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Formation of Potent Hybrid Promoters of the Mutant *llm* Gene by IS*256* Transposition in Methicillin-Resistant *Staphylococcus aureus*

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From high-level methicillin-resistant *Staphylococcus aureus* SRM551, the low-level heterogeneously resistant mutant, SRM563, was isolated by transposon mutagenesis. The transposon insertion occurred in the 3′ region of the *llm* gene in the mutant (H. Maki, T. Yamaguchi, and K. Murakami, J. Bacteriol. 176:4993–5000, 1994). Resistant revertants were generated from the mutant strain SRM563 on the plate containing methicillin at a concentration of 12.5 μg/ml or more. In some revertants, the insertion sequence IS*256* was observed to be transposed into one of five sites localized 88 to 212 bp upstream of the mutant *llm* at a frequency of 2.8×10^{-7} in the bacterial population. The IS*256* transposition created a new hybrid promoter in which the −35 region at the end of IS*256* was properly arranged in relation to the −10-like sequence upstream of *llm*. The new promoters greatly enhanced the transcription of the mutant *llm*, as judged by blotting analysis of *llm* mRNA, with concomitant elevation of the methicillin resistance. Involvement of the insertion sequence in the heteroresistance characteristics of methicillin-resistant *S. aureus* was suggested.

Various insertion sequences (ISs) are widely distributed among bacteria. They can be transposed to various loci in chromosomal DNA, which sometimes results not only in disruption of genes but also in activation of downstream genes (5). With some IS elements, such as IS*10* in *Escherichia coli*, the activation is caused by a promoter located within the element and directed outward into the adjacent gene (3). On the other hand, some elements, such as IS*1* (19) or IS*2* (10), can activate the downstream genes via the formation of a hybrid promoter in which the −35 region at the end of the element is properly positioned close to the −10 region in the host DNA. Many IS elements have been shown to contain the −35-like sequence of the promoter in their terminals (5), although not all elements have been demonstrated to be involved in the formation of functional promoters. In *Staphylococcus aureus*, IS*256* and IS*257*, which compose both ends of Tn*4001* (14) and Tn*4003* (15), respectively, belong to this category of IS elements. These elements appear to construct the hybrid promoter for the aminoglycoside resistance gene, *aacA-aphD*, in Tn*4001* (23), and that for the trimethoprim resistance gene, *dfrA*, in Tn*4003* (24), respectively. However, their transposition with concomitant activation of adjacent genes has not been observed.

Previous studies have shown that *S. aureus* became methicillin resistant via the production of a low-affinity penicillin-binding protein, designated PBP2′ or PBP2a, encoded by *mecA* of foreign origin (7, 30). The MIC of methicillin, however, varied from 3.1 μg/ml for some strains to more than 800 μg/ml for others; thus, in addition to *mecA*, other, unknown genetic factors seem to determine the resistance level (18, 25). We introduced transposon Tn*918* with a tetracycline resistance marker into the chromosome of high-level methicillin-resistant *S. aureus* (MRSA) and isolated an insertional mutant, SRM563, with reduced resistance. The mutant had a Tn*918* insert in the 3′-terminal region of *llm* and showed heterogeneous methicillin resistance similar to those of clinical isolates of low-level MRSA (17). Since the mechanism of the heteroresistance of MRSA is not known, it should be very interesting to elucidate the molecular basis of the heteroresistance of the mutant SRM563.

In the present study, we isolated high-level resistant revertants from the insertional mutant SRM563 by methicillin selection and analyzed the genetic factor which raised the resistance level in the revertant. IS*256* was found to be transposed into the area upstream of the mutant *llm*, with concomitant elevation of the resistance level in some of the revertants. The end of IS*256* had a sequence (TTGACT) (1) similar to the −35 consensus sequence (TTGACA) of the promoter (6), and this, together with one of the −10-like sequences located upstream of *llm*, formed a new hybrid promoter with strong activity, which led to an increase in the transcription of the mutant *llm*. The results of this study suggested the important role of IS transposition in the expression of heteroresistance.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. aureus* SRM551 (MIC of methicillin, 800 μg/ml) was a high-level methicillin-resistant, penicillinase-negative strain. *S. aureus* SRM563 (MIC of methicillin, 12.5 μg/ml) was a low-level heterogeneously methicillin-resistant mutant isolated by Tn*918* mutagenesis from strain SRM551 and had a Tn*918* insertion at the 3′-terminal region of *llm* (17). Bacteria were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) or in L broth (1% tryptone [Difco], 0.5% yeast extract [Difco], 0.5% NaCl [pH 7.2]) at 37°C. The strains with Tn*918* insertions were grown in medium containing 10 μg of tetracycline per ml in order to prevent transposon excision.

