Nucleotide sequence accession number. The nucleotide sequence discussed in this work will appear in DDBJ, EMBL, and GenBank under accession number ABO 15223.

RESULTS

Isolation of Tn*551* insertional mutant with reduced oxacillin resistance. From strain COL, we obtained 2000 Tn*551* insertionally inactivated mutants which grew on TSA containing erythromycin (30 μg/ml) but not cadmium (50 μg/ml) at 43°C. Among these, one Tn*551* mutant designated as COL-TS111 was isolated which showed reduced resistance against oxacillin not only in the presence of but also in the absence of Triton X-100. To provide the linkage of the Tn*551* insertion with reduced oxacillin-resistance, COL-TS111 was backcrossed into the parent COL by transduction. All of the backcrossed transductants showed similar oxacillin MICs to COL-TS111, confirming that the reduction of the oxacillin resistance level was coupled to the Tn*551* insertion. One of the backcrossed mutants was designated as COL-TS111-1. The Tn*551* was then transduced into two further MRSA strains KSA8 and NCTC10443 and one MSSA strain RN450. All transductants from both the MRSA strains reduced the oxacillin MIC although the degree of reduction varied among strains (Fig. 1), while those from RN450 did not. One each of the transductants of KSA8, NCTC10443 and RN450 was designated as KSA8-TS111, NCTC10443-TS111 and RN450-TS111 respectively. We tried to transduce the Tn*551* into other MSSA strains to elucidate whether Tn*551* increases the sensitivity to oxacillin in other MSSA strains, but failed to obtain transductants

although we confirmed that *fmtB* existed in all strains by Southern hybridization.

The Tn*551* insertion in COL was mapped by pulsed-field gel electrophoresis of *Sma*I chromosomal fragments probed with a Tn*551* specific probe. The probe hybridized with *Sma*I-I fragment (data not shown).

MICs of various antibiotics. We determined the MICs of three Tn*551*-mutants (KSA8-TS111, COL-TS111 and NCTC10443-TS111) and their parents against various antibiotics (methicillin, cefoxitin, imipenem, fosfomycin, bacitracin, vancomycin, chloramphenicol and tetracycline). The MICs of Tn*551*-insertional mutants to ß-lactam antibiotics remarkably decreased compared to those of parents (data not shown). There was no difference in susceptibility to non-ß-lactam antibiotics and protein synthesis inhibitors between mutants and their parents (data not shown).

Population analysis. The parent MRSA strains, KSA8, COL and NCTC10443, showed homogeneous resistance to oxacillin, while three respective Tn*551* mutant strains revealed heterogeneous resistance (Fig.1). The population analysis against Triton X-100 showed that the three parent strains showed homogeneous resistance while most of the populations of Tn*551*-insertional mutants did not grow in the presence of more than 0.02 % Triton X-100 (Fig.1). The frequencies of cell growth of KSA8-TS111, COL-TS111 and NCTC10443-TS111 in the presence of Triton X-100 (above 0.02%) are 10^{-2}, 10^{-5} and 10^{-5}, respectively. The MSSA strains, RN450 and RN450-TS111 showed a homogeneous resistance

against Triton X-100, and also a homogeneously population profile against oxacillin, although both strains revealed a quite similar pattern against Triton X-100 or oxacillin (data not shown).

Cloning and sequencing of the Tn*551* insertion region. One complete open reading frame (*orf*) were identified on the PCR fragment of COL (Fig.2). *orf*1 encoded a putative 2478 amino acid sequence protein with a molecular weight of 263 kDa. The isoelectric point of this putative protein was 4.29. A Shine-Dalgarno sequence of GGAGGA was found 7 bp upstream of the ATG initiation codon of *orf1*. Primer extension analysis revealed that the *orf1* transcript initiates at an adenine residue located 14 bp 5' to the *orf1* start codon (Fig.3). The sequenced portion of the first 223 bp matched perfectly with the downstream of the *glmM* region, indicating that *orf1* lies downstream of the *glmM* gene. With respect to known Tn*551* sequence, the insertion site of Tn*551* was at the 976 bp position, interrupting the N-terminal site of *orf1*. We designated *orf1* as *fmtB* (factor which affects the methicillin resistance level in the presence and absence of Triton X-100; we subsequently renamed the *fmt* gene, which was identified previously by the same screening method, as *fmtA*).

Since Tn*551* insertion sometimes result in the deletion or mutation of DNA close to insertional site and cause to affect the expression of other genes, we checked the both flanking DNA sequence on the Tn*551* insertional site in COL-TS111. DNA sequence showed that there is no deletion or mutation.

