ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1997, p. 2355–2361 Vol. 41, No. 11
0066-4804/97/$04.00+0

Cloning and Characterization of the *fmt* Gene Which Affects the Methicillin Resistance Level and Autolysis in the Presence of Triton X-100 in Methicillin-Resistant *Staphylococcus aureus*

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Received 10 February 1997/Returned for modification 18 April 1997/Accepted 26 August 1997

In methicillin-resistant *Staphylococcus aureus* (MRSA) strains, Triton X-100 reduced the oxacillin resistance level, although the degree of reduction varied from strain to strain. To study the responses of MRSA strains to Triton X-100, we isolated a Tn*551* insertion mutant of the COL strain that became more susceptible to oxacillin in the presence of 0.02% Triton X-100. The Tn*551* insertion of the mutant was transduced back to the parent strain, other MRSA strains (strains KSA8 and NCTC 10443), and methicillin-susceptible strain RN450. All transductants of MRSA strains had reduced levels of resistance to oxacillin in the presence of 0.02% Triton X-100, while those of RN450 did not. Tn*551* mutants of KSA8 and NCTC 10443 also had reduced levels of resistance in the absence of 0.02% Triton X-100. The autolysis rates of the transductants in the presence of 0.02% Triton X-100 were significantly increased. Amino acid analysis of peptidoglycan and testing of heat-inactivated cells for their susceptibilities to several bacteriolytic enzymes showed that there were no significant differences between the parents and the respective Tn*551* mutants. The Tn*551* insertion site mapped at a location different from the previously identified *fem* and *llm* sites. Cloning and sequencing showed that Tn*551* had inserted at the C-terminal region of a novel gene designated *fmt*. The putative Fmt protein showed a hydropathy pattern similar to that of *S. aureus* penicillin-binding proteins and contained two of the three conserved motifs shared by penicillin-binding proteins and β-lactamases, suggesting that *fmt* may be involved in cell wall synthesis.

The resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) to β-lactam antibiotics is mediated by penicillin-binding protein (PBP) PBP 2′ (PBP 2A), which is encoded by *mecA*, a structural gene within the *mec* locus (7, 30, 37). PBP 2′ has a low affinity for β-lactam antibiotics and is the only PBP responsible for peptidoglycan synthesis in the presence of an otherwise inhibitory concentration of β-lactam antibiotics. The expression of methicillin resistance is classified into two types: homogeneous and heterogeneous (36). The level of resistance varies among strains. *mecI-mecR1* genes, which are located upstream of the *mecA*, regulate the production of PBP 2′ (34, 35). In addition, the *blaI* gene, which regulates penicillinase synthesis in staphylococcal penicillin-resistant plasmids, also regulates the transcription of the *mecA* gene (13). However, the amount of PBP 2′ does not always coincide with the methicillin resistance level, suggesting that factors other than *mecA*-related regulatory genes are involved in methicillin resistance (5, 23).

The *fem* and *llm* genes, which are independent of the *mec* locus, are also implicated in methicillin resistance (5, 22). Inactivation of *femA* or *femB* brings about methicillin susceptibility (1, 11, 21). Inactivation of *femC* (9), *femD* (2, 38), *femE* (19), *femF* (26), *llm* (22), or sigma factor (39) brings about a lower level of resistance without affecting PBP 2′ production. Some of these genes may be involved in peptidoglycan metabolism (4, 5, 11, 21, 25, 26), but the function of these gene products has not been well determined. de Lencastre and Tomasz (6) isolated 41 Tn*551* mutants with reduced levels of methicillin resistance compared with that of the parent strain, strain COL, but the Tn*551* insertion sites have not been completely characterized. It appears that numerous factors encoded in regions other than *mec* are involved in the expression of methicillin resistance.

We previously reported that the nonionic detergent Triton X-100 (0.02%) reduced the MICs of β-lactam antibiotics for most *S. aureus* strains (17). This effect was significant for MRSA strains and slight for methicillin-susceptible *S. aureus* (MSSA) strains, although the degree of the effect varied from strain to strain. Triton X-100 converts the resistance of MRSA from homogeneous to heterogeneous. Under the same conditions, 0.02% Triton X-100 alone had no effect on cell viability, growth, the production of PBP 2′, or the binding of β-lactam antibiotics to PBPs (17). Reduction of the oxacillin resistance level was not influenced by the presence of the penicillinase plasmid or the *mecI* repressor (18). Thus, in MRSA strains, one or more factors other than *mecA* that are influenced by Triton X-100 seem to be involved in methicillin resistance.

