Table 1-continued

Strain or plasmid	Relevant characteristics	Origin or reference
E. coli		
XL-1 Blue	*rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*	(3)
	[F' *proAB lacI^{q}ZM15* Tn*10* (Tet^r)]	
BL21	*hsdS gal* (*lambdacIts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*)	(32)
Plasmids		
pUC19	cloning vector, Amp^r	(47)
pCL8	shuttle vector, Amp^r in *E. coli*, Cm^r in *S. aureus*	(35)
pLI50	shuttle vector, Amp^r in *E. coli*, Cm^r in *S. aureus*	(39)
pFLAG MAC	expression vector (Eastman Kodak Company)	
pHK4011	pUC19/4 kb *Hin*dIII-*Pst*I fragment containing Tn*551* left junction	This study
	and its flanking region of KSA8 TS111	This study
pHK4014	pUC19/8.9 kb *Xba*I fragment of KSA8	This study
pHK4357	pCL8/7.8 kb PCR fragment (*fmtB*) of COL	This study
pHK4176	pLI50/1.8 kb PCR fragment (*glmM*) of COL	This study
pHK4251	pLI50/1.8 kb *glmM* fragment + 7.8 kb fragment (*fmtB*) of COL	This study
pHK4148	pFLAG MAC/partial *fmtB* fragment (repeated sequence)	This study
pHK4203	pFLAG MAC/*femD* fragment	This study

Table 1-continued

Strain or plasmid	Relevant characteristics	Origin or reference
E. coli		
XL-1 Blue	*rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZM15* Tn*10* (Tet^{r})]	(3)
BL21	*hsd*S *gal* (*lambdac*I*ts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*)	(32)
Plasmids		
pUC19	cloning vector, Amp^{r}	(47)
pCL8	shuttle vector, Amp^{r} in *E. coli*, Cm^{r} in *S. aureus*	(35)
pLI50	shuttle vector, Amp^{r} in *E. coli*, Cm^{r} in *S. aureus*	(39)
pFLAG MAC	expression vector (Eastman Kodak Company)	
pHK4011	pUC19/4 kb *Hin*dIII-*Pst*I fragment containing Tn*551* left junction	This study
	and its flanking region of KSA8 TS111	This study
pHK4014	pUC19/8.9 kb *Xba*I fragment of KSA8	This study
pHK4357	pCL8/7.8 kb PCR fragment (*fmtB*) of COL	This study
pHK4176	pLI50/1.8 kb PCR fragment (*glmM*) of COL	This study
pHK4251	pLI50/1.8 kb *glmM* fragment + 7.8 kb fragment (*fmtB*) of COL	This study
pHK4148	pFLAG MAC/partial *fmtB* fragment (repeated sequence)	This study
pHK4203	pFLAG MAC/*femD* fragment	This study

Table 2. MICs of oxacillin for COL and their derivatives

strain	MIC of oxacillin (μg/ml)
COL	512
COL-TS111	0.13
COL-TS111 pHK4357 (*fmtB*)	0.13
COL-TS111 pHK4176 (*femD*)	128
COL-TS111 pHK4251 (*femD* + *fmtB*)	256

Table 3. Effect of N-acethylglucosamine and glucosamine on the MIC of oxacillin

substrate		MIC of oxacillin (μg/ml)		
		COL	COL *fmtB*::Tn*551*	COL *glmM*::Tn*551*
none		512	0.25	4
N-acetylglucosamine	(1 mM)	512	64	64
	(10 mM)	1024	64	64
	(100 mM)	1024	128	128
Glucosamine	(1 mM)	512	16	64
	(10 mM)	1024	32	64