Primers and probes. Oligonucleotides were synthesized with Oligo 1000 (Beckman, Fullerton, Calif.). Primers A (5′-GCTTAATTGGAGCCGTTCTT-3′) and B (5′-TATTTTTAGAAACGTCATCC-3′) were synthesized according to the nucleotide sequence of IS*256* (1). The other primers and a probe used in this study are shown in Fig. 2. The primers for primer extension and the probe for Northern analysis were end labeled with [γ-^{32}P]ATP (222 TBq/mmol; Du Pont, NEN, Boston, Mass.) by using the Megalabel kit (Takara, Kyoto, Japan).

Transformation of *S. aureus*. Chromosomal DNA of *S. aureus* was prepared from protoplast (27). *S. aureus* was transformed with chromosomal DNA having a Tn*918* insertion as described previously (29). Briefly, competent recipient cells prepared by treatment with helper phage 55 and 0.1 M $CaCl_2$ were incubated with DNA for 2.5 min in an ice bath and for 2.5 min at 37°C. The tetracycline-

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resistant transformants were selected on tryptic soy agar containing 10 μg of tetracycline per ml.

PCR and DNA sequencing. PCR amplification was carried out with GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems, Foster City, Calif.) by using the TaKaRa Taq kit for PCR (Takara) as described previously (17) except that 1.25% formamide was added to prevent nonspecific amplification. Direct sequencing of PCR products was performed with ABI 373 by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (both from Perkin-Elmer Applied Biosystems) according to the manufacturer's protocol.

Determination of transcription start site of *llm*. Total RNA was extracted from exponentially growing *S. aureus* cells as described previously (17). The 5′ end of the *llm* mRNA was determined by primer extension reaction with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, Md.) according to the manufacturer's instructions. The end-labeled 30-mer primer of 0.033 pmol was hybridized to 20 μg of total RNA at 65°C for 60 min in 100 μl of the hybridization buffer and was cooled to room temperature. The extension reaction was carried out at 37°C for 60 min. After ethanol precipitation, the reaction product was subjected to urea-polyacrylamide gel electrophoresis. As a control, by using a 17-mer primer corresponding to the 5′ terminal of the 30-mer primer for the primer extension reaction, a DNA sequencing reaction was performed by the dideoxy chain termination method with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio) and [^{35}S]deoxycytidine 5′-α-thiotriphosphate (222 TBq/mmol; Amersham International plc, Little Chalfont, Buckinghamshire, England), and the reaction product was also subjected to electrophoresis on the same gel. Signals were detected by autoradiography.

Northern blotting. Twenty micrograms of total RNA prepared as described above was electrophoresed on a formaldehyde-agarose gel and transferred to a FLASH nylon membrane (Stratagene, La Jolla, Calif.). The membrane was prehybridized in hybridization buffer (0.5 M sodium phosphate buffer [pH 7.2] containing 7% sodium dodecyl sulfate [SDS], 1% bovine serum albumin, and 1 mM EDTA) (2) for 1 h at 60°C and was hybridized in the same buffer with radiolabeled probe at 60°C overnight. Next, the membrane was washed with 2× standard saline citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS and then with 0.2× SSC plus 0.1% SDS for 30 min each time at room temperature; finally, it was washed twice with 0.2× SSC plus 0.1% SDS at 60°C for 15 min each time. The membrane was subjected to autoradiography.

Susceptibility test. MICs were determined by using serial twofold dilutions of antibiotic in L agar (L broth solidified with 1.5% agar). The overnight culture of bacteria in tryptic soy broth was diluted to about 10^6 CFU per ml. A bacterial suspension of 1 μl was inoculated onto the agar and incubated at 37°C for 20 h before the MIC was scored.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession no. D82063.