In this study, we isolated a Tn*551* insertion mutant from MRSA COL in which the level of 0.02% Triton X-100-induced sensitization to oxacillin was increased. The Tn*551* insertion was transduced to two other MRSA strains. The rate of autolysis of the mutants increased compared to that of the parent strain in the presence of 0.02% Triton X-100. The DNA sequence of the Tn*551* insertion region was determined and analyzed.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant properties	MIC (μg/ml) of oxacillin[a]				Resistance expression (class)[b]	
		Without Triton X-100		With Triton X-100		Without Triton X-100	With Triton X-100
		24 h	48 h	24 h	48 h		
Parental							
KSA8	*mec*	512	512	16	32	Homo (4)	Hetero (3)
COL	*mec*	512	512	1	2	Homo (4)	Hetero (2)
COL(pRN3208)	COL with pRN3208 [Rep(Ts)]	512	512	1	2	ND	ND
NCTC 10443	*mec*	256	512	0.25	0.5	Homo (4)	Hetero (1)
RN450	Laboratory strain	0.13	0.13	0.03	0.03	ND	ND
Tn*551* mutants							
KSA8-TS339	*fmt*::Tn*551*, transduced from COL-TS339	16	32	0.5	0.5	Hetero (3)	Hetero (1)
COL-TS339	Mutagenized *fmt*::Tn*551*	64	512	0.25	0.25	Homo (4)	Hetero (1)
COL-TS339-1	*fmt*::Tn*551*, backcross of COL-TS339	64	512	0.25	0.25	Homo (4)	Hetero (1)
NCTC 10443-TS339	*fmt*::Tn*551*, transduced from COL-TS339	16	64	0.13	0.13	Hetero (3)	Hetero (1)
RN450-TS339	*fmt*::Tn*551*, transduced from COL-TS339	0.13	0.13	0.03	0.03	ND	ND

[a] MICs were determined by the microdilution method after 24 or 48 h of incubation.
[b] The class of the population analysis profile was determined by the criteria described in Materials and Methods. Homo, homogeneous; Hetero, heterogenous; ND, not determined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The MRSA strains used in this study are listed in Table 1. Bacteria were grown in Trypticase soy broth (TSB; Difco) at 37°C unless otherwise noted. Tn*551* insertion mutants and their transductants were grown in TSB containing erythromycin (30 μg/ml). KSA8, COL, and NCTC 10443 were homogeneously resistant to oxacillin. COL and COL(pRN3208) were kindly provided by Alexander Tomasz. NCTC 10443 and BB270 were kindly provided by Keiichi Hiramatsu and Brigitte Berger-Bächi, respectively.

Transposon mutagenesis. Mutagenesis with Tn*551* was performed as described previously (19). Strain COL harboring pRN3208, a plasmid that has a temperature-sensitive origin of replication, that encodes cadmium resistance, and that contains Tn*551* with an erythromycin resistance determinant, was grown at 30°C for about 18 h. The culture was plated at various concentrations on Trypticase soy agar (TSA) plates containing 100 μg of erythromycin per ml, and the plates were incubated at 43°C for 48 h. The colonies that grew on TSA containing erythromycin at 43°C were selected and replated on three TSA plates containing 100 μg of erythromycin per ml, 50 μg of cadmium per ml, or neither. After a 24-h incubation at 43°C, the colonies that grew in the presence of erythromycin but not cadmium were used for screening in this study. The approximate curing efficiency of the plasmid was 10^{-5}.

Screening mutants with increased susceptibility to oxacillin in the presence of Triton X-100. Tn*551*-mutagenized *S. aureus* cells were grown on TSA plates, and loopfuls of cells ($>10^7$ cells) were replica plated onto TSA containing 0.02% Triton X-100 and oxacillin (16 μg/ml). Under the experimental conditions, the parent strain could grow at a frequency of 10^{-5} to 10^{-6} on TSA containing 0.02% Triton X-100 and oxacillin, and virtually every plated cell formed a colony after 48 h of incubation at 37°C. The strains that did not grow on this plate were again tested by the same method. The strains that grew on TSA containing erythromycin but not 0.02% Triton X-100 and oxacillin were isolated as oxacillin-susceptible mutants in the presence of 0.02% Triton X-100.

DNA manipulations. Routine DNA manipulations, DNA digestion with restriction enzymes, DNA ligations, gel electrophoresis, Southern blotting of DNA, and hybridization were performed essentially as described previously (31). Pulsed-field gel electrophoresis was performed as described previously (14). *Sma*I-digested DNA fragments were separated in 1% agarose gel in TBE buffer (Tris-borate-EDTA) by using a Bio-Rad CHEFII apparatus. Hybridization was performed by a chemiluminescent procedure (ECL direct labeling kit or 3′-oligolabelling kit; Amersham Life Science, Bucks, United Kingdom).

Cloning of Tn*551* insertion region. To clone the Tn*551* insertion region from KSA8 chromosomal DNA, we first cloned the *Hin*dIII junction fragment containing the left portion of Tn*551* and the flanking region from strain KSA8-TS339 by using the oligonucleotide probe (5′-AGC GCC TAC GGG GAA TTT GT-3′) derived from the known Tn*551* sequence. A *Hin*dIII library of KSA8-TS339 chromosomal DNA was ligated into the pUC19 vector and was transformed into *Escherichia coli* XL-1 Blue. The clone which contained the plasmid carrying the left portion of Tn*551* and its flanking region was identified from this library by colony hybridization. The cloned junction fragment was in turn used to probe a *Hin*dIII library from wild-type strain KSA8 cloned into pUC19. The plasmid containing the 3.6-kb *Hin*dIII fragment was isolated as a Tn*551* insertion region from the wild type.

