

Table 1-continued

Strain or plasmid	Relevant characteristics	Origin or reference
<i>E. coli</i>		
XL-1 Blue	<i>rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI<sup>q</sup>ZM15 Tn10</i> (Tetr)]	(3)
BL21	<i>hsdS gal1 (lambda)citS857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</i>	(32)
Plasmids		
pUC19	cloning vector, Amp <sup>r</sup>	(47)
pCL8	shuttle vector, Amp <sup>r</sup> in <i>E. coli</i> , Cm <sup>r</sup> in <i>S. aureus</i>	(35)
PLI50	shuttle vector, Amp <sup>r</sup> in <i>E. coli</i> , Cm <sup>r</sup> in <i>S. aureus</i>	(39)
pFLAG MAC	expression vector (Eastman Kodak Company)	
pHK4011	pUC19/4 kb HindIII-PstI fragment containing Tn551 left junction and its flanking region of KSA8 TS111	This study
pHK4014	pUC19/8.9 kb XbaI fragment of KSA8	This study
pHK4357	pCL8/7.8 kb PCR fragment ( <i>fntB</i> ) of COL	This study
pHK4176	PLI50/1.8 kb PCR fragment ( <i>glnM</i> ) of COL	This study
pHK4251	PLI50/1.8 kb <i>glnM</i> fragment + 7.8 kb fragment ( <i>fntB</i> ) of COL	This study
pHK4148	pFLAG MAC/partial <i>fntB</i> fragment (repeated sequence)	This study
pHK4203	pFLAG MAC/ <i>fntD</i> fragment	This study

Table 1-continued

Strain or plasmid	Relevant characteristics	Origin or reference
<i>E. coli</i>		
XL-1 Blue	<i>rec1 endA1 gyrrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacYZNM15 Tn10</i> (Tetr)]	(3)
BL21	<i>hsdS gal (lambdactets857 ind1 sam7 nin5 lacUV5-r7 gene 1)</i>	(32)
Plasmids		
pUC19	cloning vector, Amp <sup>r</sup>	(47)
pCL8	shuttle vector, Amp <sup>r</sup> in <i>E. coli</i> , Cm <sup>r</sup> in <i>S. aureus</i>	(35)
PLI50	shuttle vector, Amp <sup>r</sup> in <i>E. coli</i> , Cm <sup>r</sup> in <i>S. aureus</i>	(39)
pFLAG MAC	expression vector (Eastman Kodak Company)	
PHK4011	pUC19/4 kb <i>HindIII-PstI</i> fragment containing Tn551 left junction and its flanking region of KSA8 TS111	This study
PHK4014	pUC19/8.9 kb <i>XbaI</i> fragment of KSA8	This study
PHK4357	pCL8/7.8 kb PCR fragment ( <i>fntB</i> ) of COL	This study
PHK4176	PLI50/1.8 kb PCR fragment ( <i>glmM</i> ) of COL	This study
PHK4251	PLI50/1.8 kb <i>glmM</i> fragment + 7.8 kb fragment ( <i>fntB</i> ) of COL	This study
PHK4148	pFLAG MAC/partial <i>fntB</i> fragment (repeated sequence)	This study
PHK4203	pFLAG MAC/ <i>FemD</i> fragment	This study

Table 2. MICs of oxacillin for COL and their derivatives

strain	MIC of oxacillin ( $\mu\text{g/ml}$ )
COL	512
COL-TS111	0.13
COL-TS111 pHK4357 ( <i>fmtB</i> )	0.13
COL-TS111 pHK4176 ( <i>femD</i> )	128
COL-TS111 pHK4251 ( <i>femD</i> + <i>fmtB</i> )	256

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

substrate	MIC of oxacillin ( $\mu\text{g/ml}$ )		
	COL	COL <i>fmtB::Tn551</i>	COL <i>glmM::Tn551</i>
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