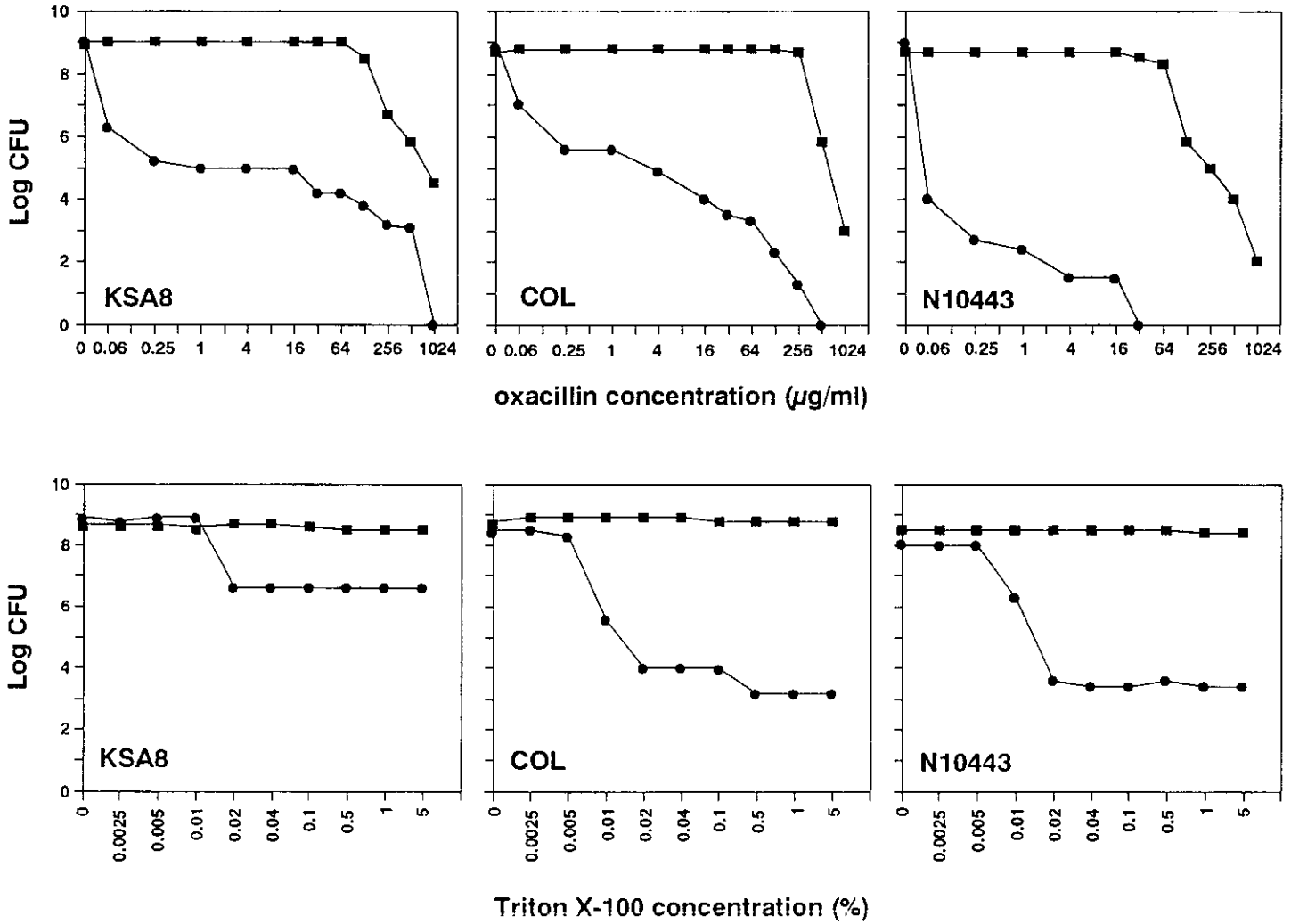
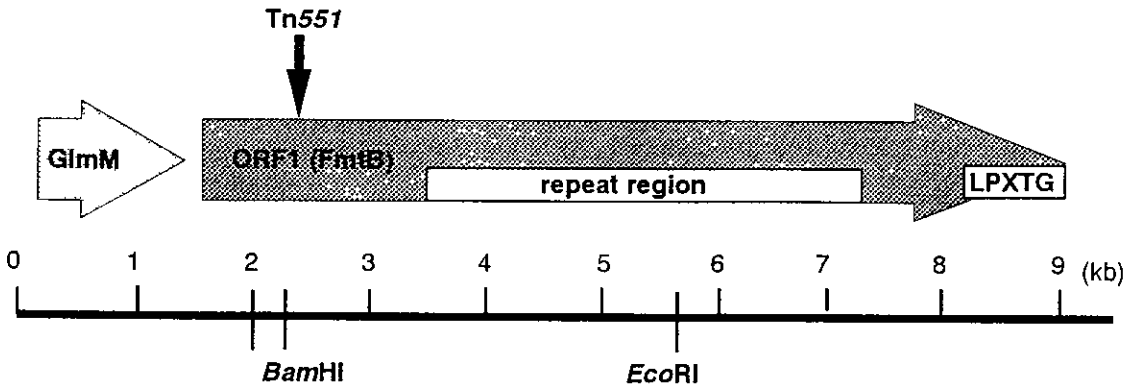

Log CFU
KSA8
COL
N10443
0 0.06 0.25 1 4 16 64 256 1024
oxacillin concentration (μg/ml)
0 0.0025 0.005 0.01 0.02 0.04 0.1 0.5 1 5
Triton X-100 concentration (%)

Fig. 1

Fig.2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ORF1 (FmtB)
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1
2
3
4
5
6
7
8
9
(kb)
BamHI
EcoRI