Since in a preliminary trial complementation with a multicopy plasmid was unsuccessful, we used an integration vector to complement the mutation caused by Tn*551* with a single copy of the gene. We cloned the fragment (3.6-kb *Hin*dIII fragment) into an integration vector, pCL83, and designated the recombinant plasmid pHK4082. BB270, which is tetracycline susceptible (Tc^S), was used as a host to test the complementation because all other strains in this study are tetracycline resistant (Tc^r) (pCL83 has a Tc^r gene on its plasmid). First, the Tn*551* region inserted into COL-TS339 was transferred to BB270 by transduction, and then the transductant was transformed with pHK4082. Integration of *fmt* into this strain was carried out as described previously (20). We determined the MICs of oxacillin, methicillin, and imipenem for these strains in the presence and absence of 0.02% Triton X-100.

The DNA sequences of both strands were determined by the dideoxy chain termination method with the AutoRead sequencing kit (Pharmacia Biotech). A nested set of deletions for sequencing was constructed by using exonuclease III and mung bean nuclease (Takara kilo-sequence deletion kit; Takara Biomedicals, Kusatsu, Japan) by the method of Henikoff (10).

MICs and population analysis. The MICs of various antibiotics in the presence or absence of 0.02% Triton X-100 were determined by a microdilution method as described previously (17). TSB was used, and the initial suspensions, prepared by diluting overnight cultures of each strain, contained 10^5 cells per 100 μl. The MIC was determined after 24 and 48 h of incubation. Population analysis profiles were determined as described previously (36). Briefly, aliquots of an overnight culture were plated onto TSA containing various concentrations of oxacillin (from 0.06 to 1,024 μg/ml), with or without 0.02% Triton X-100. Colonies were counted after 48 h of incubation at 37°C. We determined the critical parameter for assignment of the colonies into separate populations for population analysis, based on the classification of methicillin by Tomasz and colleagues (6, 36), as follows: class 1, oxacillin MIC of less than 0.5 μg/ml for most cells; class 2, oxacillin MIC of 1 to 4 μg/ml; class 3, oxacillin MIC of 8 to 128 μg/ml; and class 4, oxacillin MIC of greater than 256 μg/ml.

Autolysis assay. Strains KSA8 and COL and their Tn*551* insertion mutants at the exponential phase of growth (optical density at 660 nm, 1.0) were harvested by centrifugation at 10,000 × *g* for 10 min and were washed twice with phosphate-buffered saline. The cells were resuspended in 30 mM phosphate buffer (pH 6.8), with or without 0.02% Triton X-100, to an optical density at 660 nm of 0.7. The suspension was incubated at 37°C with shaking, and autolysis was monitored by measuring the turbidity at 30-min intervals.

Amino acid analysis of peptidoglycan. Peptidoglycan was isolated as described previously (21). Samples for amino acid analysis were prepared by hydrolyzing peptidoglycan with 4 N HCl at 100°C for 15 h. Samples were then analyzed with an L-8500 amino acid analyzer (Hitachi, Tokyo, Japan).

Susceptibility of heat-inactivated cells to bacteriolytic enzymes. The susceptibility of heat-inactivated cells to bacteriolytic enzymes was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels containing heat-inactivated KSA8 or the mutant of KSA8 with the Tn*551* insertion (0.5 mg of cells [dry weight] per 1 ml). Heat-inactivated cells were prepared as follows: overnight cultures were washed with distilled water three times and were then boiled for 1 h. The cells were freeze-dried and stored at −20°C until use. Lysostaphin (0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 μg/ml), 51 kDa of *N*-acetylglucosaminidase (64, 32, 16, 8, and 2 μg/ml) (32), and 62 kDa of L-alanine amidase (4, 2, 1, 0.5, 0.25, and 0.125 μg/ml) (33) were used as bacteriolytic enzymes. Ten microliters of each sample was applied to the wells. After electrophoresis, the gels were washed with distilled water for 30 min with constant

agitation and were incubated at 37°C in 0.1 M sodium phosphate buffer (pH 6.8). After 12 h of incubation, visible bands were detected with an Immuno Viewer MU (Jookoo Sangyo Co., Ltd., Tokyo, Japan) and the minimal concentration that showed a visible band was defined as the minimal bacteriolytic dose (MBD).

PBP analysis. PBP analysis was performed as described previously (17). Membrane fractions were prepared from KSA8 and its mutant with the Tn*551* insertion grown in the presence or absence of 0.02% Triton X-100 and reacted with [^{3}H]benzylpenicillin (10 to 30 Ci/mmol; Amersham International, Bucks, United Kingdom). After electrophoresis, PBPs were detected by fluorography.

Chemicals and reagents. Triton X-100 was purchased from Nacalai Tesque, Kyoto, Japan. Oxacillin, methicillin, bacitracin, and vancomycin were from Sigma Chemical Co., St. Louis, Mo. Cefoxitin, imipenem, chloramphenicol, fosfomycin, and tetracycline were from Daiichi Seiyaku (Tokyo, Japan), Banyu Seiyaku (Tokyo, Japan), Sankyo (Tokyo, Japan), Wako Chemicals (Osaka, Japan), and Lederle Japan (Tokyo, Japan), respectively.

