JOURNAL OF BACTERIOLOGY, Mar. 1994, p. 1460–1467 Vol. 176, No. 5
0021-9193/94/$04.00+0
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The *femC* Locus of *Staphylococcus aureus* Required for Methicillin Resistance Includes the Glutamine Synthetase Operon

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Tn*551* insertional inactivation of *femC* is known to reduce methicillin resistance levels in methicillin-resistant and -susceptible *Staphylococcus aureus*. By use of cotransductional crosses, *femC* was mapped close to *thrB* on the *Sma*I-A fragment of the *S. aureus* NCTC 8325 chromosome. The Tn*551* insertion *femC*::Ω2005 was found to interrupt an open reading frame coding for a putative protein of 121 amino acids which is highly similar to the glutamine synthetase repressors (GlnR) of *Bacillus* spp. Downstream of *femC*, an open reading frame highly similar to *Bacillus* sp. glutamine synthetases (GlnA) was found. Northern (RNA) blots probed with putative *glnR* or *glnA* fragments revealed that 1.7- and 1.9-kb transcripts characteristic of wild-type cells were replaced by less abundant 7.0- and 7.2-kb transcripts in the *femC*::Ω2005 mutant. Total glutamine synthetase activity was also decreased in the mutant strain; the addition of glutamine to defined media restored the wild-type methicillin resistance phenotype of the *femC* mutant. This result suggests that the Ω2005 insertion in *glnR* has a polar effect on *glnA* and that *glnR* and *glnA* are transcribed together as an operon. These results suggest that the loss of wild-type levels of glutamine synthetase and the consequent decrease in glutamine availability cause a decreased level of methicillin resistance.

Methicillin resistance in staphylococci is mediated by the *mec* determinant, containing *mecA*, which codes for low-affinity penicillin-binding protein PBP2a or PBP2′. It has been suggested that PBP2a is a transpeptidase or an enzyme involved in a peptidoglycan synthesis system which operates at high concentrations of methicillin (4, 7). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains give rise to populations of cells that vary in their levels of susceptibility to methicillin. This phenomenon has been termed heterogeneity (15, 31). The level of resistance and degree of heterogeneity are strain dependent and are affected by external factors (for a review, see reference 5).

Since the discovery of a chromosomal locus outside the *mec* determinant at which mutations affect methicillin resistance in *S. aureus* (1), a total of four such loci have been characterized and mapped (3). These loci are said to contain *fem* (factors essential for methicillin resistance) genes (2) or *aux* genes (auxiliary genes) (34). Tn*551* insertional inactivation of either *femA* or *femB* abolishes methicillin resistance completely, while inactivation of either *femC* or *femD* reduces the basal resistance level (3, 13, 16).

Three *fem* factors, *femAB* and *femD*, are in some way involved in peptidoglycan synthesis. *femAB* is important in the formation of the pentaglycine bridge linking muropeptides (19), and *femD* may be involved in the formation of a common peptidoglycan precursor (6). The exact function of these genes is still in question.

We now present evidence that the Tn*551* insertion *femC*::Ω2005 is in *glnR*, the gene coding for the repressor of the glutamine synthetase (GS) operon, *glnRA*. This insertion results in a polar effect on *glnA* transcription, leading to a reduction in GS activity and probably glutamine availability, which causes the decrease in methicillin resistance observed in the *femC*::Ω2005 mutant.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and plasmid pTZ18R were used in the cloning of the *femC* region. Transductions of *femC*::Ω2005 were carried out with bacteriophage 80α, and transductants were selected on 20 mg of erythromycin per liter. All bacteria were grown in LB (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) at 30 or 37°C and 200 rpm. Defined media with gradients of methicillin and with or without glutamine were incubated at 37°C. The defined medium of Townsend and Wilkinson (35) was used with the following modifications. Our defined medium contained (in milligrams per liter) aspartic acid (45), cystine (2), isoleucine (3), methionine (3), tyrosine (5), adenine (5), cytosine (5), guanine (5), uracil (5), and thymine (20). Highly methicillin-resistant colonies of BB589 were selected for by plating on LB plates containing 256 or 512 mg of methicillin per liter. E-tests (AB BIODISK, Solna, Sweden) were carried out on Mueller-Hinton agar with an inoculum turbidity equivalent to a 0.5 McFarland standard (25).