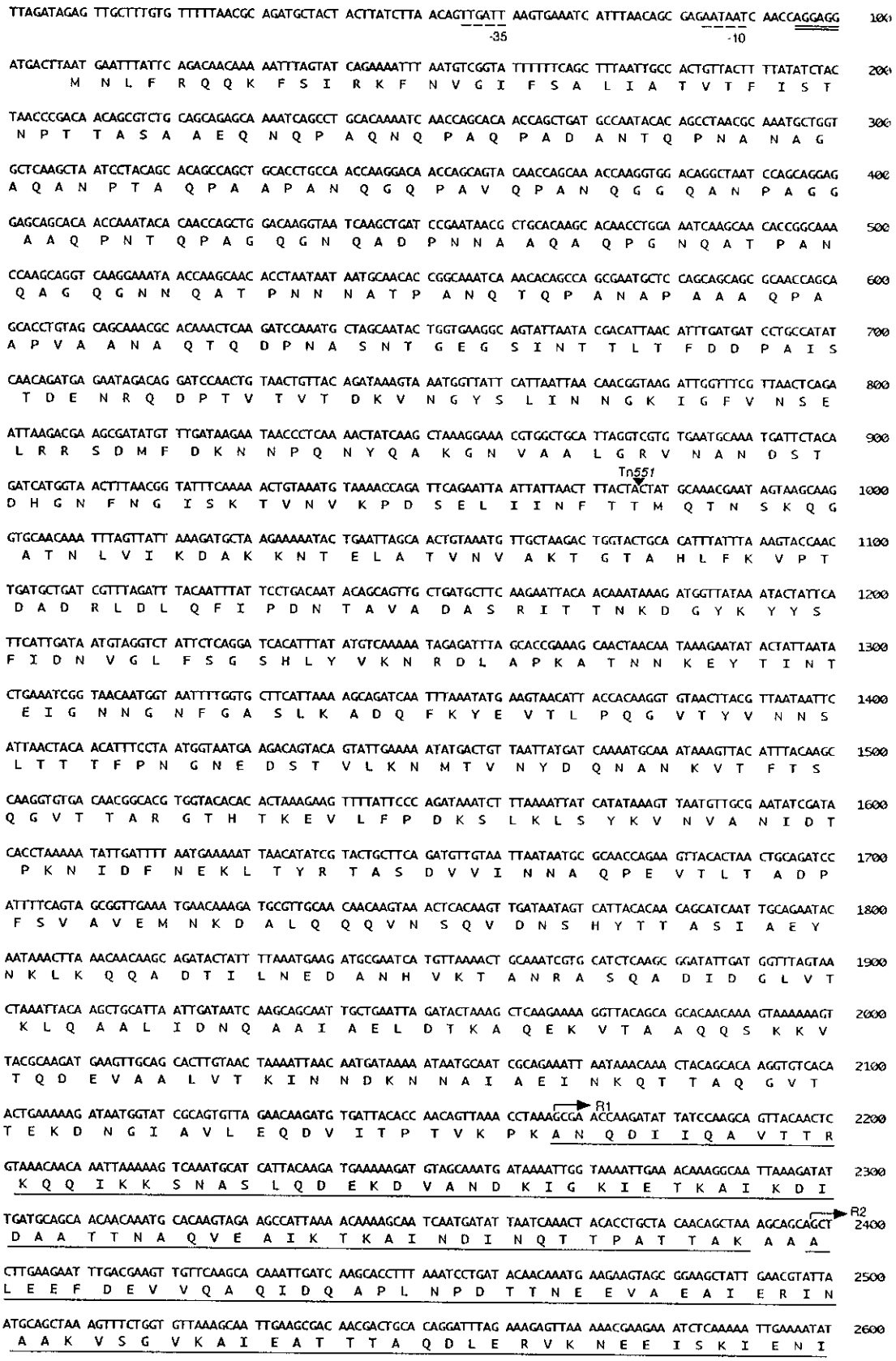