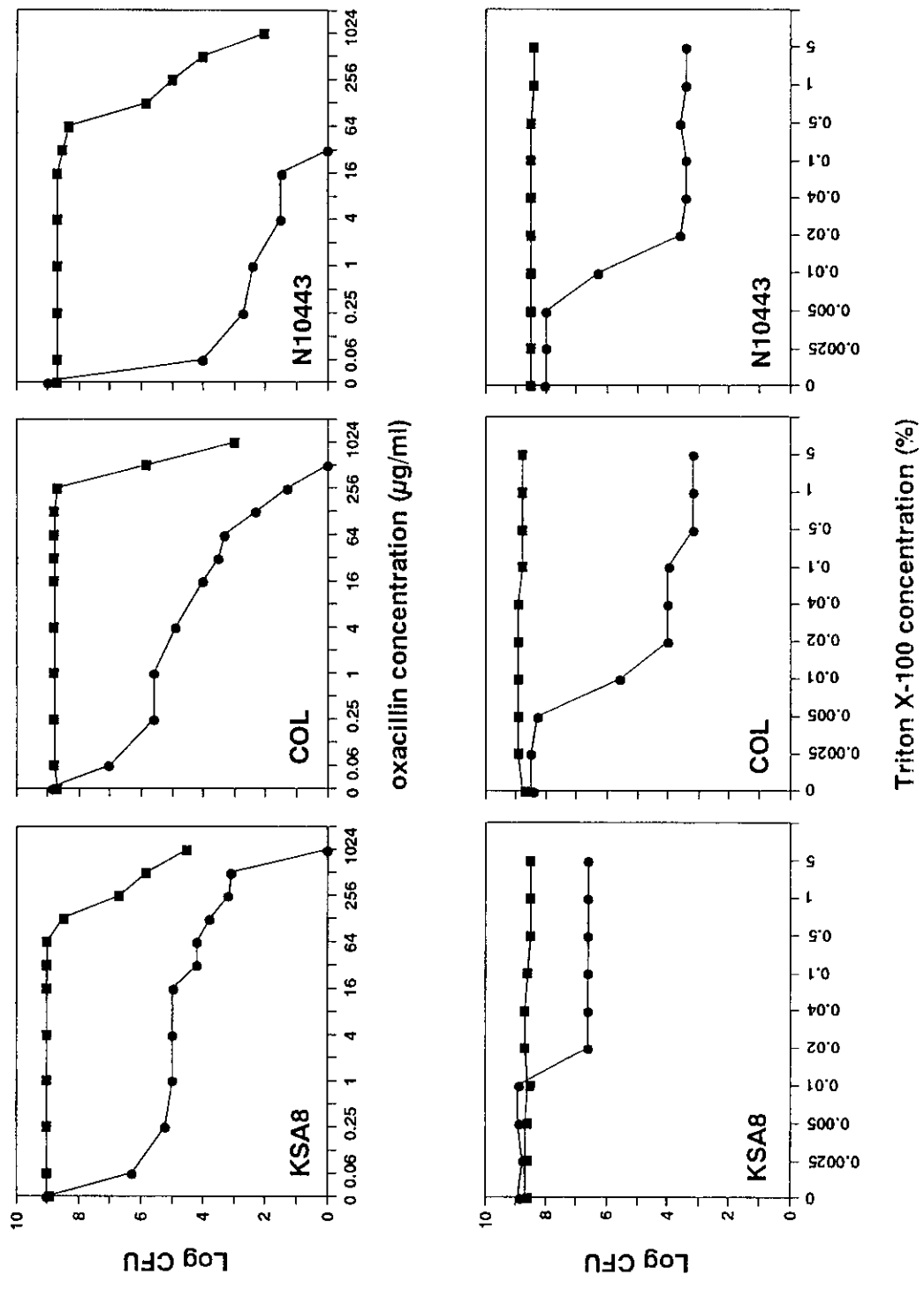
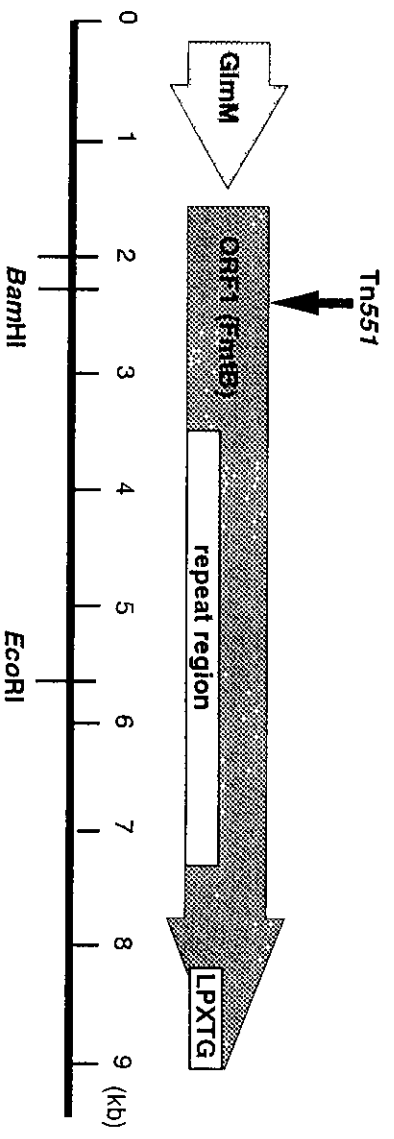