***fmtB* encodes a cell surface protein with a multiple repeat domain.** Fig.1 is a schematic representation of the ORF in the *fmtB* region in COL. In the central part of FmtB, 17 tandemly repeated sequences beginning at position 687 and ending at position 1993 are present. Each repeat consists of 75 amino acids. From the 1st to 12th repeat, 4 amino acids are perfectly conserved (Fig. 5), whereas the last 5 repeats show less similarity. The cell wall sorting motif, LPXTG, was found at the C-terminus of FmtB in COL. When the deduced FmtB was compared with known protein sequences in the BLAST and FASTA network search services (DDJB), this protein showed a similarity in the portion of multiple repeats with EF protein in *Streptococcus suis* (36) and Lmp1 in *Mycoplasma hominis* (23).

Complementation test. Table 2 shows that wildtype *fmtB* from COL was unable to restore the oxacillin resistance in COL-TS111, although FmtB expression was found in this strain (COL-TS111 pHK4357) by Western blotting. While, unexpectedly partial complementation was obtained in the mutant with *glmM* alone and with the large fragments covering both *glmM* and *fmtB*.

Identification and localization of FmtB protein by immunoblotting. We explored localization of protein reacting with antiserum raised against the repeated region of FmtB by Western blotting. We detected a protein band of high molecular mass (approximate size, 310 kDa in COL whole cell lysate) (Fig.6). When *fmtB* gene was incorporated into the mutant, the protein band reacted with anti-FmtB serum was found, showing the same molecular size with that of the parent strain, COL. These

results suggested that the immunoreactive high molecular mass protein was the FmtB protein, although the molecular weight estimated from SDS-PAGE did not fully correspond with that calculated from the nucleotide sequence.

Various fractions of the COL strain were investigated for FmtB protein. Fig. 6 shows that the immunoreactive band was mainly present in the cell wall fraction. We detected a small amount of this protein in culture supernatant, but not in membrane and cytoplasmic fraction.

GlmM expression in *fmtB*-mutant. Since the *glmM* fragment partially restored the wild type phenotype in the *fmtB*-inactivated mutant, Western analysis was performed with the antiserum raised against GlmM. We found a band reacting with the antiserum in COL, and the size of this band (50 kDa) was similar to the molecular weight (49.2 kDa) estimated from the nucleotide sequence of *glmM* (Fig.7). The immunoreactive band was also present in *fmtB*::Tn*551* mutant of COL. While, the band was absent in *femD*::Tn551 mutant. The amount of FemD protein in the *fmtB*-mutant was similar to that of its parent.

Effect of N-acetylglucosamine and glucosamine on the MIC of oxacillin in the mutants. Since *glmM* restored the mutation in *fmtB*-inactivated mutant, it rasised the possibility that *fmtB*-inactivation affects the early step of cell wall synthesis, like *glmM* encoding for a phosphoglucosamine mutase shown to alter methicillin resistance (45). Therefore, we measured MIC of oxacillin in the presence of N-acetylglucosamine or glucosamine, which is the substrate for synthesizing the cell

wall peptidoglycan. To campare *fmtB*-mutation with the *glmM*-mutation, a *glmM*-inactivated mutant was constructed from COL strain by transducing Tn*551* from BB591 into COL. MIC of oxacillin COL-TS111 (*fmtB* inactivation) was 0.25 μg/ml, while that of *glmM*-mutant was 4 μg/ml. In the presence of N-acetylglucosamine (above 1 mM to 100 mM) or glucosamine (above 1mM to 10 mM), the MICs of both mutants increased, especially *fmtB*-mutant increased the MIC about 256 fold in the presence of 10 mM N-acetylglucosamine. We used an glucosamine hydrochloride, so above 100 mM of glucosamine, the solution was acidic to inhibith the cell growth.

DISCUSSION

Several factors, like *fem* series, which affect the methicillin resistance in MRSA have been identified. Since FmtB has a cell wall sorting motif and was detected in cell wall fraction by immunoblotting, it is first reported that cell wall sorting protein is associated with methicillin resistance in MRSA.

fem mutants have besides enhanced susceptibility to methicillin also other phenotypic alterations (2, 6, 13, 25). FemA or FemB mutants have reduced sensitivity to lysostaphin and a reduced glycine content of peptidoglycan (13, 25, 38). FemB, GlnR and GlmM mutants show a suppressed rate of autolysis compared to their parents (6). Since analysis of amino acid components of peptidoglycan, cell wall susceptibility to bacteriolytic enzymes, autolysis rate and PBP profiles of the mutants in this study did not reveal any differences to the

parents (data not shown), FmtB inactivation did not cause the marked alteration of peptidoglycan. Enhanced susceptibilities to oxacillin or Triton X-100 was observed in the three MRSA mutants, but not in the MSSA mutant, RN450-TS111, suggesting that the observed inhibition of FmtB expression in three MRSA mutants was important for the alteration of this phenotype.