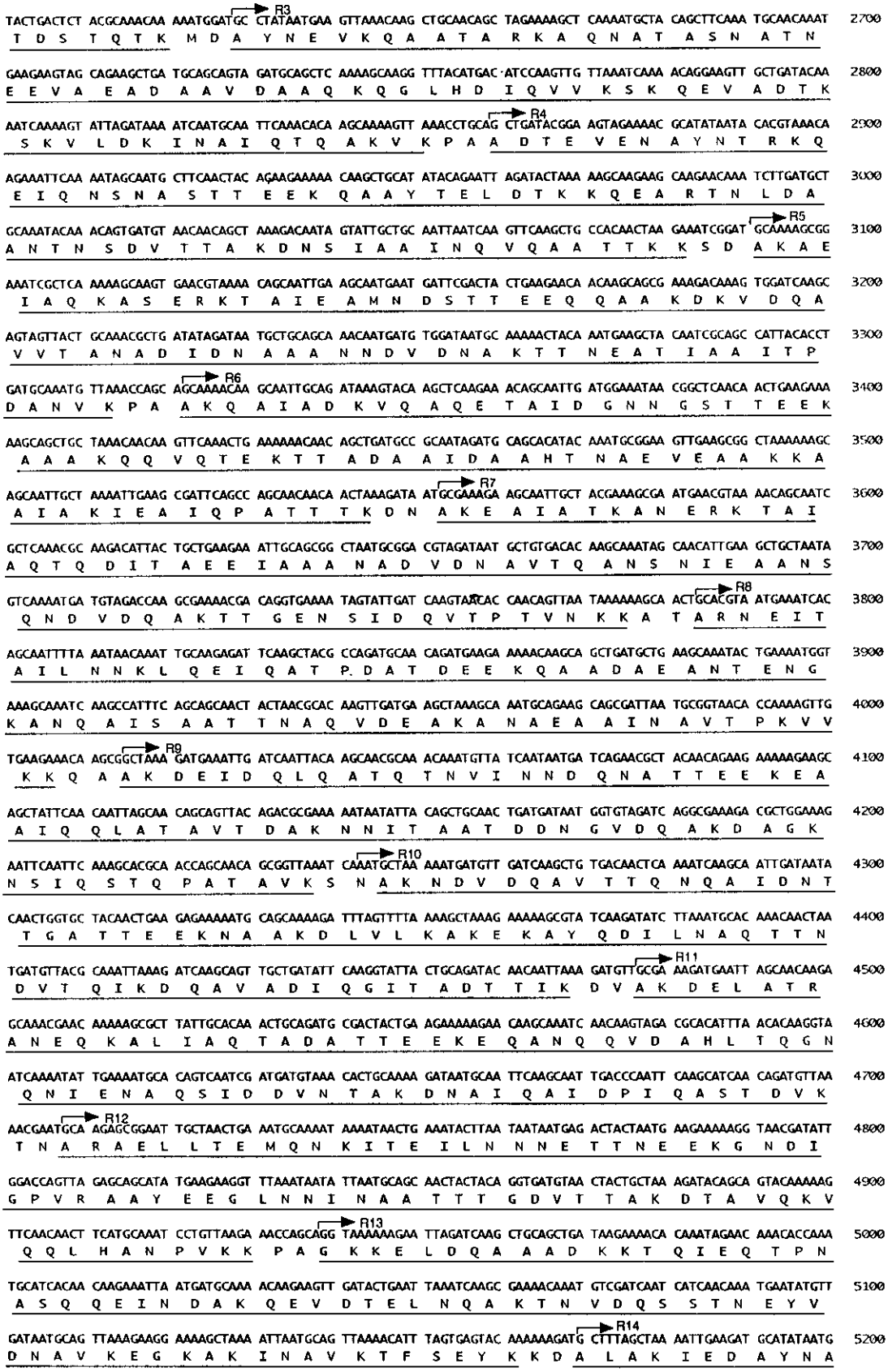