RESULTS

IS*256* transposition in the high-level methicillin-resistant revertants. From the heterogeneously methicillin-resistant mutant SRM563, a spontaneous revertant was isolated which grew well on the plate containing 400 μg of methicillin per ml. In the preliminary experiment to identify the mutation which caused the elevation of the resistance, the *llm* region of the revertant was transferred back to *S. aureus* SRM551 by transformation followed by tetracycline selection. Contrary to the expectation that *llm* with Tn*918* would reduce the resistance level, the transformants obtained grew well on the plate with 25 μg of methicillin per ml, indicating that the mutation was linked with *llm*. Further examinations by PCR with a pair of primers, N and R (Fig. 1A), revealed that the 1.8-kb DNA fragment was amplified in the revertant while the 499-bp fragment was amplified in strains SRM551 and SRM563, as expected from the sequence data (Fig. 1B). Therefore, a fragment of 1.3 kb was inserted into the area amplified with primers N and R. Next, an additional 154 colonies of strain SRM563 that grew especially well on plates containing methicillin concentrations of 12.5 to 100 μg/ml for a day or 200 to 400 μg/ml for 2 days were surveyed by PCR with the same primers, and 23 colonies were found to give the amplified fragment of 1.8 kb while the others gave a fragment of the same size as that given by strain SRM563. The insertion occurred in one in 3.6×10^6 bacterial cells in the population (frequency, 2.8×10^{-7}) when the revertants were isolated on plates containing 25 μg of methicillin per ml.

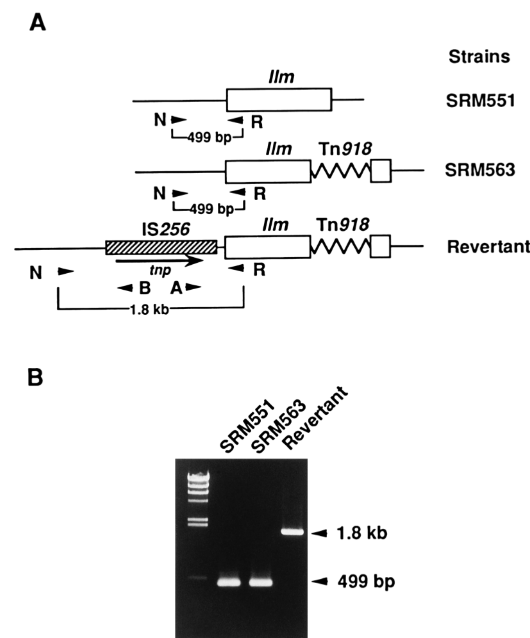


FIG. 1. Structure of the *llm* region and PCR amplification of the region upstream of *llm*. (A) The *llm* gene, Tn*918*, IS*256*, and the putative transposase gene (*tnp*) are represented by an open box, a zigzag line, a diagonally striped box, and an arrow, respectively. Primers used for PCR are shown by arrowheads indicating their orientations. The sizes of the products of PCR using primers N and R were 499 bp and 1.8 kb. (B) Agarose gel (1%) separation of DNA fragments amplified by PCR using primers N and R. Bacteriophage lambda DNA digested with *Hin*dIII was loaded on the left side as a molecular-size marker.

As described previously, during storage at 4°C for a week, colonies of the *llm* mutant SRM563 became transparent while those of the parental strain SRM551 remained opaque, a result which was due to the increased autolysis of the mutant. Colonies of all the revertants with the insertion, like those of the parental strain, did not become transparent. Thus, the autolysis rate, as well as the methicillin resistance level, reverted in part to the level of the parent upon IS insertion.

