transducing Tn*551* in COL-TS339 into BB270 (13). Integrated DNA region containing *fmtA* was transduced into BB270-TS339 strain, then the MIC of oxacillin of the transductants were determined.

Muropeptide analysis. KSA8, KSA8-TS339 and KSA8-TS339C were further investigated, because the reduction of resistance level in KSA8-TS339 was most remarkable among the strains tested. Preparation of murein and reduction of muropeptide were described elsewhere (22, 27). Digested muropeptides were fractionated by reverse-phase high-pressure liquid chromatography (RP-HPLC) as described elsewhere (22, 27).

Recombinant FmtA protein. To produce recombinant FmtA protein, the DNA fragment corresponding to signal peptide-processed FmtA was amplified by PCR with two primers, and cloned in-frame downstream from the His-tag (6xHis) sequence in the pET28c expression vector (Novagen, Madison, WI) to generate pHK4171. The plasmid was verified by sequencing. The plasmid was electroporated into *E. coli* HMS174, and the transformant (HK4172) was used for purification of recombinant protein. HK4172 was grown at 37°C with shaking until OD660 reached to 0.5, then IPTG (final concentration 1 mM) was added to induce the expression of fusion protein. After 3 h incubation, bacteria were collected by centrifugation and resuspended in 20 mM Tris-HCl containing 0.5 M NaCl and 50 mM imidazole (pH 7.9) (buffer 1), then disrupted by Ultrasonic disruptor

(TOMY SEIKO, Tokyo). After centrifugation to remove the cell debris, the supernatant was applied to a TSKgel AF-chelate 5PW column (7.5 by 20 mm), pretreated with 50 mM $NiSO_4$, then equilibrated with buffer 1. Bound proteins were one step eluted with 20 mM Tris-HCl containing 0.5 M NaCl and 1 M imidazole (pH 7.9). Fractions containing recombinant FmtA proteins were detected by Coomassie brilliant blue stain after SDS-PAGE.

PBP assay. Recombinant FmtA protein was used for PBP assay. 0.5 μg (10 pmol) recombinant protein, dialyzed against 50 mM Tris-HCl (pH 7.5), was incubated with various concentrations of [^{3}H]-benzylpenicillin (20, 50, 100 pmol) (10 to 30 Ci/mmol; Amersham International, Bucks, United Kingdom) at 37°C for 15 min, then the reaction was stopped by the addition of the sample loading buffer. Samples were applied to the well, then electrophoresed in 10% polyacrylamide gel. PBP activity was detected by fluorography.

Antiserum. The purified recombinant FmtA protein (50 μg) emulsified with an equal volume of Freund's complete adjuvant (Difico Laboratories, Detroit, Mich.) was subcutaneously injected to a rabbit (weight 2.5 kg). After two and four weeks, recombinant protein (50 μg) emulsified with equal volume of Freund's incomplete adjuvant was subcutaneously injected to the rabbit. Subsequently, the rabbit was injected intravenously with 50 μg purified

protein in PBS. The antiserum for immunoblotting was used in 1,000 fold dilution.

Fractionation of proteins from *S. aurues* cells. *S. aureus* cells growing to late exponentially phase were collected by centrifugation at 10,000 X *g*. After washing with PBS, cells were suspended in PBS containing phenylmethylsulfonyl fluoride (1 mM) and digested with lysostaphin (final concentration 100 μg/ml) for 30 min at 37°C. Then, the supernatants obtained after centrifugation at 10,000 X *g* were used as whole cell lysate fraction. Various fractions of *S. aureus* cells were prepared as follows: (i) culture supernatant obtained by centrifugation at 10,000 X *g* for 30 min and concentrated 60 times by 80 % saturated ammonium sulfate precipitation, (ii) cell wall extract, (iii) membrane extract and (iv) cytoplasmic fraction obtained as follows. Cells were suspended in digestion buffer (30 % raffinose in 50 mM Tris [pH 7.5] with 0.145 M NaCl) containing 1 mg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.), 100 μg of DNase (Sigma), and phenylmethylsulfonyl fluoride (1 mM). Cell mixture was incubated for 1 h at 37°C. Protoplasts were removed by centrifugation at 8,000 x *g* for 10 min, and the supernatant was used as cell wall extracts. The protoplasts were disrupted by addition of 50 mM Tris-HCl (pH 7.5), then centrifuged at 100,000 x *g* for 60 min. Supenatant was used as cytoplasmic fraction, and precipitation suspended with 50 mM Tris-HCl (pH 7.5) was used as membrane extract.