Fig.3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E E V A E A D A A V D A A Q K Q G L H D I Q V V K S K Q E V A D T K
R4
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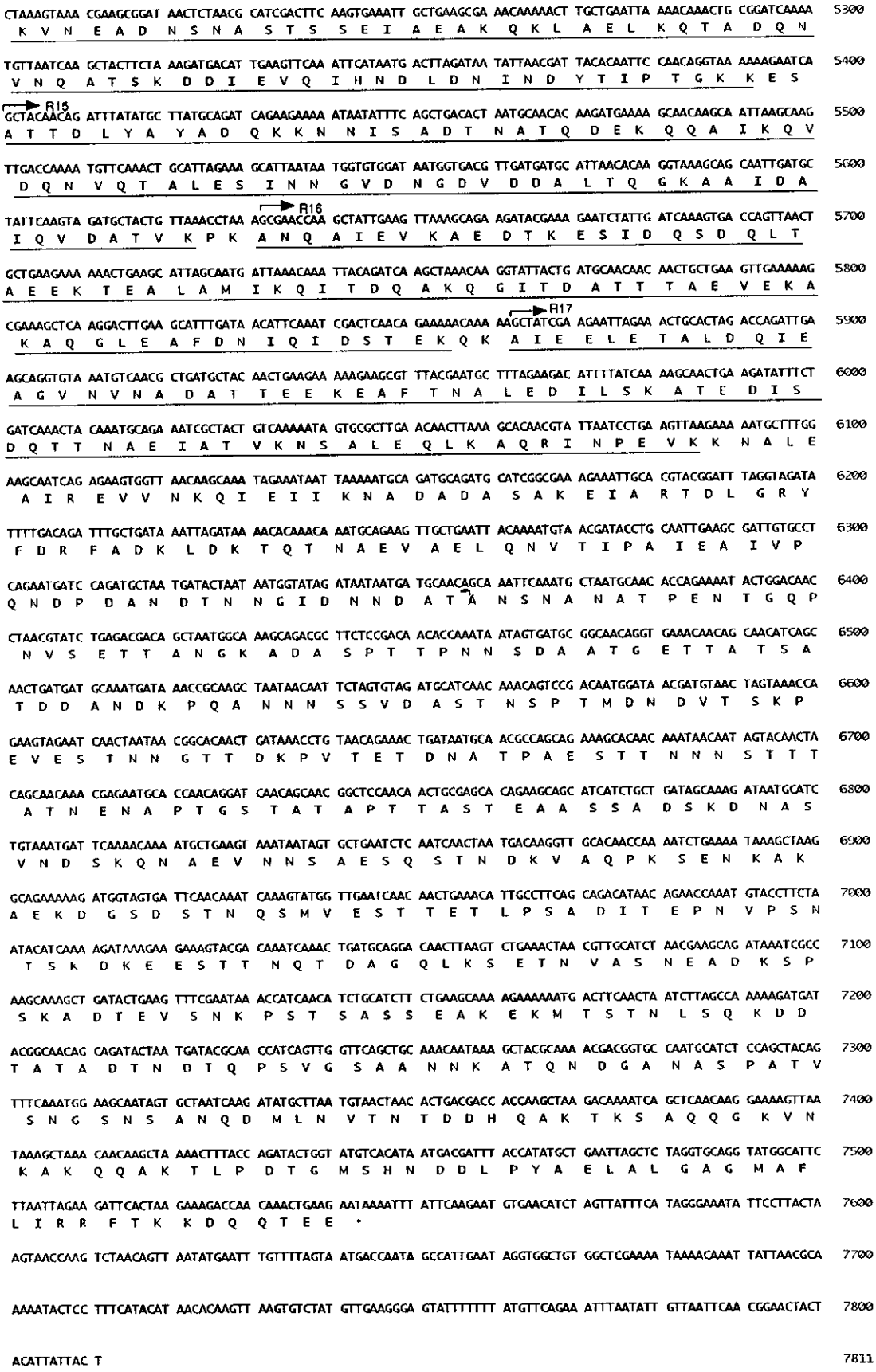
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R16
R17

