, contributed to the increase in the autolysis rate was excluded by the facts that strain SRM564, which carried Tn*918* at a site other than *llm* and was derived from strain SRM551, showed the same autolysis rate as the parent. The transformants of two clinical isolates, SR17164 which exhibited no reduction of

TABLE 1. Influence of *llm* mutation on methicillin resistance level of *S. aureus*

Bacterial strain[a]	MIC (μg/ml) for[b]:			
	Parent		Transformant[c]	
	Methicillin	Imipenem	Methicillin	Imipenem
SRM551[d,e]	800	50	12.5	0.39
SR17003[d,e]	800	50	25	0.78
SR17004	800	50	200	6.25
SR17005	800	50	12.5	0.2
SR17026	400	25	50	0.39
SR17049	>800	100	200	6.25
SR17164[d]	800	100	800	50
SR17170	800	50	12.5	0.2
SR17183	800	50	12.5	0.39
SR6606	800	50	100	6.25
SR6626	800	50	200	6.25
SRM1371[d,e]	400	12.5	12.5	0.39
SRM1561[d,e]	>800	100	50	0.78
SR3665	800	50	12.5	0.39
SR3716	>800	100	25	0.78
SR5613	400	25	25	0.39
SR5640	100	6.25	6.25	0.1
SR3618[d]	12.5	0.39	6.25	0.1
SR3704	25	0.39	12.5	0.2
SR3712[d,e]	12.5	0.2	6.25	0.1
SR17238[d,e]	6.25	0.2	1.6	0.05
SRM401[d,e]	6.25	0.1	6.25	0.2
SRM719[d,e]	6.25	0.2	3.13	0.05
RN2677[d]	1.56	0.025	1.56	0.025
SRM106[d,e]	12.5	0.39	6.25	0.2

[a] All strains except RN2677 were methicillin resistant. Strain SRM106 was a transformant of RN2677 with *mecA*.
[b] MICs were determined by the agar dilution method.
[c] *llm* mutation of *S. aureus* SRM563 was introduced into parents by genetic transformation.
[d] Autolysis rates were determined.
[e] Heterogeneous resistance of the transformant was confirmed by the observation that more than 0.001% of total population could give rise to colonies on plates containing 100 μg of methicillin per ml.

resistance, and SRM401, and that of SRM106 showed lower autolytic rates than the parents (Fig. 6B).

While the transformant of SR17164 showed a generation time twice as long as that of its parent (about 60 and 30 min, respectively), generation times for SRM551 and SRM401 were similar to those for their transformants. Thus, there seemed no correlation between autolysis and growth rates.

Production of PBP2'. When grown in the presence of 1 μg of cefazolin or flomoxef per ml, strain SRM551 produced 2.3- or 2.6-fold more PBP2', respectively, than under noninductive conditions as described previously (28). The induced levels of PBP2' in the mutant SRM563 were similar to those of the parent, but the noninduced level was 1.7-fold higher. In the Tn*918* transformants of SRM401 and SRM1561, noninduced levels of PBP2' were 1.2- and 0.7-fold those of parents, respectively. Thus, reduction of the resistance level of the mutant was not caused by the decrease of PBP2' production. No significant differences of profiles of the other PBPs were observed between strain SRM551 and its mutant SRM563 (data not shown).

DISCUSSION

Erythromycin transposon Tn*551* was used for transposon mutagenesis in studies on methicillin-resistant *S. aureus*. This transposon could be inserted into various sites of the *S. aureus* chromosome to allow isolation of *fem* and *mecA* mutants (2, 20, 29). These mutants revealed that a set of genes, in addition to *mecA*, was involved in the expression of methicillin resistance and also of high-level methicillin resistance. The transposon insertion was demonstrated to occur at specific sites rather than random ones when its insertion sites were examined within a relatively short DNA span (18). Thus, some genetic determinants which affect methicillin resistance might undergo hardly any Tn*551* mutagenesis. To identify these determinants, we used a different transposon, Tn*918* (7), which is thought to have different insertion site specificity. Another advantage of using Tn*918* is that the transposon is precisely excised to regenerate the intact gene in which it had resided, as shown with Tn*916* which was almost identical to Tn*918* (12).

Until now, the genes shown to affect methicillin resistance were *fem* genes. The *llm* gene identified in the present study differs from *femA* and *femB* in the nucleotide sequence (2). In addition, the *llm* product is hydrophobic and those of *femA* and *femB* are hydrophilic, suggesting that their locations differ when functioning. The mutation of *llm* reduced a high level to a low level of resistance but not to a susceptible level and did