Fig. 1

Fig.2



TTAGATAGAG TTGCTTTGTG TTTTAAACGC AGATGCTACT ACTTATCTTA ACAGTTGATT AAGTGAATC ATTTAACAGC GAGAATAATC AACCCAGGAG 100

-35 -10

ATGACTTAAT GAATTTATTC AGACAACAAA AATTTAGTAT CAGAAAAATTT AATGTCGGTA TTTTTCAGC TTTAATYGCC ACTGTTACTT TTATATCTAC 200  
M N L F R Q Q K F S I R K F N V G I F S A L I A T V T F I S T

TAACCCGACA ACAGCGTCTG CAGCAGAGCA AAATCAGCCT GCACAAAATC AACCCAGACA ACCAGCTGAT GCCAATACAC AGCCTAACGC AAATGCTGGT 300  
N P T T A S A A E Q N Q P A Q N Q P A Q P A D A N T Q P N A N A G

GCTCAAGCTA ATCCTACAGC ACAGCCAGCT GCACCTGCCA ACCAAGGACA ACCAGCAGTA CAACCAGCAA ACCAAGGTGG ACAGGCTAAT CCAGCAGGAG 400  
A Q A N P T A Q P A A P A N Q G Q P A V Q P A N Q G G Q A N P A G G

GAGCAGCACA ACCAAATACA CAACCAGCTG GACAAGTAA TCAAGCTGAT CCGAATAACG CTGCACAAGC ACAACCTGGA AATCAAGCAA CACCGGCAAA 500  
A A Q P N T Q P A G Q G N Q A D P N N A A Q A Q P G N Q A T P A N

CCAAGCAGGT CAAGGAAATA ACCAAGCAAC ACCTAATAAT AATGCAACAC CGGCAAATCA AACACAGCCA GCGAATGCTC CAGCAGCAGC GCAACCAGCA 600  
Q A G Q G N N Q A T P N N N A T P A N Q T Q P A N A P A A A Q P A

GCACCTGTAG CAGCAAACGC ACAAACCTCAA GATCCAAATG CTAGCAATAC TGGTGAAGGC AGTATTAAATA CGACATTAAC ATTTGATGAT CCTGCCATAT 700  
A P V A A N A Q T Q D P N A S N T G E G S I N T T L T F D D P A I S

CAACAGATGA GAATAGACAG GATCCAACCTG TAACGTGTAC AGATAAAGTA AATGGTATT CATTAAATTA CAACGGTAAG ATTGGTTTCG TTAACCTAGA 800  
T D E N R Q D P T V T V T D K V N G Y S L I N N G K I G F V N S E

ATTAAGACGA AGCGATATGT TTGATAAGAA TAACCTCAA AACTATCAAG CTAAGGAAA CGTGGCTGCA TTAGTCTGTG TGAATGCAA TGATTCTACA 900  
L R R S D M F D K N N P Q N Y Q A K G N V A A L G R V N A N D S T

GATCATGGTA ACTTTAACGG TATTTCAAAA ACTGTAATG TAAAACCAGA TTCAGAATTA ATTATTAAC TTA<sup>Tn551</sup>CTACTAT GCMAACGAAT AGTAAGCAAG 1000  
D H G N F N G I S K T V N V K P D S E L I I N F T T M Q T N S K Q G

GTGCAACAAA TTTAGTTATT AAAGATGCTA AGAAAAATAC TGAATTAGCA ACTGTAATG TTGCTAAGAC TGGTACTGCA CATTATTTTA AAGTACCAAC 1100  
A T N L V I K D A K K N T E L A T V N V A K T G T A H L F K V P T