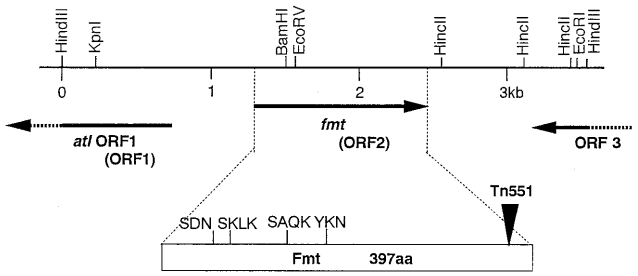

FIG. 1. Restriction map of the *fmt* region of *S. aureus* KSA8. The solid arrows represent the ORFs and the direction of transcription. The lower box shows the *fmt* product with the active-site motifs and insertional site of Tn*551*.

RESULTS

Isolation of Tn*551* insertion mutants. Among 3,000 erythromycin-resistant colonies of Tn*551* insertion mutants of strain COL, one mutant which could not grow on TSA containing 0.02% Triton X-100 and oxacillin was isolated. This mutant was designated COL-TS339, and the oxacillin MIC for this mutant was reduced in the presence of 0.02% Triton X-100 (Table 1). In the absence of 0.02% Triton X-100, the MIC of oxacillin at 24 h was lower for the mutant than for the parent strain, probably because of the slow growth rate of the mutant in the presence of oxacillin. The MIC of oxacillin at 48 h was the same for the mutant as for the parent strain in the absence of 0.02% Triton X-100.

To associate the Tn*551* insertion with reduced oxacillin resistance in the presence of 0.02% Triton X-100, strain COL-TS339 was backcrossed into the parent strain (strain COL) by transduction. Oxacillin MICs for all transductants were similar to that for the Tn*551* mutant, implying that the phenotype was derived from the Tn*551* insertion in the chromosome. One of the transductants with the Tn*551* insertion was designated COL-TS339-1.

To investigate whether the Tn*551* insertion influenced other strains, the Tn*551* insertion in COL-TS339 was transduced into strains KSA8, NCTC 10443, and RN450. Oxacillin MICs for all erythromycin-resistant transductants of KSA8 and NCTC 10443 were lower than those for the respective parent strains in the presence and absence of 0.02% Triton X-100, while the oxacillin MIC was not changed for any of the transductants of RN450. The results for three representative transductants, designated KSA8-TS339, NCTC 10443-TS339, and RN450-TS339 respectively, are presented in Table 1. Compared to the MIC for COL-TS339, after 48 h of incubation, the MICs for KSA8-TS339 and NCTC 10443-TS339 were still reduced.

Localization of Tn*551* insertion. To analyze the Tn*551* insertion in the chromosome, we performed Southern hybridization. Chromosomal DNAs isolated from COL, COL-TS339, KSA8, and KSA8-TS339 were digested with *Hin*dIII and *Eco*RI separately, resolved by conventional gel electrophoresis, and hybridized with Tn*551* DNA. The probe hybridized with three *Hin*dIII fragments of 5.2, 2.6, and 1.3 kb and with an *Eco*RI fragment of 25 kb (data not shown). Since there are two *Hin*dIII sites and no *Eco*RI site within Tn*551*, the hybridization pattern suggested that Tn*551* inserted at a single site. To map the Tn*551* insertion in *S. aureus* COL, chromosomal DNA was digested with *Sma*I, resolved by pulsed-field gel electrophoresis, and hybridized with Tn*551* DNA. Tn*551* inserted in the *Sma*I-B fragment (data not shown).

Cloning and sequencing of the gene with the Tn*551* insertion. To clone the Tn*551* insertion sites, we first cloned the 5.2-kb *Hin*dIII fragment from KSA8-TS339 which contained the left portion of Tn*551* and the flanking chromosomal DNA by using an oligonucleotide-specific probe containing the left portion of Tn*551*, as described in Materials and Methods. The cloned fragment was in turn used as a probe to clone the 3.6-kb *Hin*dIII fragment from parent strain KSA8 (Fig. 1).

The sequence of the 3.6-kb *Hin*dIII fragment of KSA8 was determined (Fig. 2). One complete open reading frame (ORF; ORF2) and two partial ORFs (ORF1 and ORF3) were identified (Fig. 1). ORF1 and ORF2 were transcribed divergently, with the two ATG codons separated by 450 bp. ORF2 and ORF3 were also transcribed divergently, with the two stop codons separated by 824 bp. Potential Shine-Dalgarno sequences were found preceding ORF1 and ORF2. ORF2, in which the Tn*551* insertion extended from nucleotides 1234 to 2425, could potentially encode a protein of 397 amino acid residues with a molecular mass of 46 kDa. The sequenced portion of ORF1 matched perfectly the 208 bp of the sequence of an N-terminally truncated ORF which, along with two other ORFs, clusters with the autolysin gene *atl* (27).