Construction of *fmtA* promoter fusion. The putative *fmtA* promoter region was ligated into *BamHI* and *Hind*III sites within the *xylE* transcriptional fusion vector, pSL24 (Table 1). The DNA fragment containing the promoter region was amplified with two oligonucleotide primers (5'-AATAAGCTTACACACGCATGTATAACTAGT-3' and 5'-ACAGGATCCAGAACCAAT GCTAGAAGGATC-3') generating *BamHI* or *Hind*III sites from KSA8 chromosomal DNA for direct cloning into the upstream of the promoterless *xylE* reporter gene of pSL24, to generate pHK4125. The putative *orf1* promoter region, different transcriptional direction to *fmtA* (Fig. 1), was ligated into *BamHI* and *EcoRI* sites within pSL24. The DNA fragment was amplified with two oligonucleotide primers (5'-AATGAATTCACACACGCATGTATAACTAGT-3' and 5'-ACAGGATCCAGAA CCAATGCTAGAAGGATC-3') generating *BamHI* or *EcoRI* sites, then cloned into pSL24 to generate pHK4147. Both PCR fragments were verified by sequencing. The recombinant plasmids were electroporated into RN4220 and then transduced to strain COL by using phage 80 alpha.

Catechol 2,3-dideoxygenase assays. *S. aureus* COL strain having pHK4125 (HK9610) or pHK4147 (HK9605) was grown to various growth phase (OD_{660}= 0.4, 0.8, 1.0, 3.4, 5.0), and quantitative assays were performed as spectrophotometrically as described by Zukowski et al (37). When necessary, antibiotics were added to the medium initially, and cells were grown to reach OD660= 1.0. One unit is defined as increase of 12 OD_{375} per 1 min. Specific activity is defined as miliunits per milligram of

protein. This experiment was repeated at three times, then the mean and S.D. were calculated.

Effect of oxacillin on the amount of FmtA protein by immunoblotting analysis. Some portion (500 μl) of *S. aureus* COL overnight culture was inoculated into fresh TSB (25 ml) with or without antibiotics and grown at 37°C until OD660 reached to 1.0. Whole cell lysates were prepared as described above. The samples were separated in a 10% polyacrylamide gel and transferred to a nitrocellulose membrane by mini-transblot cell (Bio-Rad Laboratories, Tokyo, Japan). Immunoblotting was performed with the anti-FmtA serum or anti-62kDa *N*-acetylmuramyl-L-alanine amidase (62kDa-AM) serum as the first antibody, followed by peroxidase-conjugated goat F (ab')2 anti-rabbit IgG (ICN Pharmaceuticals, Inc., Aurora, Ohio). 62kDa-AM was the product of autolysin, ATL (19), and was previously reported that the amount of 62kDa-AM protein was not affected in the presence of ß-lactam antibiotics (20), so we used 62kDa-AM as standard. Detection was performed according to the mannual supplied with Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, Mass.). Immunoreactive bands corresponding to FmtA or 62kDa-AM were quantitated with an image scanner (NIH Image 1.52) connected to a computer, and the ratio of density of FmtA or 62kDa-AM in the experimental sample to the density in a sample prepared from the strain grown in the absence of oxacillin was calculated. This experiment was repeated three times, then the mean of each samples were calculated.

Chemicals and reagents. Oxacillin, methicillin, bacitracin and vancomycin were from Sigma Chemical Co, St Louis, MO. Erythromycin, chloramphenicol and fosfomycin were from Wako Chemicals (Tokyo, Japan). Cefoxitin and tetracycline were from Daiichi Seiyaku (Tokyo, Japan) and Lederle Japan (Tokyo, Japan), respectively.

RESULTS

***Cat* insertional mutant.** We obtained a mutant, COL-TS339C, from COL by inactivating *fmtA* by *cat* insertion (Fig.1). This mutant revealed a reduced MIC of oxacillin compared with that of the parent. The MIC was similar to that of COL-TS339 which carries a Tn*551* insertion at the C-terminal of *fmtA*. To eliminate the possibility of other mutation during construction, we transduced the *cat* gene from COL-TS339C into COL. All transductants reduced their MIC of oxacillin as was observed in COL-TS339C (Table 2). The *cat* gene was also transduced into another MRSA strains, KSA8, and the transductant was designated as KSA8-TS339C. It also showed reduced MIC to oxacillin. The MIC of each *cat*-insertional mutant to oxacillin was identical to that of respective Tn*551*-insertional mutants.