YGATGCTGAT CGTTTAGATT TACAATTTAT TCCTGACAAT ACAGCAGTTG CTGATGCTTC AAGAATTACA ACAATAAAG ATGGTTATAA ATACTATTCA 1200  
D A D R L D L Q F I P D N T A V A D A S R I T T N K D G Y K Y Y S

TTCAATTGATA ATGTAGTCT ATTCTCAGGA TCACATTTAT ATGTCAAAAA TAGAGATTA GCACCCGAAAG CAACTAACAA TAAAGAATAT ACTATTAATA 1300  
F I D N V G L F S G S H L Y V K N R D L A P K A T N N K E Y T I N T

CTGAAATCGG TAACAATGGT AATTTTGGTG CTTCATTAATA AGCAGATCAA TTTAAATATG AAGTAACATT ACCACAAGGT GTAACCTACG TTAATAATTC 1400  
E I G N N G N F G A S L K A D Q F K Y E V T L P Q G V T Y V N N S

ATTAACATACA ACATTTCTTA ATGGTAATGA AGACAGTACA GTATTGAAAA ATATGACTGT TAATTATGAT CAAAATGCAA ATAAAGTTAC ATTTACAAGC 1500  
L T T T F P N G N E D S T V L K N M T V N Y D Q N A N K V T F T S

CAAGGTGTGA CAACGGCAGC TGGTACACAC ACTAAGAAG TTTTATTCCT AGATAAATCT TTAATAATYAT CATATAAAGT YAATGTTGCG AATATCGATA 1600  
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P K N I D F N E K L T Y R T A S D V V I N N A Q P E V T L T A D P

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F S V A V E M N K D A L Q Q Q V N S Q V D N S H Y T T A S I A E Y

AATAAACTTA AACACAAGC AGATACTATT TTAATGAAG ATGCGAATCA TGTAAAATCT GCAAAATCGTG CATCTCAAGC GGATATTGAT GGTTAGTAA 1900  
N K L K Q Q A D T I L N E D A N H V K T A N R A S Q A D I D G L V T

CTAAATFACA AGCTGCATTA ATTGATAATC AAGCAGCAAT TGCTGAATTA GATACTAAG CTCAAGAAAA GGTTACAGCA GCACAACAAA GTAAAAAAGT 2000  
K L Q A A L I D N Q A A I A E L D T K A Q E K V T A A Q Q S K K V

TACGCAAGAT GAAGTTGCAG CACTTGTAACT TAAAATTAAC AATGATAAAA ATAATGCAAT CGCAGAAAT AATAAACAAA CTACAGCACA AGGTGTACA 2100  
T Q D E V A A L V T K I N N D K N N A I A E I N K Q T T A Q G V T

ACTGAAAAAG ATAATGGTAT CGCAGTGTTA GAACAAGATG TGATTACACC AACAGTTAAA CCTAAAGCGA ACCAAGATAT TATCCAAGCA GTTACAACCTC 2200  
T E K D N G I A V L E Q D V I T P T V K P K A N Q D I I Q A V T T R

GTAAACAACA AATTAATAAG TCAATGCAT CATTACAAGA TGAATAAGAT GTAGCAATG ATAAATTTGG TAAAATTGAA ACAAGGCAA TTAAGATAT 2300  
K Q Q I K K S N A S L Q D E K D V A N D K I G K I E T K A I K D I

TGATGCAGCA ACAACAATG CACAAGTAGA AGCCATTAATA ACAAAAGCAA TCAATGATAT TAATCAAATC ACACCTGCTA CAACAGCTAA AGCAGCAGCT 2400  
D A A T T N A Q V E A I K T K A I N D I N Q T T P A T T A K A A A

CTTGAAGAAT TTGACGAAGT TGTTCAAGCA CAAATGATC AAGCACCTTT AAATCTGAT ACAACAATG AAGAAGTAGC GGAAGCTATT GAACGTATTA 2500  
L E E F D E V V Q A Q I D Q A P L N P D T T N E E V A E A I E R I N

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A A K V S G V K A I E A T T T A Q D L E R V K N E E I S K I E N I