Since the known insertion sequence of about 1.3 kb in *S. aureus* was IS*256*, primers A and B within IS*256* (Fig. 1A) were synthesized according to the sequence data (1) and PCR was performed with the primer pair A and R or with primers N and B. All the resistant revertants with the 1.3-kb insert tested (24 strains) gave the amplified fragment (data not shown), confirming that IS*256* was inserted in the same direction in all these revertants. Nucleotide sequences of the junction regions of IS*256* and chromosomal DNA were then determined by direct sequencing of the amplified fragments given by 19 of 24 strains. The data revealed that IS*256* insertion occurred at one of five sites (Ins1 to Ins5 in Fig. 2) localized from 88 to 212 bp upstream of *llm*. The numbers of the revertants with IS*256* insertion at Ins1, Ins2, Ins3, Ins4, or Ins5 were 6, 4, 2, 5, or 2, respectively. Every IS*256* insert was flanked by an 8-bp duplication. These 8-bp sequences, considered to be target sequences for transposition of IS*256*, were distinct from one another. They were also distinct from the 8-bp direct repeats flanking IS*256* on the chromosome reported previously (4). No obvious site specificity for IS*256* transposition was observed.

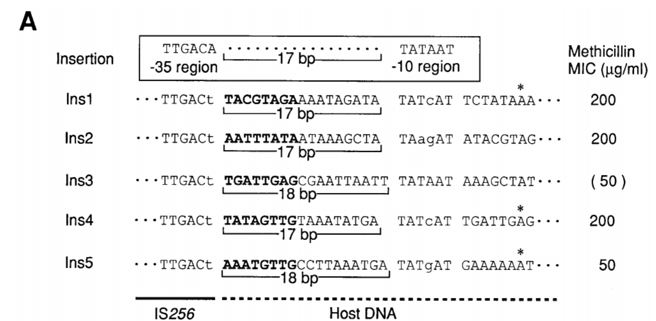
```
                                                        •••TCCTT
-300  TAATTGACCACTAGCTATTGTAAGTGAAAACTTACAATTTGTCATTAGTTTACATATAAA
        primer N
-240  ATTAATGTATGATATAGACTTTGATGTTAAAATGTTGCCTTAAATGATATGATGAAAAAA
                                    Ins5
-180  TGAATAATAGGCGATATATAAGAAATGAATCGTATAGTTGTAAATATGATATCATTGATT
                                       Ins4      primer PE2    Ins3
-120  GAGCGAATTAATTTATAATAAAGCTATAAGATATACGTAGAAAATAGATATATCATTCTA
              Ins2                      Ins1
 -60  TAAAGACAATATTAAATAAATATAACGTTAAAAACAATTAATATCGATGAAGGTGAATAA
                                                         SD
   1  ATGGTTACATTATTACTAGTTGCAGTAACAATGATTGTCAGTTTGACGATAACACCAATT
                    primer PE1                   probe F
  61  GTTATTGCAATATCGAAAAGATTAAATTTAGTTGATAAACCAAATTTTAGAAAAGTACAC

 121  ACTAAACCTATTTCAGTTATGGGTGGTACAGTGATTCTCTTTTCATTTTTAATAGGTATT

 181  TGGATTGGTCATCCT•••
        primer R
```

FIG. 2. Nucleotide sequence of the region upstream of *llm* where IS*256* insertion occurred. The *llm* coding region is boldfaced. The underlined insertion sites of IS*256* (Ins1 to Ins5) were duplicated upon the insertion. The putative ribosomal binding site (Shine-Dalgarno sequence [SD]) is also underlined. Arrows indicate sequences of primers N and R, used for PCR; primers PE1 and PE2, used for primer extension analysis; and a 48-mer probe, F, used for Northern analysis. Arrows pointing left indicate that the sequences are complementary to those of the corresponding region.

Formation of hybrid promoters. Sequences similar to the −10 consensus region of the promoter were found 17 or 18 bp downstream of IS*256* at all five insertion sites identified. Together with these −10-like sequences, the outward-facing −35 region at the end of IS*256* generated constructs similar to the *E. coli* σ^{70} consensus promoter (Fig. 3A) that *S. aureus* RNA polymerase could recognize (20). We next determined the transcription start site of *llm* with an IS*256* insertion at Ins1, Ins4, or Ins5. As shown by the example in Fig. 3B, the primer extension product was detected at the site 7 bp downstream of the −10-like region of the hybrid promoter in all the strains tested, while no products were detected at the corresponding site of the predicted promoter *P* within IS*256* (1). The construct created by IS*256* insertion was revealed to function as a hybrid promoter.