The putative 397-amino-acid protein identified above showed amino acid sequence identity with carboxypeptidase in *Streptomyces* sp. strain R61 (29% identical residues, with 144-amino-acid overlaps) (15), low-molecular PBPs in *Bacillus subtilis* (30% identical residues, with 72-amino-acid overlaps) (28), *Streptomyces lactamdurans* (30% identical residues, with 46-amino-acid overlap) (3), *Synechocystis* sp. (42% identical residues, with 40-amino-acid overlap) (16), and several β-lactamases (data not shown). The putative protein shared two of the three conserved motifs with PBPs and β-lactamase: (FXXX)SXXK (located at positions 63SKLK66 and 127SAQK130), which is responsible for binding to β-lactam antibiotics, and S(Y)XN (located at positions 46SDN48, 186YKN188, and 354YYN356), which is considered to have a proton shuttle function (8). The other motif, K(H)T(S)G, was not found in the sequence. The hydropathy pattern of the putative protein showed similarity with the patterns of *S. aureus* PBPs 1, 2, 2a, and 4 (data not shown). Taken together, we suggest that the putative 397-amino-acid protein is related to the enzyme involved in cell wall synthesis. We designated the gene *fmt* (factor which affects the methicillin resistance level and autolysis in the presence of Triton X-100).

To establish whether the cloned 3.6-kb *Hin*dIII fragment could restore the mutation caused by the Tn*551* insertion, we conducted a complementation test by integrating the 3.6-kb *Hin*dIII fragment into BB270 *fmt*::Tn*551* chromosome by using integration vector pCL83. Table 2 indicates that this fragment restored to BB270 *fmt*::Tn*551* the wild-type susceptibility to β-lactams.

Downstream of the *fmt* gene, the partial ORF (ORF3; 130 amino acids) showed homology with cytochrome *c* oxidase polypeptide III of *B. subtilis* (55% identical residues, with 130-amino-acid overlaps), a thermophilic bacterium (52% identical