Fig.3

TACTGACTCT ACGCAAACAA AAATGGATGC <sup>R3</sup>CTATAATGAA GTTAAACAAG CTGCAACAGC TAGAAAAGCT CAAAATGCTA CAGCTTCAA TGCAACAAAT 2700  
T D S T Q T K M D A Y N E V K Q A A T A R K A Q N A T A S N A T N

GAAGAAGTAG CAGAAGCTGA TGCAGCAGTA GATGCAGCTC AAAAGCAAGG TTTACATGAC ATCCAAGTTG TTAATCAAA ACAGGAAGTT GCTGATACAA 2800  
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

AAATCAAAAGT ATTAGATAAA ATCAATGCAA TTCAAAACACA AGCAAAAGTT AAACCTGCAG <sup>R4</sup>CTGATACGGA AGTAGAAAAC GCATATAATA CAGCTAAACA 2900  
S K V L D K I N A I Q T Q A K V K P A A D T E V E N A Y N T R K Q

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E I Q N S N A S T T E E K Q A A Y T E L D T K K Q E A R T N L D A

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A N T N S D V T T A K D N S I A A I N Q V Q A A T T K K S D A K A E

AAATCGCTCA AAAAGCAAGT GAACGTAAAA CAGCAATTGA AGCAATGAAT GATTGACTA CTGAAGAACA ACAAGCAGCG AAAGACAAGG TGGATCAAGC 3200  
I A Q K A S E R K T A I E A M N D S T T E E Q Q A A K D K V D Q A

AGTAGTTACT GCAAACGCTG ATATAGATAA TGCTGCAGCA AACCAATGATG TGGATAATGC AAAAACTACA AATGAAGCTA CAATCGCAGC CATTACACT 3300  
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A N E Q K A L I A Q T A D A T T E E K E Q A N Q Q V D A H L T Q G N

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→ R15  
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CGAAGCTCA AGGACTTGAA GCATTGATA ACATTCAAAT CGACTCAACA GAAAAACAAA → R17  
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A G V N V N A D A T T E E K E A F T N A L E D I L S K A T E D I S

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TGTAATGAT TCAAAACAAA ATGCTGAAGT AAATAATAGT GCTGAATCTC AATCAACTAA TGCAAGGTT GCACAACCAA AATCTGAAAA TAAAGCTAAG 6900  
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T S K D K E E S T T N Q T D A G Q L K S E T N V A S N E A D K S P

AAGCAAAGCT GATACTGAAG TTTGCAATAA ACCATCAACA TCTGCATCTT CTGAAGCAA AGAAAAATG ACTTCAACTA ATCTTAGCCA AAAAGATGAT 7200  
S K A D T E V S N K P S T S A S S E A K E K M T S T N L S Q K D D

ACGGCAACAG CAGATACTAA TGATACGCAA CCATCAGTTG GTTCAGCTGC AAACAATAAA GCTACGCAA ACGACGGTGC CAATGCATCT CCAGCTACAG 7300  
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TTTCAAATGG AAGCAATAGT GCTAATCAAG ATATGCTTAA TGTAAC TAAC ACTGACGACC ACCAAGCTAA GACAAAAATCA GCTCAACAAG GAAAAATGTA 7400  
S N G S N S A N Q D M L N V T N T D D H Q A K T K S A Q Q G K V N

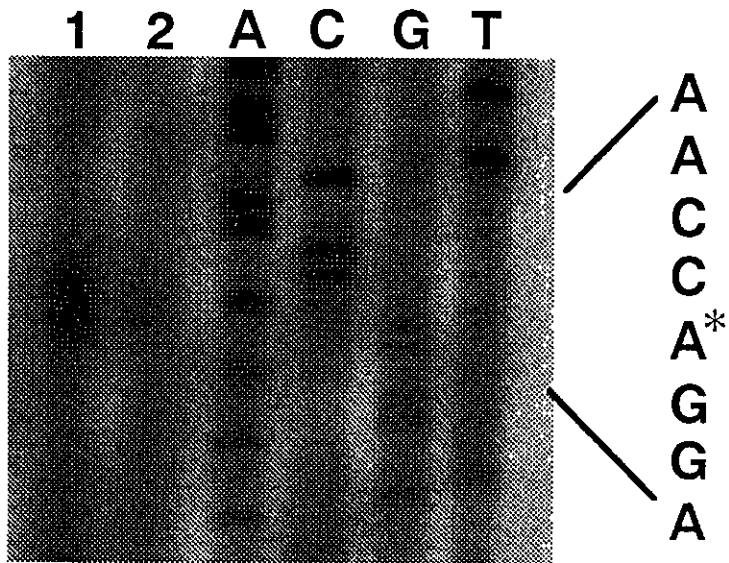
TAAAGTAAA CAACAAGCTA AAACCTTACC AGATACTGGT ATGTCACATA ATGACGATTT ACCATATGCT GAATTAGCTC TAGGTGACGG TATGGCATT C 7500  
K A K Q Q A K T L P D T G M S H N D D L P Y A E L A L G A G M A F