Site-directed mutagenesis of FmtA. Mutagenized *fmtA* sequences (46S change to 46C, 186Y to 186C, 63S to 63C, and 127S to 127C), which were constructed by PCR-site specific mutagenesis using pHK4130 as a template, were first cloned

into pGEM-T easy vector. After we confirmed the DNA sequence of these genes, they were cloned into pCL83 to generate pHK4188, pHK4157, pHK4159 and pHK4186 respectively (Table 1). We performed Western blotting with whole lysates of BB270, BB270-TS339 and BB270-TS339 harboring plasmid encoding mutant or wild type *fmtA* using anti-FmtA serum as primary serum. As expected, an immunoreactive band was observed with similar molecular mass in all strains except BB270-TS339 (data not shown), indicating the expression of mutagenized FmtA in vivo. The MIC of oxacillin of BB270-TS339 having pHK4188, pHK4157 or pHK4186 were similar to that of BB270 (128 μg/ml), while MIC of BB270-TS339 having pHK4159 was similar to that of BB270-TS339 (16 μg/ml) (Fig.2). So, only mutagenized FmtA with 127S altered to 127C did not restore the mutation, while the other three mutant *fmtA* genes restored the mutation.

Muropeptide analysis of the mutants. Muropeptide profiles of KSA8, KSA8-TS339 and KSA8-TS339C were established by RP-HPLC (Fig.3). The profile of KSA8 was similar to that of other wild type strains described earlier (22, 27). Like BB270, the highest peak was observed in the dimeric fraction, and a large amount of oligomers was observed in the profile of KSA8, consistent with the typical high degree of cross-linking of staphylococcal peptidoglycan. The two *fmtA*-inactivated mutants showed a similar muropeptide profile. Compared to the wild type, both mutants showed a slightly reduced amount of oligomeric muropeptides, as can be seen from the

increase especially of the monomers relative to all other fractions and the clear decrease of high molecular weight oligomers eluting at large retention time (Fig.3, Table 4). A detailed resolution of the monomer fractions is shown in Fig.3. All peaks were identified by comparison with standard samples based on mass spectrometry and/or amino acid analysis (22, 27). In KSA8-TS339 and KSA8-TS339C, the relative amount of the major monomeric component (M4, monomer pentapeptide with five glycine peak) to that of M9 (peak 7, glutamic acid instead of glutamine in M4) and D5 (dimer from M4 and M9) was reduced, compared to that of parent strain; while these changes were not drastic, they were clearly most pronounced in KSA8-TS339C (*fmtA::cat*).

Penicillin binding activity of recombinant FmtA. When the membrane fraction of COL were used for penicillin binding assay, we failed to detect a band corresponding to FmtA (data not shown). Since FmtA lacks one of three motifs which were typically found in ß-lactamases and PBPs, FmtA may have a very weak binding affinity to penicillin. We therefore assessed the penicillin binding activity of purified recombinant FmtA (rFmtA) with 6 x His tag at the N-terminal. 0.5 μg of rFmtA was incubated with ^{3}H-benzylpenicillin, subjected to polyacrylamide gel electrophoresis followed by fluorography. We did not detect any radioactive band (data not shown), suggesting that FmtA lacks covalent penicillin binding affinity.

Identification and localization of FmtA by immunoblotting. Using anti-FmtA serum, we found an immunoreactive band in whole lysate of COL cells corresponding to the molecular mass of FmtA calculated from DNA sequence, which was missing in whole lysate of COL-TS339C (Fig.4). To investigate the cellular localization of FmtA, we performed Western blotting analysis with various fractions of COL cells. Fig4 shows that FmtA was predominantly found in the membrane fraction but not in supernatant, cell wall, or cytoplasmic fraction.

Transcriptional fusion studies. (i) promoter activity during growth: Fig. 5 showed that the promoter activity of *fmtA* in exponentially growing cells was higher than that of cells in stationary phase. When the cells entered the stationary phase, the transcription of *fmtA* was reduced.
(ii) Effect of oxacillin: In the presence of 1 μg/ml oxacillin, the *fmtA* promoter activity was similar to that without oxacillin (Table 5). The activity was slightly enhanced by addition of 10 μg/ml oxacillin, and increased at 2.5 times by addition of 100 μg/ml (1/4 MIC) oxacillin. On the contrary, the *orf1* promotor activities in the presence of oxacillin, even in 100 μg/ml oxacillin, was quite similar to that without oxacillin (Table 5).
(iii) Effect of various antibiotics: An increase of XylE activity was observed by addition of 1/4 MIC of oxacillin (100 μg/ml), methicillin (200 μg/ml), cefoxitin (64 μg/ml), fosfomycin (8 μg/ml) and bacitracin (32 μg/ml), while the activity in the presence of 1/4 MIC of vancomycin (0.5

μg/ml) and tetracycline (32 μg/ml) was similar to that of the control (Fig.6). Especially, after addition of ß-lactams and fosfomycin, the activity was 2 to 3 times higher than that of the control.

Immunoblotting analysis. When the cells were exposed to oxacillin (1 to 100 μg/ml), the amount of FmtA protein was increased in a dose dependent manner (Table 6). Especially, the amount of FmtA protein in the presence of 100 μg/ml oxacillin showed two times higher than that without oxacillin, in accordance with the results of the promoter activity assay using the XylE activity as a reporter system. On the contrary, the amount of 62kDa-AM protein was not changed by addition of oxacillin.