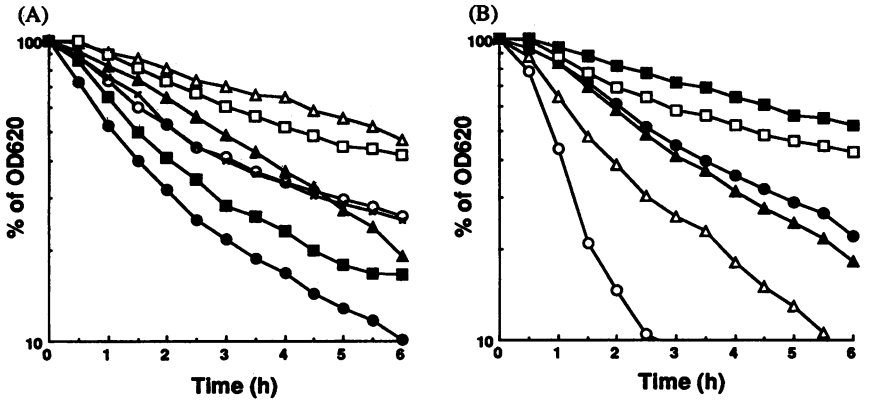

FIG. 6. Autolysis of *S. aureus* strains and their *llm* mutants in the presence of Triton X-100. Open and closed symbols represent parent strains and *llm* mutants, respectively. (A) *S. aureus* strains whose autolysis rate increased after mutation of *llm*. Symbols: ○ and ●, SRM551 and SRM563; △ and ▲, SR3618 and its mutant; □ and ■, RN2677 and its mutant; ×, SRM564. (B) *S. aureus* strains whose autolysis rate decreased after mutation of *llm*. Symbols: ○ and ●, strain SR17164 and its mutant; △ and ▲, strain SRM401 and its mutant; □ and ■, SRM106 and its mutant. OD_{620}, optical density at 620 nm.

not diminish the low-level resistance very much. In contrast, the *femA* or *femB* mutants of low-resistance strains lost their resistance trait and became susceptible (1). On the other hand, *femC* and *femD* were very similar to *llm* in that strains changed from high to low resistance upon the mutation of these genes (3, 20). Autolysis rates of the mutants, however, increased for *llm* mutation and decreased for *femC* and *femD* mutations (8). The nucleotide sequence of *llm* differed from that recently determined for *femC* (14).

Comparison of the nucleotide sequences of the 3′-terminal region of *llm* in nine strains revealed that nucleotide substitutions occurred at the third position of the 3′-terminal codons at high frequency. In addition, all nine strains had one of two sequences, the SRM401 or the SRM551 type, and no strain had an intermediate sequence. It may be that, because of the limited number of origins from which methicillin-resistant strains emerged as shown recently (22, 30), all of the resistant strains examined originated from strains with either an SRM401- or an SRM551-type sequence. The susceptible strains tested might have been derived from resistant strains by loss of *mecA* (19), since for the sequence determination, we selected the strains giving the 532-bp PCR product that the most resistant strains gave with primers N1 and C3. It was unclear why the nucleotide substitutions occurred at high frequency in the 3′-terminal region and how many sequence variations the *S. aureus* species had. In spite of the variability of the nucleotide sequence, only C-terminal aspartate in the SRM551-type sequence was replaced by glutamate in the SRM401 type within the region where sequence comparison was made. The amino acid conservation suggested that strains having an *llm* mutation could barely survive in the population. A possible explanation for this is that the *llm* mutants tend to die more rapidly because of their high autolysis rate or that the *llm* product is important for growth. The possibility that the product was indispensable could not be ruled out, since Tn*918* insertion occurred close to the C terminus, which might result in the production of a partly functional protein.

Transformation of various strains of methicillin-resistant *S. aureus* with *llm* mutant DNA revealed that the reduction of resistance and increase of autolysis rate occurred concurrently in most transformants. Though the physiological basis for the responses of these strains to *llm* mutation was unclear, the reduction of resistance and increase of autolysis rate seemed to be associated with each other in most strains. The correlation between high-level methicillin resistance and autolysis rate has received considerable attention in connection with the mechanism of high-level resistance. In *femC* and *femD* mutants of the high-resistance strain, the reduction of methicillin resistance occurred with a concomitant decrease of the autolysis rate (8). In contrast, an earlier report described a low-resistance strain derived from a high-resistance one showing a higher autolysis rate (15). Furthermore, autolysis of bacteria exposed to penicillin was reported to be a postmortem event (25). These facts, taken together with our present data, suggested that a lower level of resistance and a higher autolysis rate do not always occur together. There are presumably several genes which when inactivated result in a reduction of resistance. Inactivation of different genes might cause different physiological alterations: some could increase and others could decrease the autolysis rate.

The intact product of *llm* was needed to keep methicillin resistance in most of the high-level methicillin-resistant strains but not in strain SR17164, as shown in Table 1. Furthermore, the population analysis shown in Fig. 5 indicated that high-resistance colonies emerged very easily from low-resistance *llm* mutant on a plate containing a high concentration of methicillin. Their resistance levels were not affected by *llm* mutation. Taking these facts into consideration, there seem to be several different genes which were assumed to give high-level methicillin resistance and to differentiate high-resistance from low-resistance strains (29, 38). However, *llm* did not seem to be one of these genes, because all *S. aureus* strains (including susceptible ones) had *llm* and seemed to express it.

The physiological function of the *llm* product remains to be determined. Two observations suggested that this protein is involved in the metabolism of cell surface constituents such as peptidoglycan. First, mutation of the gene resulted in reduction of methicillin resistance and increase of the autolysis rate. The products of *femA* and *femB* with similar influence on methicillin resistance were demonstrated to be involved in the biosynthesis of the pentaglycine cross bridge of peptidoglycan (9, 18, 26). Second, the hydrophobic nature of the protein suggested that it functions on the membrane as in the case of peptidoglycan biosynthesis enzymes such as PBPs. Elucidation of its physiological role should provide a better understanding of cell wall biosynthesis and the mechanism of high-level methicillin resistance.

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