TAAATTAGAA GATTCACTAA GAAAGACCAA CAACTGAAG AATAAAATTT ATTCAAGAAT GTGAACATCT AGTTATTTC A TAGGGAAAAA TTCCTTACTA 7600  
L I R R F T K K D Q Q T E E

AGTAACCAAG TCTAACAGTT AATATGAATT TGTTTTAGTA ATGACCAATA GCCATTGAAT AGGTGGCTGT GGCTCGAAAA TAAAAACAAT TATTAACGCA 7700

AAAACTCC TTTCAATCAT AACCAAGTT AAGTGTCTAT GTTGAAGGGA GTATTTTTT ATGTTGAGAA ATTTAATATT GTTAATTCAA CGAACTACT 7800

ACATTATTAC T 7811



**Fig. 4**

R1  
R2  
R3  
R4  
R5  
R6  
R7  
R8  
R9  
R10  
R11  
R12

ANQD I I QAVT TRKQQK KKSNA  
LEEFDEVVQ AQIDQAPLNP  
AYNFKQAAT ARKAQMATAS  
ADTEVENAYN TRKQEI QNSN  
AKAIAQKAS ERKTAIEAMN  
AKQAIAADKVVQ AQETAIDGNN  
AKKAIATKAN ERKTAIAQTQ  
ARNBITAILN NKLLQEQATP  
AKDEIDQLQA TQTNVINNDQ  
AKNDVDQAVT TQNQAIDNTT  
AKDELATRAN EQKALIAQTA  
ARAE LLTEMQ KNITEILNNA

NDKI GKIETK  
IERNAAKVS  
DAAVDAAQKQ  
YTELDTKKQE  
KDKVDQAVVT  
KQQVQTEKTT  
NADVDDNAVTD  
DAEANTENGG  
IQQEATAVTD  
KDLV LKAKKEK  
NQQVD AHLTQ  
IGPVRAAYEE

KDV  
VAE  
VAE  
KQA  
QQA  
KAA  
IAA  
KQA  
KEA  
KNA  
KEQ  
KGN

Q  
TTN  
N  
STT  
STT  
STT  
LTA  
DDE  
KNA  
KEQ  
KGN

A  
D  
N  
A  
D  
G  
D  
D  
N  
G  
D  
D  
E

R1  
R2  
R3  
R4  
R5  
R6  
R7  
R8  
R9  
R10  
R11  
R12

AIKDIIDAAAT  
GVKAI EATTT  
GLHDIQVVKS  
ARTNLDAAANT  
ANAIDNAAAT  
ADAIDAAHT  
ANSNIEAANS  
ANQAISAATTT  
AKNNITAAATD  
AYQDILLVAQT  
GNQNIENAAQS  
GLNNINAAAT

NAQVEAI  
AQDEERVV  
KQEVADTK  
NSDVTTAK  
NNDVDNAK  
NAEVEAAK  
QNDVDQA  
NAQVDEAK  
DNGVDQA  
TNDVTQI  
IDDVNTAK  
TGDVTTAK

TK  
NE  
SK  
DN  
TT  
KA  
TT  
KAN  
DA  
DDQ  
DN  
DDT

AIND  
EISK  
VLDK  
SIAA  
NEAT  
AIAK  
GENS  
AEAA  
GKNS  
AVAD  
AIQA  
AVQK

QTT  
ITD  
AIQT  
QVQA  
AITP  
AIQP  
DVTP  
AVTP  
STQP  
IGITA  
DPIQA  
POLHA

ATT  
ST  
QAKV  
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DANV  
ATTT  
TVNK  
KVVK  
ATAV  
DTTI  
STDV  
NPVK