DNA manipulations. All DNA manipulations, gel electrophoresis, blotting of DNA, and hybridization procedures were performed essentially as described by Maniatis et al. (20). Restriction enzymes, a random-primer labeling kit, T4 DNA ligase, and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim Biochemica and were used according to the directions of the manufacturer.

Cloning of *femC*. A *Hin*dIII library of *femC*::Tn*551* mutant BB589 was ligated into pTZ18R and transformed into *E. coli* DH5α. Plasmid pBBB104 (Fig. 1a) containing a 2.5-kb *Hin*dIII fragment which included a *femC*::Tn*551* junction fragment was isolated from this library by probing with the *Hin*dIII-*Ava*I J_R fragment of Tn*551* (12). The 1.5-kb *Ava*I-*Hin*dIII *femC* frag-

TABLE 1. *S. aureus* strains and plasmids used in this study

Strain or plasmid	Description	Reference or origin
Strains		
BB255	NCTC 8325	3
BB906	NCTC 8325 Ω2005(*femC*::Tn*551*)	This study, by transduction of Ω2005 with phage 80α from BB589 into BB255
BB270	NCTC 8325 *mec*	3
BB589	NCTC 8325 *mec* Ω2005(*femC*::Tn*551*)	3
BB895	NCTC 8325 *mec hmr*[a] Ω2005(*femC*::Tn*551*)	This study, from a BB589 colony picked from an LB plate containing 256 mg of methicillin per liter
BB905	NCTC 8325 *mec hmr* Ω2005(*femC*::Tn*551*)	This study, from a BB589 colony picked from an LB plate containing 512 mg of methicillin per liter
BB399	DU4916 *mec*	11
BB913	DU4916 *mec* Ω2005(*femC*::Tn*551*)	This study, by transduction of Ω2005 with phage 80α from BB589 into BB399
ISP851	NCTC 8325 *lys115 trp103 thrB106 uraA141 ilv129 nov142 pig131 ala126 tmn3106*	Obtained from P. Pattee
Plasmids		
pBBB104	Vector: pTZ18R; cloning site: *Hin*dIII; insert: 2.5-kb *Hin*dIII chromosomal fragment from BB589 containing the Tn*551* J_R fragment and the *femC* junction fragment[b]	
pBBB105	Vector: pTZ18R; cloning site: *Bam*HI; insert: 1.9-kb *Sau*3A chromosomal fragment from BB270 containing the ORF of *femC*[b] (*glnR*) and partially cloned ORF1 and *glnA*	

[a] *hmr*, high (level of) methicillin resistance.
[b] See Fig. 1.

ment from plasmid pBBB104 was subsequently used to probe a *Sau*3A library from MRSA strain BB270 cloned into the *Bam*HI site of plasmid pTZ18R. Plasmid pBBB105 containing an insert of 1.9 kb covering the Ω2005 insertion site and thus the *femC* region was isolated (Fig. 1b).

RNA manipulations. Solutions used in total RNA isolation were prepared in water treated with 0.2% diethyl pyrocarbonate. RNA was isolated from cultures grown in LB to an optical density at 580 nm (OD_{580}) of 1.0. Sodium azide was added to cultures to a final concentration of 0.025 mM before cells were