DISCUSSION

Penicillin binding proteins and ß-lactamases possess three conserved motifs, SXXK, SXN, and K(H)T(S)G, which are thought to be responsible for catalytic activities of these proteins (11, 24, 25, 30). Serine of SXXK motif is the active site residue which covalently binds to ß-lactam antibiotics, and the other two motifs are also considered to be involved in interaction with ß-lactams (11, 12, 17). Site-directed mutagenesis of conserved amino acids in each three motifs of PBPs led to loss of penicillin binding activity (34). FmtA shows similarity to *Streptomyces* DD-carboxypeptidase, low molecular PBP in *Bacillus subtilis* and several class C ß-lactamases, though it lacks one of

three conserved motifs, K(H)T(S)G. Our penicillin binding assay revealed that recombinant FmtA had no stable, covalent penicillin binding activity. However, FmtA of wild type restored the mutation of *fmtA*-inactivated mutants. These results suggest that stable penicillin binding activity of FmtA is not essential for function of FmtA in maintaining methicillin resistance phenotype of MRSA. Among the tested mutations in conserved motifs of FmtA, serine in 127SAQK130 appears to be important for FmtA function (Fig. 2). Since only the second pair of SXXK/SXN motifs (127SAQK and 186YKN) has the correct orientation and approximately the spacing found in PBPs and ß-lactamases (11, 12, 17), it supports our finding although the mutagenesis of tyrosine in 186YKN had no effect on the complementation. These results indicate that only SXXK motif (127SAQK) of FmtA, which generally found in PBPs and ß-lactamses with other two motifs, is important for function of FmtA.

HPLC-muropeptide analysis is a powerful tool for investigating cell wall structure, although some important parameter like glycan chain length variations, secondary wall polymer properties or degree of O-acetylation are usually not analyzed (22, 27). In KSA8-TS339, there was a slight increase of the non-amidated monomers, dimers and trimers, related to the slight decrease of the degree of crosslinking, when compared with those of the parent, KSA8 strain. Thus, the HPLC profile in KSA8-TS339 resembled that of a *glnR* mutant as reported previously (26) although the alteration of HPLC profile in the *glnR* mutant was more

drastic than that observed in the *fmtA* mutant. The same effects were found in the muropeptide profile of KSA8-TS339C, but the effects were more exaggerated. This might be related to the fact that FmtA inactivation occured at the very C-terminal of the protein for strain KSA8-TS339, while the *cat* interposition took place close to its N-terminal in strain KSA8-TS339C (Fig. 1). *glnR* codes a glutamine synthetase repressor (7), and inactivation of *glnR* resulted in an increase of non-amidated monomers, dimers, and trimers, and a reduced cell wall cross-linking (26). FmtA does not have homology with glutamine synthetase or its associated enzymes. It was reported that *S. aureus* grown in the presence of penicillin decreased the amidation of glutamate residues and the degree of cross-linking, indicating that penicillin affects the structure of stem pentapeptide other than cross-linking (16, 32). Penicillin does not inhibit the glutamine synthetase directly, so that the block of amidation in glutamate residues should be caused by the indirect effect(s) of penicillin. The alteration of peptidoglycan by *fmtA*-inactivation may be a indirect effect, and the precise function of FmtA remains to be elucidated.

Promoter analysis of *fmtA* demonstrated that the transcriptional level of *fmtA* increased when the cells were exposed to ß-lactam antibiotics, especially at high concentration of ß-lactam antibiotics (Table 5). Immunoblotting analysis also confirmed this finding (Table 6). It has been reported that the expression of PBP2' and ß-lactamases are regulated by *mecR1*/*mecI* and *blaR1*/*blaI*,

respectively (9, 29, 31). These genes are located close to upstream of *mecA* or *blaZ*, and induce the expression of *mecA* or *blaZ* products in the presence of ß-lactam antibiotics. There is no possible region like *mecR1*/*mecI* or *blaR1*/*blaI* at the upstream of *fmtA*. Transcription of *fmtA* was not induced at the low concentration (1 μg/ml) of oxacillin, so FmtA may be involved in the expression for high level of methicillin resistance. Enhancement of FmtA expression was also found in the presence of certain non-ß-lactam cell wall inhibitors, especially fosfomycin, so this enhancement is not specific for ß-lactam antibiotics, but at least for several cell wall synthesis inhibitors. These results imply that the induction of *fmtA* transcripts is mediated by unknown signal transducing system(s) other than *mecR1*/*mecI* or *blaR1*/*blaI*. Further biochemical research will be needed to elucidate the function of FmtA and mechanism of its regulation.

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