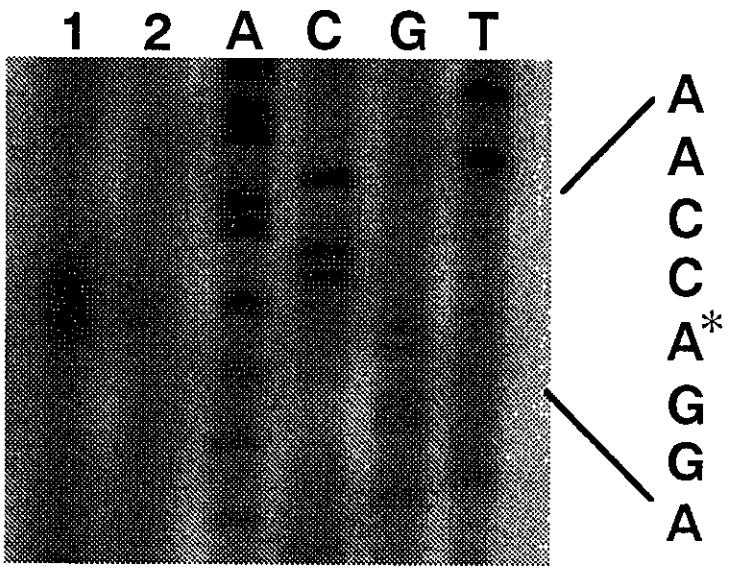

Fig. 4

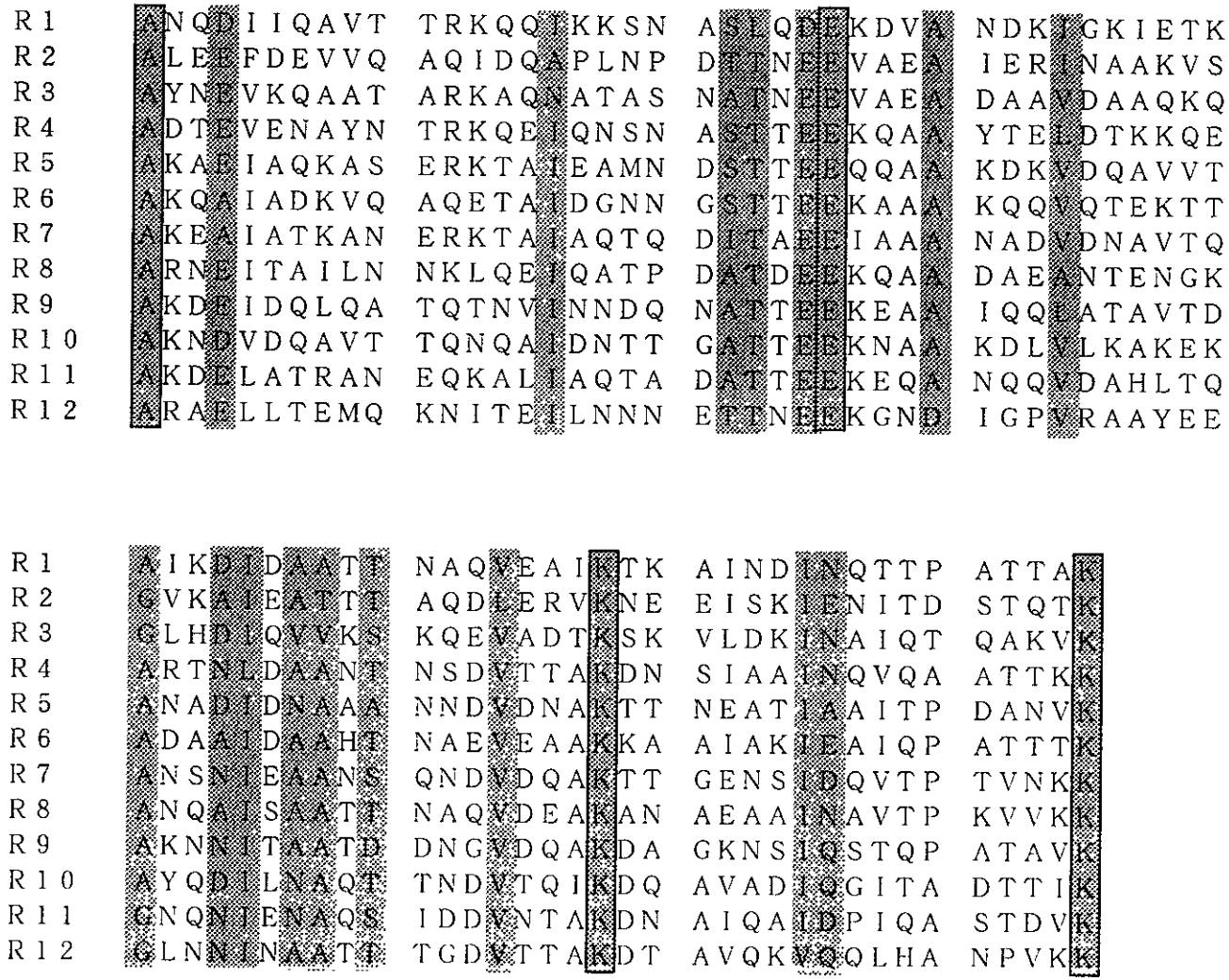
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Fig. 5