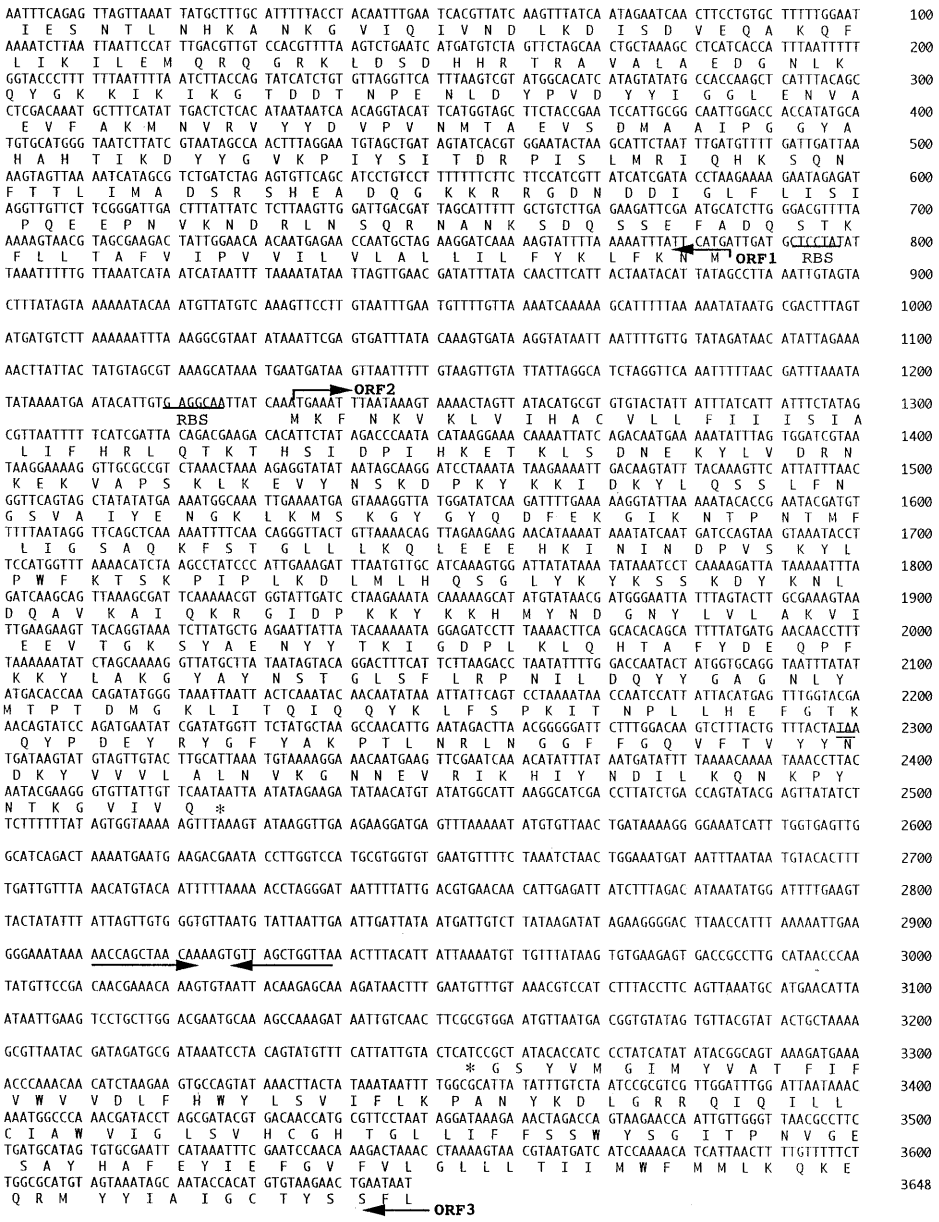

FIG. 2. Nucleotide sequence of *fmt* region from *S. aureus* KSA8. The ribosome-binding sites are underlined. One complete ORF (ORF2) defining *fmt* and two partial ORFs (ORF1 and ORF3) were found. The stop codon is marked by asterisks. Tn*551* insertional sites are double underlined. Palindromic sequences are indicated by arrows.

residues), *Escherichia coli* (44% identical residues), and several other species.

MICs of various antibiotics. We also checked whether *fmt* inactivation causes a change in the susceptibilities to other antibiotics. Table 3 indicates that *fmt*::Tn*551* mutants had increased susceptibilities to β-lactams in the presence or absence of 0.02% Triton X-100 compared with the susceptibilities of the parent strains, while the susceptibilities of the mutants to other antibiotics were not altered. Susceptibilities to two non-β-lactam cell wall synthesis inhibitors (fosfomycin and bacitracin) were increased by the addition of 0.02% Triton X-100, although the MICs for the parent and the mutant strain were the same in the presence or absence of 0.02% Triton X-100.

Population analysis. To further study the effect of the *fmt* mutation, strains KSA8, COL, and NCTC 10443 and their

TABLE 2. MICs for BB270 and its derivative strains

Strain	Time (h)	MIC (μg/ml)[a]					
		OXA		MET		IMP	
		Without TRX	With TRX	Without TRX	With TRX	Without TRX	With TRX
BB270	24	128	0.25	256	4	16	0.008
	48	256	0.25	512	4	32	0.008
BB270 *fmt*::Tn*551*	24	16	0.06	32	0.5	0.25	0.004
	48	32	0.13	64	1	1	0.004
BB270(pHK4082) *fmt*::Tn*551*	24	128	0.25	256	4	16	0.008
	48	256	0.5	512	4	32	0.016

[a] Abbreviations: TRX, Triton X-100; OXA, oxacillin; MET, methicillin; IMP, imipenem. MICs were determined after 24 and 48 h of incubation.

TABLE 3. MICs of various antibiotics for parent and *fmt*::Tn*551* mutant[a]

Strain	TRX	MIC (μg/ml)										
		MET		CFX		IMP		FOM	BC	VCM	CP	TC
		24 h	48 h	24 h	48 h	24 h	48 h					
KSA8	−	2,048	4,096	512	1,024	64	64	128	128	2	8	128
	+	32	128	64	128	0.5	2	128	32	2	8	128
KSA8 *fmt*::Tn*551*	−	16	32	16	32	0.25	1	128	128	2	16	128
	+	4	8	4	4	0.015	0.015	64	32	2	8	128
COL	−	1,024	1,024	256	256	16	32	1,024	128	2	8	256
	+	4	8	16	16	0.015	0.06	128	8	2	8	256
COL *fmt*::Tn*551*	−	128	512	128	128	1	32	1,024	128	2	8	256
	+	1	2	1	2	0.008	0.008	128	8	2	8	256
NCTC 10443	−	256	512	128	128	8	16	128	128	2	8	128
	+	2	4	2	4	0.008	0.015	16	4	2	8	128
NCTC 10443 *fmt*::Tn*551*	−	16	64	64	64	0.25	1	128	128	2	8	128
	+	1	2	1	2	0.004	0.008	8	4	2	8	128

[a] Abbreviations: TRX, Triton X-100; MET, methicillin; CFX, cefoxitin; IMP, imipenem; FOM, fosfomycin; BC, bacitracin; VCM, vancomycin; CP, chloramphenicol; TC, tetracycline.

respective mutants with the Tn*551* insertion were characterized by population analysis. As indicated in Fig. 3, strains KSA8, COL, and NCTC 10443 were homogeneously resistant (class 4) in the absence of 0.02% Triton X-100. In the presence of 0.02% Triton X-100, these MRSA strains were less resistant to oxacillin (KSA8, class 3; COL, class 2; NCTC 10443, class 1). In the presence of 0.02% Triton X-100, the *fmt*::Tn*551* (i.e., TS339) mutants showed reduced levels of resistance to oxacillin compared with the levels of resistance of the parent strains. In the absence of 0.02% Triton X-100, the *fmt*::Tn*551* mutants of KSA8 and NCTC 10443 had reduced levels of resistance (KSA8-TS339, class 3; NCTC 10443-TS339, class 3) compared to the levels of resistance of the respective parent strains, whereas the resistance profile of the *fmt*::Tn*551* mutation in strain COL did not change (class 4).