Methicillin resistance levels and hybrid promoter activities. The *llm* regions of the revertants with IS*256* insertions at a site other than Ins3 were transferred back to *S. aureus* SRM551 by transformation. Methicillin MICs against the transformants and the Ins3-type revertants were 50 or 200 μg/ml, while the MIC against strain SRM563 was 12.5 μg/ml (Fig. 3A). These results clearly showed that IS*256* transposition raised the resistance level. Furthermore, resistance levels of the Ins5-type transformant and the Ins3-type revertant were lower than those of the other types of strains. The former two types of strains had an 18-bp spacer sequence between the −35 and −10 regions of the hybrid promoter, which resulted in lower activity of the promoter, as described below. In addition, some revertants showed higher resistance levels than the corresponding transformants which were transformed with DNA of the revertants, suggesting the occurrence of a second mutation in these strains which elevated the resistance and was not linked to *llm*.

In order to examine the activity of the newly formed hybrid promoters, Northern analysis of Ins1-, Ins4-, and Ins5-type strains was performed with the 48-mer probe F complementary to the 5′ region of *llm* (Fig. 4). The amounts of *llm* transcript from the hybrid promoters were much larger than those from the wild-type promoters in strains SRM551 and SRM563. The amount of the transcript in the Ins5-type strain was a little less than the amounts in the Ins1- and Ins4-type strains, which appeared to correspond well to the lower resistance level of the former and which appeared to be due to the presence of the 18-bp instead of the 17-bp spacer between the −35 and −10 regions of the promoter. Since the *llm* transcripts from the hybrid promoters were a little smaller than that from the wild-type promoter in SRM563, the wild-type promoter was apparently located upstream of the sites where the hybrid promoters were formed, although its precise location has not been determined yet.



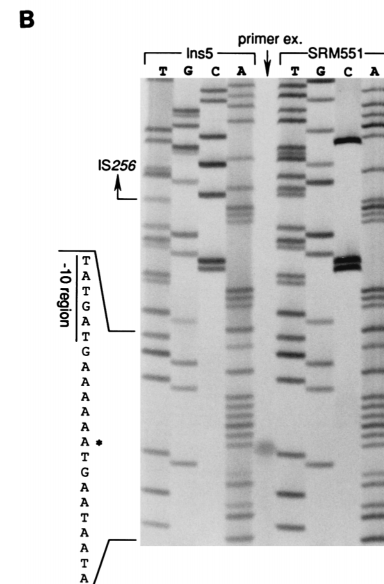


FIG. 3. Hybrid promoters formed by IS*256* insertions into the region upstream of *llm* and determination of the transcription start site. (A) Junction sequences of IS*256* insertions upstream of *llm* are shown. Open box, *E. coli* σ^{70} consensus promoter; lowercase letters, nucleotides of putative promoters differing from the consensus; boldface letters, 8-bp target sequences. The MICs of methicillin against the transformants with an IS*256* insertion at Ins1, Ins2, Ins4, or Ins5 are given on the right. The MIC in parentheses is that against two revertants with an Ins3 insertion that were independently isolated on plates containing 12.5 or 25 μg of methicillin per ml. The MIC against *S. aureus* SRM563, the parent strain of the revertants, was 12.5 μg/ml. The transcription start sites of the Ins1-, Ins4-, and Ins5-type hybrid promoters were determined by primer extension analysis with primers PE1, PE1, and PE2, respectively, and are indicated by asterisks. (B) Example of primer extension analysis of the revertant with the insertion at Ins5. The primer extension product with total RNA prepared from the revertant is shown as "primer ex." Lanes T, G, C, and A show the sequence analysis of the region upstream of *llm* in the revertant (Ins5) or *S. aureus* SRM551. The nucleotide sequences around the transcription start site (indicated with an asterisk) are given on the left.

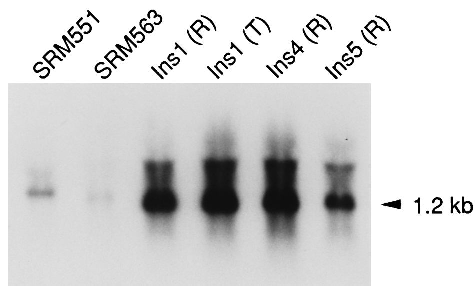


FIG. 4. Detection of mRNA of *llm*. Northern analysis was performed with 20 μg of total RNA of each strain and with the 48-mer probe F (see Fig. 2). R, revertant; T, transformant.