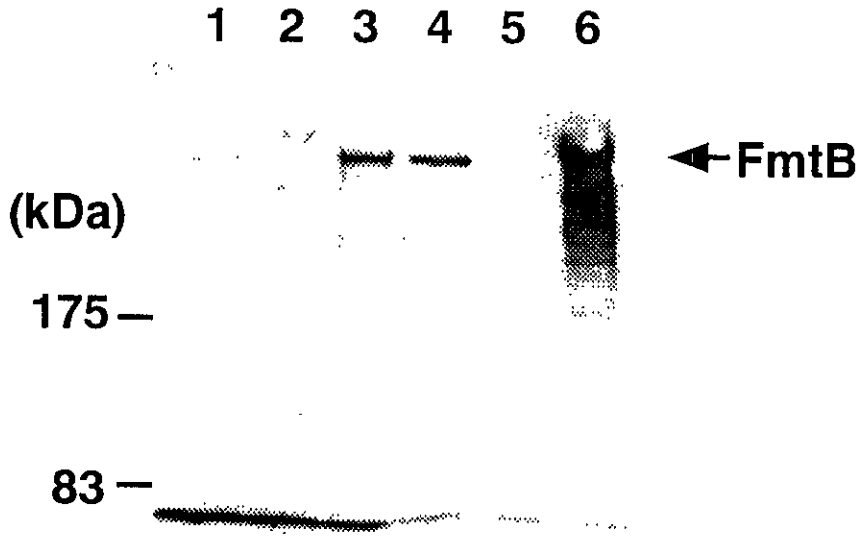

Fig. 6

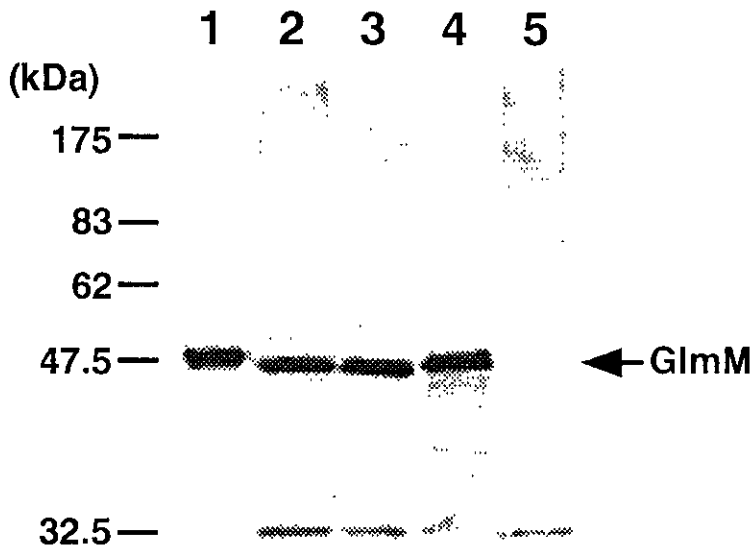

Fig. 7

Characterization of *fmtA*, a gene that modulates the expression of methicillin resistance in *Staphylococcus aureus*

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ABSTRACT

fmtA is a factor which affects methicillin resistance level in methicillin-resistant *Staphylococcus aureus* (MRSA). The FmtA protein has two of three conserved motifs which are typically found in PBPs and ß-lactamases. Complementation experiment revealed that serine at position 127 in the SAQK motif was important for restoration of methicillin resistance phenotype. To investigate the function of FmtA, HPLC analysis of cell wall muropeptides was performed with a *fmtA*-inactivated mutant and its parent. The mutant showed a slightly reduced cross-linking and partially reduced amidation of glutamate residues in the peptidoglycan of the mutant. The transcription of *fmtA* was dose dependently increased by addition of ß-lactam antibiotics, fosfomycin and bacitracin. This increase was remarkable at high concentration of the antibiotics. FmtA is localized in the membrane fraction, and recombinant FmtA lacks penicillin-binding activity. These results show that FmtA mutation affects cell wall synthesis, causing to reduce the methicillin resistance in MRSA.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has an extra penicillin binding protein 2' or 2A (PBP2' or PBP2A), which shows low affinity for ß-lactam antibiotics and functions under the otherwise inhibitory concentration of ß-lactam antibiotics (6, 21). PBP2' is encoded by *mecA* within the *mec* element (33). *MecI/R1* or *BlaI/R1* has been identified as a regulatory factor for PBP2' expression (9, 29, 31), though the expression of PBP2' was not always corresponding with the resistance level of ß-lactams (15).

The series of *fem* and *aux* factors, *llm*, *fmt*, *sigB* were identified as factors which affect the methicillin resistance level (1, 4, 5, 14, 36). These genes are located on chromosomal DNA outside the *mec* element. Most of them were thought to be associated with peptidoglycan synthesis. FemA is involved in formation of glycines 2 and 3 of the pentaglycine side chain of the peptidoglycan precursor, and FemB is involved in formation of glycines 4 and 5 of the side chain (8, 27). GlnR (FemC) is a glutamine synthetase repressor and inactivation of this gene affects the amidation of the isoglutamine in the peptidoglycan stem-pentapeptide (7, 26). GlmM (FemD) has a phosphoglucosamine mutase activity and is involved in an early step in peptidoglycan synthesis (35). MurE (FemF) is associated with the addition of lysine to the stem peptide (18). These factors are thought to be important in cell wall synthesis in general and to function in accordance with PBP2' in the presence of methicillin. However, many

factors other than the *fem* series are considered to be involved in peptidoglycan synthesis (5). Investigation of these genes is important to understand the variety of resistance levels toward methicillin in clinical *S. aureus* strains.

Recently, we found a novel gene, *fmtA* (we renamed *fmt* to *fmtA*), which affects the methicillin resistance level (13). Inactivation of *fmtA* resulted in reduction of the methicillin resistance level in MRSA; especially, homogeneous resistance was converted to heterogeneous. Also, *fmtA* mutation results in increase of an autolysis rate. Complementation experiment revealed that *fmtA* alone restored the mutation indicated that the reduction of methicillin resistance by Tn*551* insertion was not due to a polar effect on downstream gene. Therefore, *fmtA* is thought to be responsible for the methicillin resistance. The putative protein, FmtA, has SXXK and S(Y)XN motifs which are typically found in ß-lactamases and low-molecular PBPs. In this study, we demonstrated that FmtA mutation affects cell wall synthesis, and its expression is enhanced in the presence of ß-lactam antibiotics.