Characterization of Tn*551* mutants. To further characterize the effect of the Tn*551* insertion, the rates of autolysis of the mutants and their parent strains were analyzed. As indicated in

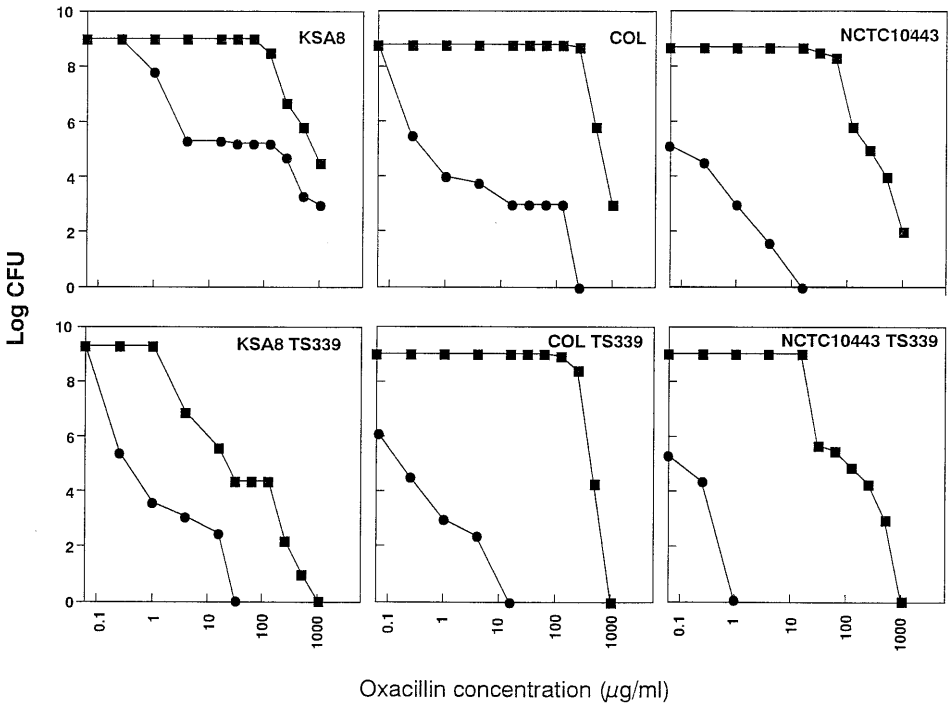

FIG. 3. Population analysis of the parent strains and their mutants with Tn*551* insertions. Overnight cultures of bacteria were plated on TSA containing serial dilutions of oxacillin in the absence (solid squares) or presence (solid circles) of 0.02% Triton X-100 and were incubated at 37°C for 48 h.

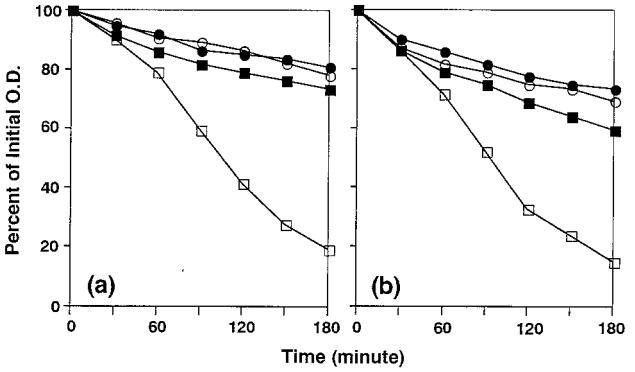

FIG. 4. Autolysis of *S. aureus* KSA8 and its mutants with Tn*551* insertions (a) or COL and its mutants with Tn*551* insertions (b). Cells grown to the exponential phase were harvested and suspended in 30 mM sodium phosphate buffer (pH 6.8) without (solid symbols) or with (open symbols) 0.02% Triton X-100. The suspensions were incubated at 37°C, and the turbidity was measured at 660 nm at 30-min intervals. Symbols: ● and ○, parent strain; ■ and □, TS339 mutant. O.D., optical density.

Fig. 4, although KSA8-TS339 had only a marginally higher lysis rate than the parent strain in the absence of 0.02% Triton X-100; in the presence of the detergent, the mutant strain lysed more rapidly. Similar results were obtained with strain COL and its *fmt*::Tn*551* mutant.

To determine whether the Tn*551* insertion altered the peptidoglycan composition, we analyzed the amino acid compositions of the peptidoglycans isolated from KSA8 and its mutant. There were no significant differences except that the molar ratio of the glycine content was slightly reduced (10 to 15%) in the Tn*551* mutant (data not shown). Other amino acids were not changed by the Tn*551* insertion in terms of the molar ratio of glutamic acid.

Since the parent and its mutant showed no remarkable change in amino acid composition, we further analyzed the cell wall structure by determining the susceptibilities of the parent and its mutant to bacteriolytic enzymes (lysostaphin, the 51-kDa *N*-acetylglucosaminidase), and the 62-kDa L-alanine amidase by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described in Materials and Methods. The MBDs of these enzymes for the mutant were similar to those for the parent strain. As a comparison, the MBD of lysostaphin for the *femA* and *femB* mutants was four to eight times higher than that for the parent strains (data not shown).