Fig. 5

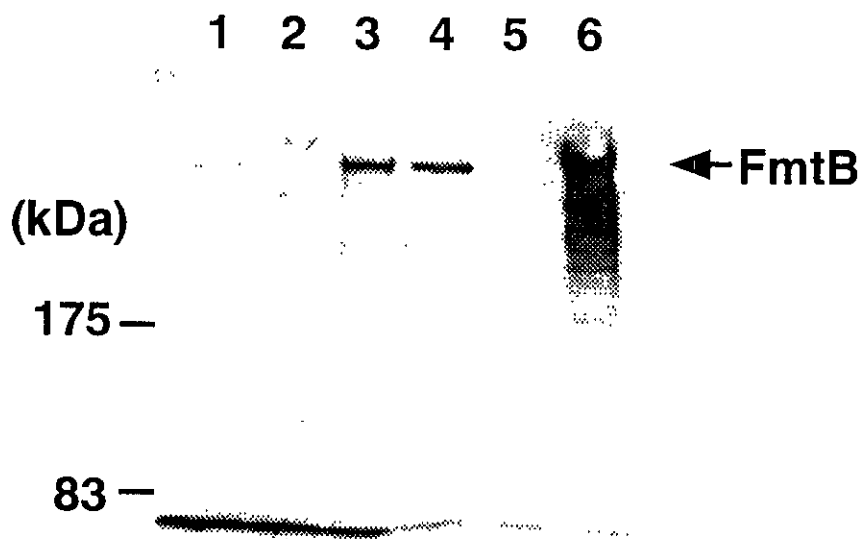


Fig. 6

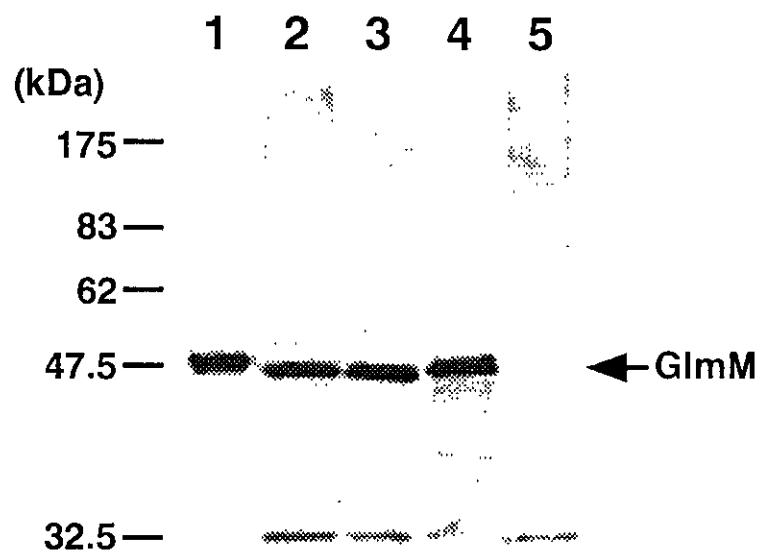


Fig. 7

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  $\beta$ -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  $\beta$ -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  $\beta$ -lactam antibiotics and functions under the otherwise inhibitory concentration of  $\beta$ -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  $\beta$ -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). GlnM (FemD) has a phosphoglucosamine mutase activity and is involved in an early step in peptidoglycan synthesis (35). MureE (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 Tn551 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  $\beta$ -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  $\beta$ -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  $\mu\text{g/ml}$ ), chloramphenicol (CP) (10  $\mu\text{g/ml}$ ) or ampicillin (100  $\mu\text{g/ml}$ ) 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 Mannheim 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 Mannheim, and PCR was performed with the GeneAmp PCR System 2400 (Perkin Elmer).

**Construction the *cat* insertional mutant.** In COL-TS339, Tn551 was inserted at the C-terminal of *fmtA* (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 *EcoRI*-

*SalI* fragment from pHK4033 was cloned into pCL52.1 (thermosensitive plasmid; Table 1) to generate pHK4079. Then, the *cat* gene from *Sau3AI* digested *E. coli*-*S. aureus* shuttle vector, pLI50 (3), was ligated into pHK4079, using the *BamHI* 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 β-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 cysteine 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 succeeded 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