MATERIAL AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. *Staphylococcus* and *Escherichia* were grown in either Trypticase soy broth (TSB) or Brain Heart Infusion broth (BHI), (both from Beckton Dickinson Microbiology systems, Cockeysville, Md)

and Luria Bertani broth (LB), respectively. When needed, erythromycin (EM) (30 μg/ml), chloramphenicol (CP) (10 μg/ml) or ampicillin (100 μg/ml) were added to the medium.

DNA manipulations. Routine DNA manipulations, DNA digestion with restriction enzymes, shrimp alkaline phosphatase, DNA ligations, gel electrophoresis, Southern blotting of DNA and hybridization, and DNA sequencing were performed essentially as described (23). Restriction enzymes and shrimp alkaline phosphatase were purchased from Boehringer Manheim Biochemica, Tokyo, Japan, and T4 DNA ligase was from New England BioLab, Beverly, MA. Hybridization was performed by means of a chemiluminescent procedure (ECL direct labelling kit or 3'-oligolabelling kit; Amersham Life Science, Bucks, UK). DNA sequences of both strands were determined by the dideoxy chain termination method with Autoread sequencing kit (Pharmacia Biotech., Tokyo, Japan). PCR reagents were from Boehringer Manheim, and PCR was performed with the GeneAmp PCR System 2400 (Perkin Elmer).

Construction the *cat* insertional mutant. In COL-TS339, Tn*551* was inserted at the C-terminal of *fmt*A (Fig.1), which raised the possibility that the C-terminally truncated FmtA still possesses a partial enzyme activity. Therefore, we constructed a mutant which possesses inactivated *fmtA* gene by *cat* insertion in its N-terminal region. A plasmid pHK4080 containing the *cat* inserted at N-terminal of *fmtA* was first constructed. A 3.5 kb *Eco*RI-

*Sal*I fragment from pHK4033 was cloned into pCL52.1 (thermosensitive plasmid; Table 1) to generate pHK4079. Then, the *cat* gene from *Sau3A*I digested *E. coli-S. aureus* shuttle vector, pLI50 (3), was ligated into pHK4079, using the *BamH*I site at N-terminal of *fmtA* (Fig.1) to generate pHK4080. The recombinant plasmid was electroporated into RN4220 to generate HK9710 strain. HK9710 was grown at 42°C in the presence of antibiotics (3 μg/ml of tetracycline and 10 μg/ml of chloramphenicol) to select strains with the plasmid integrated into chromosomal DNA. A single colony was then incubated at 30°C in the presence of chloramphenicol for 24 to 48 h to allow excision of the integrated plasmid. Then, the strain growing in the presence of chloramphenicol, but not in the presence of tetracycline was isolated. The *cat* gene was transduced by phage 80 alpha to KSA8 and COL strains. The *cat* insertional inactivation of *fmtA* in the transductants as confirmed by Southern hybridization.

Site-directed mutagenesis of *fmtA*. Since FmtA protein has two of three motifs (S[Y]XN, SXXK) which are typically found in low-molecular PBPs and ß-lactamases, we tried to investigate whether *fmtA* with a point mutation in these motifs could restore the mutation. To know which motifs are important for having its activity, complementation experiment was performed by measuring the MIC of oxacillin in each strains, having mutated FmtA. Serine or tyrosine residue in two S(Y)XN motifs (position at 46SDN48 and 186YKN188) and two SXXK motifs (position at 63SKLK66 and

127SAQK130) were replaced with cystein by PCR-site specific mutagenesis described previously (10). *fmtA* cloned into pGEM-T easy vector (pHK4130) was used as the template for mutagenesis. Synthetic oligonucleotide primers used for mutagenesis are listed in Table 3. For mutagenesis, two fragments were amplified with two sets of primers (primer 1 and reverse primer, or primer 2 and forward primer). Forward (antisense strand) and reverse (sense strand) primers were constructed from either side of polycloning site of pGEM-T easy vector. Primer 1 and 2, constructed from opposite strands, were overlapped within 20 nt. Following PCR, the two fragments were mixed, and denatured at 94°C for 30 min, then stand at 37°C for 1 h to anneal both fragments in the overlapping region. Then, 2nd PCR was performed using the forward and reverse primers after the restoration of single strand region to double strand by DNA polymerase. Consequently, the desired PCR-amplified fragment was cloned into PCR cloning vector, pGEM-Teasy (Promega), followed by verification of the sequence of the mutagenized gene. Since we suceeded to restore the mutation of *fmtA*-inactivated mutant using a single-copy integration vector (pCL83)(3), but failed using a multi-copy vector (13), we cloned DNA fragments containing mutant *fmtA* into pCL83, then electroporated into *S. aureus* 316, which allowed to integrate pCL83 into chromosomal DNA (3, 13). Since pCL83 has a tetracycline resistance marker, COL and KSA8 strain were resistance to tetracycline, so tetracycline susceptible strain, BB270-TS339, was used for complementation experiment. BB270-TS339 was constructed by