In addition, we also compared the PBP profiles of strain KSA8 and its Tn*551* mutant. There were no significant differences between the parent and the mutant grown in the presence or absence of 0.02% Triton X-100 in the level of production of PBP 2′, including other PBPs (data not shown).

DISCUSSION

Sequencing analysis indicated that *fmt* is different from other factors known to affect methicillin resistance, including *femA*, *femB*, *femC*, *femD*, *llm*, and sigma factor (1, 9, 11, 22, 38, 39). The fact that *fmt* was mapped in the *Sma*I-B fragment also indicates that the *fmt* gene is distinct from genes in the *mec* locus (located in the *Sma*I-G fragment) and from *femE* (located in the *Sma*I-A fragment). de Lencastre and Tomasz (6) had reported that four Tn*551* insertions in COL affecting methicillin resistance were also mapped within the *Sma*I-B fragment. However, further mapping demonstrated that *fmt* was distinct from these insertions. Their insertions were mapped in 5.0-, 5.2-, 7.3-, and 3.0-kb *Hin*dIII fragments, respectively, whereas the *fmt* gene was mapped in a 3.6-kb *Hin*dIII fragment. Furthermore, population analysis indicated that the methicillin resistance pattern of one of these mutants (COL *femF*::Tn*551*) was heterogeneous (26), whereas that of COL with *fmt*::Tn*551* is homogeneous (same as the oxacillin resistance pattern; data not shown). These results suggest that *fmt* is a novel factor affecting methicillin resistance. The deduced Fmt protein shares limited sequence similarity with carboxypeptidases, β-lactamase, and low-molecular-mass PBPs. The Fmt protein had a hydropathy pattern similar to that of other *S. aureus* PBPs, especially PBP 4, even though it had limited amino acid homology with them (data not shown) (PBP1 for A. Wada, 1994, accession no. D28879, DDBJ) (12, 24). Like other PBPs in *S. aureus*, the putative Fmt protein has within the first 25 amino acids of the N terminus a highly hydrophobic region which could serve as a signal peptide for translocation through the cytoplasmic membrane. At the C-terminal end, a hydrophobic region that was typical among the low-molecular-mass PBPs and that may function as a membrane anchor was also found. In this study, we found that the Tn*551* mutation does not cause the apparent alteration of the peptidoglycan structure, unlike the *fem* mutations. However, it is interesting that inactivation of *fmt* caused the cells to lyse more rapidly in the presence of 0.02% Triton X-100 (Fig. 4), suggesting that *fmt* is responsible for cell wall stability in the presence of detergent. By PCR amplification, we found that *fmt* was present in all strains tested, including MRSA and MSSA strains, indicating that the *fmt* gene is common among *S. aureus* strains (data not shown). These results suggest that Fmt is involved in cell wall biosynthesis, although the exact biological function of Fmt remains to be explored. It should also be noted that the association of *fmt* with the level of methicillin resistance in clinically isolated MRSA strains is unknown.

Triton X-100 is commonly used to extract membrane-bound enzymes and proteins without a loss of activity. Raychaudhuri and Chatterjee (29) have reported that Triton X-100 affected some membrane-bound enzymes differentially in *S. aureus* cells grown in the presence of 0.008% Triton X-100. On the basis of the assumption that several factors other than *mec* play a role in methicillin resistance, we speculate that one or more Triton X-100-sensitive factors common among MRSA and/or MSSA strains are involved in the resistance. When *fmt* was inactivated, the level of oxacillin resistance was lowered in the presence of Triton X-100, suggesting that *fmt* is involved in the expression of oxacillin resistance in the presence of Triton X-100. Triton X-100-insensitive factors are also implicated, since the bacteria that inactivated *fmt* still showed heterogeneous resistance to oxacillin. Interestingly, population analysis revealed that two of three MRSA strains with inactivated *fmt* had reduced resistance levels in the absence of Triton X-100 (Fig. 3). Similarly, inactivation of the *llm* gene caused different responses among strains. For one strain the MICs of β-lactams were reduced, whereas for the others they were not (22). These results suggest that the factors affecting oxacillin resistance vary according to the genetic background of the respective MRSA strains. Further biochemical and genetic research on *fmt* and other factors is required to elucidate the mechanism of expression of oxacillin resistance.

ACKNOWLEDGMENTS

We thank Alexander Tomasz, Brigitte Berger-Bächi, and Keiichi Hiramatsu for generous gifts of strains and DNA probes. We thank Yuji Watanabe and Yasuyuki Higashi, Fujisawa Pharmaceutical Co., Ltd., for the amino acid analysis. We also thank Mitsuko Takahashi for technical assistance.

A part of this study was carried out in the Research Facility, Hiro-

shima University School of Dentistry and the Center for Molecular Medicine, Hiroshima University School of Medicine.

This work was supported by a grant-in-aid (grant no. 0755715) for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

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