The region of FmtB which showed a similarity to EF protein in *S. suis* (36) and Lmp1 in *M. hominis* (23) is within repeated sequences of 75 amino acids (Fig. 5). These proteins are thought to be associated with the pathogenesis (23, 36). Like EF protein, the molecular mass of FmtB calculated from sequence analysis was different from that estimated from SDS-PAGE (Fig. 6). It has been observed that highly charged proteins often migrate anomalously in SDS gels (10, 36). Production of proteins with multiple repeats has been reported in some bacteria (8, 14, 34, 43, 44). Multiple repeats are thought to be involved in binding of the protein to specific ligands (8, 14, 34, 43, 44). In some cases the amino acid sequences in repeats are highly conserved, whereas in other cases show limited homology with variations (15, 18, 44). The repeat variation has commonly been observed in the M protein of *Streptococcus pyogenes* (15, 18), the glucosyltransferase of *Streptococcus downei* and *S. mutans* (44), and the toxin A of *Clostridium difficile* (44). The 17 repeated region of FmtB also shows weak similarity among each repeated sequences, but the role of the repeated region remains unknown.

fmtB alone did not restore the TS111 mutation and *glmM* overexpression partially restored the mutation of *fmtB*-inactivated mutants, it raises the possibility that Tn*551* in

fmtB region caused a polar effect on the upstream or downstream of *fmtB*. As shown in Fig.1, *glmM*, which is a factor for methicillin resistance, was located close to the upstream of *fmtB*. Glanzmann et al. reported that *glmM* makes an operon with other two unknown genes (11). Northern analysis revealed that *glmM* transcribed with two other genes or only *glmM* itself, but further big band was not found (11). We also found same bands probing with *glmM* internal fragment in COL-TS111 mutant (data not shown). We demonstrated that the equal amount of GlmM protein was expressed in *fmtB*-mutant by immunoblotting and also transcriptional strat of *fmtB* was found. On the downstream of *fmtB*, there is no clear palindromic sequence, but next *orf* was found about 1 kbp apart from *fmtB* and transcrives divergently. Although we failed to detect the band by Northern blotting using several internal fragments of *fmtB*, these results may indicate that *fmtB* is trascribed separately with other genes. However, why *fmtB* in trans did not complement the mutation remains unknown. We found the FmtB protein in all fractions (supernatant, cell wall cytoplasm) in COL-TS111 having pHK4357 (*fmtB*) (data not shown), while the parent strain only found in cell wall (Fig.6). The overexpression of FmtB in COL-TS111 having pHK4357 may not be precisely localized and functioning.

When we compared the *glmM* mutant with the *fmtB* mutant in COL strain, we found reproducible difference with their susceptibilities to oxacillin. The *fmtB*-inactivated mutant (COL-TS111) was more susceptible to ß-lactam antibiotics than *glmM*-inactivated mutant (COL *glmM*::Tn*551*). Furthermore, it has been reported that the autolysis rate of *glmM* mutant was slower than that of the parent (6), while that of *fmtB*-mutant was

similar to that of the parent. However, GlmM overexpression, GlcNAc and Glc restored the *fmtB*-mutation. Also, N-acetylglucosamine and glucosamine restored the *glmM*-mutation. N-acetylglucosamine and glucosamine are substrate for the glycan strands of peptidoglycan. *glmM* encodes for the phosphoglucosamine mutase, which catalyze the reaction of glucosamine-1-phosphate from glucosamine-6-phosphate, the first step for the formation of peptidoglycan precursor, UDP-*N*-acetylglucosamine (17). Therefore, the function of *fmtB* is thought to be close to that of *glmM*, indicating that *fmtB* is associated with the early step of cell wall synthyesis, though their precise functions are different. Further biochemical research into GlmM and FmtB will elucidate their relationship, and their association with methicillin resistance.

ACKNOWLEDGEMENTS

We thank Alexander Tomasz and Keiichi Hiramatsu for generously donating the strains. We also thank Yuji Watanabe and Yasuyuki Higashi, Fujisawa Pharmaceutical Co., Ltd., for amino acid analysis.

This work was supported by a grant-in-aid for Encouragement of Young Scientists (grant no.10770119) from the Ministry of Education, Science, Sports and Culture of Japan, and Health Sciences Research Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health and Welfare of Japan.

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Fig.6. Western blot of fractions from *S. aureus* strain COL. The fractionationation method was described in Materials and Methods. Samples were prepared from strain COL (lane 1-4), COL-TS111 (lane 5), COL-TS111 having pHK4357 (*fmtB*) and resolved by SDS-PAGE in 5 % polyacrylamide gel, then subjected to Western blotting. Immunodetection was made using anti-FmtB serum as primary serum. Lanes: 1, culture supernatant; 2, cytoplasmic fraction; 3, cell wall extract; 4 to 6, whole cell lysate.

Fig. 7. Western blot of whole cell lysates of COL and its derivative strains. Samples were resolved by SDS-PAGE in 10 % polyacrylamide gel, then subjected to Western blotting. Immunodetection was done using anti-GlmM serum as primary serum. Lanes: 1, recombinant GlmM; 2, COL; 3, COL-TS111; 4, COL (*glmM*::Tn*551*) having pHK4176; 5, COL (*glmM*::Tn*551*).

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