DISCUSSION

IS*256* was originally found flanking the aminoglycoside resistance determinant, yielding the composite transposon Tn*4001* (14) or a Tn*4001*-like element (9, 28). Both can transpose into various loci on the chromosome (12, 16) and are involved in the dissemination of the resistance gene among staphylococci, enterococci, and streptococci (11, 13, 26). In Tn*4001*, IS*256* was found to provide the −35 region for the promoter of the aminoglycoside resistance gene, thus constructing a hybrid promoter (23). In this case, transposition of IS*256* independent of a transposon had not yet been observed. On the other hand, IS*256* itself was present in multiple copies on the chromosome (9, 22) and was observed to increase in copy number and to transpose independently of Tn*4001* or the Tn*4001*-like element upon transformation or conjugation (21, 26). Our preliminary Southern analysis also suggested that there are multiple copies of IS*256* in most clinical isolates of MRSA and the parent strain, SRM551, and that its copy number increases in the *llm* mutant upon transformation (unpublished data). In those cases where independent transposition was observed, however, IS*256* was not shown to enhance the expression of certain genes. The present study has demonstrated that independent transposition of IS*256* without contact with external plasmids or bacteria occurred to form new, strong hybrid promoters, which increased the expression of the mutant *llm* gene, thereby making the bacteria more methicillin resistant.

The most distinct traits of hybrid promoters formed by IS*256* insertion were as follows. First, the promoters could be generated at multiple sites in structures different from one another, at least five in our experiment. This feature appeared to increase the probability of promoter formation and to produce great variety in activity levels among newly formed promoters. Indeed, differences in activity, which were observed among the hybrid promoters formed, were reflected in the resistance level recovered. The length of space between the −10 and −35 regions seemed to affect the activity. Second, the hybrid promoters were created at sites where there had been no wild-type promoter. In some cases, such as that of IS*2*, the −35 sequence within the IS element substituted for the −35 region of the original promoter and formed the hybrid promoter with the wild-type −10 region (10). Accordingly, the identical transcript was produced from the hybrid promoter. On the other hand, IS*256* in this study newly generated the hybrid promoters with sequences similar to that of the consensus −10 region. So even if the *llm* transcription from the wild-type promoter was subjected to some regulation, although nothing was known about that, the transcription from the hybrid promoter may not have been subjected to any regulation. Indeed, activities of the hybrid promoters, which are much higher than that of the wild-type promoter, may likely be ascribed to this trait. These characteristics of the hybrid promoters would help the bacteria to survive in a new environment, i.e., in the presence of methicillin.

The frequency of formation of the hybrid promoters by IS*256* (2.8×10^{-7}) was much higher than that by IS*2* in *E. coli* (less than 10^{-10}) (10). Aside from the multiple insertion sites of IS*256*, this might be due to the traits of the element itself, as well as to the lower GC content in *S. aureus* chromosomal DNA, which were favorable to transposition and to promoter formation. Among the IS*256* transpositions that occurred, we could detect only those resulting in the potent hybrid promoter formation which required IS*256* insertion at suitable sites in the proper direction. Thus, IS*256* seemed to transpose much more often than we had estimated, although there are only a few reports describing its transposition because it is difficult to detect.

Heteroresistance is one of the characteristics that low-level resistant strains of MRSA exhibit (8). However, its mechanism is not yet understood. Our mutant, SRM563, used in this study also showed heteroresistance, and the transposition of IS*256* causing transcriptional activation of the gene *llm* was shown to contribute to the heteroresistance. These findings suggested that IS transposition was also at least partly involved in the heteroresistance of clinical MRSA strains by activating or inactivating some genes.

Finally, in the strain SRM563, the *llm* gene was disrupted in the 3′-terminal coding region by Tn*918* insertion, and the product of the gene was deduced to be a protein truncated in the C-terminal region, since the stop codon was located close to the junction in Tn*918* (17). Thus, the possibility of the truncated protein being partly functional was noted previously (17). The present study strongly suggests that this was indeed the case, since the increase in the transcription of the mutant *llm* elevated the resistance level.

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