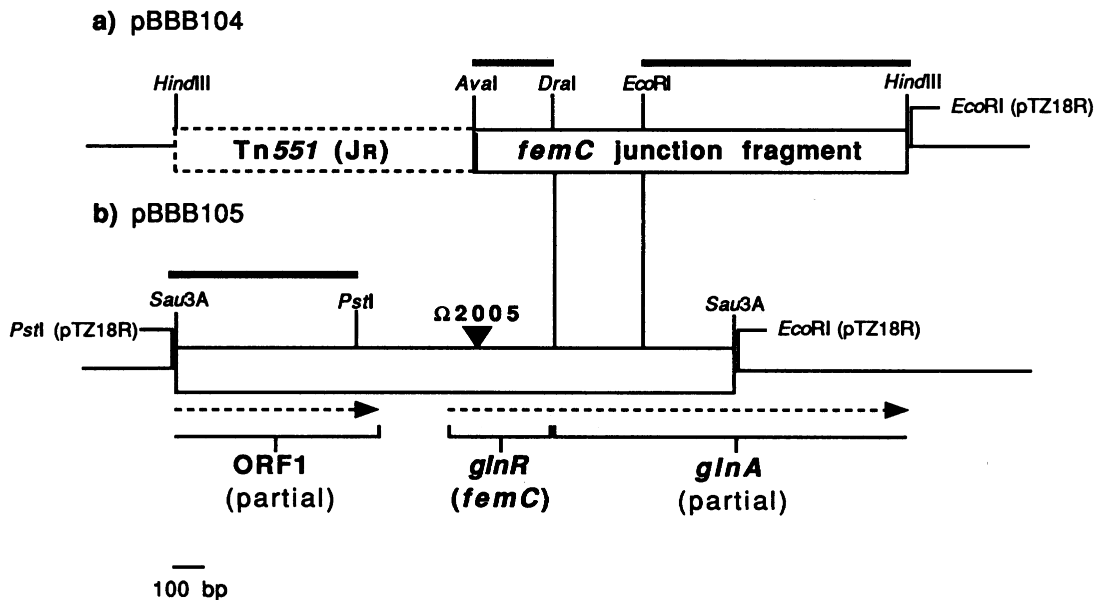

FIG. 1. Restriction map, organization, and sequence overlap of a 2.5-kb *Hin*dIII *femC*::Tn*551* junction fragment cloned from BB589 and a 1.9-kb *Sau*3A fragment cloned from BB270 and covering *femC* (*glnR*). Lines are drawn from the *Dra*I and *Eco*RI sites of pBBB104 to the same sites in pBBB105 to show the alignment and sequence overlap of the two cloned fragments. (a) pBBB104. The right junction of Tn*551* (Tn*551* J_R, 1.0 kb) in relation to the cloned *femC* junction fragment (1.5 kb) is shown. The 0.3-kb *Ava*I-*Dra*I (*glnR*) and 0.8-kb *Eco*RI (*glnA*) probes are indicated by solid bars. (b) pBBB105. *glnR* is shown in relation to partially cloned *glnA*. The position of partially cloned ORF1 is also shown. The proposed direction of transcription of *glnR* and partially cloned ORF1 and *glnA* (broken arrows) and the position of Tn*551* insertion site Ω2005 in *glnR* are indicated. The 0.6-kb *Pst*I ORF1 probe is indicated by a solid bar.

harvested by centrifugation. The cell pellet was resuspended in 500 μl of STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris [pH 6.8]), and the suspension was placed in a 1.5-ml Eppendorf tube and immediately frozen in an ethanol-dry ice bath. When all samples were isolated, they were thawed at 100°C for 1 min, and lysostaphin was added to a final concentration of 125 mg/liter. The cells were incubated on ice for 10 min, placed at 100°C for 1 min, and centrifuged at 12,000 × *g* for 2 min. The cell debris pellet was removed with a sterile toothpick, and 200 μl of neutral phenol-chloroform-isoamyl alcohol (20) was added to the tube, which was then vortexed for 10 s. After centrifugation, the aqueous phase was removed and placed in a new Eppendorf tube. The nucleic acids were precipitated by the addition of sodium acetate (pH 4.8) to a final concentration of 0.3 M and 1 volume of isopropanol. The pellet was resuspended in 100 μl of DNase digestion buffer (20 mM sodium acetate [pH 4.5], 10 mM $MgCl_2$, 10 mM NaCl) containing DNase (30 U) and RNase inhibitor (30 U). After incubation at 37°C for 30 min, 50 μl of neutral phenol-chloroform-isoamyl alcohol was added, and the mixture was vortexed (5 s) and centrifuged. The upper phase was placed in a new Eppendorf tube, and the RNA was precipitated as described above. The RNA pellet was then resuspended in 50 μl of diethyl pyrocarbonate-treated water, and the concentration was determined photometrically at 260 nm.

RNA gels were made of 1.5% agarose in 1.0% MOPS buffer (200 mM morpholinepropanesulfonic acid, 50 mM sodium acetate trihydrate, 10 mM EDTA; final pH, 7.0) and 1% formaldehyde. The gels were run in 1.0% MOPS buffer at 120 V for 0.5 h and then at 150 V for 1 h. RNA samples were denatured by the addition of 1 volume of denaturation mixture (68% deionized formamide, 5% formaldehyde, 14% 10× MOPS buffer, 4% 10-mg/ml ethidium bromide) and heating at 65°C for 10 min; samples were placed on ice for 5 min before being loaded. Molecular weights were determined with RNA molecular weight marker I from Boehringer or rRNAs from samples. The gels were then soaked in sterile 0.025 M sodium phosphate buffer (pH 6.5), and RNA was transferred to GeneScreen (NEN Research Products, Boston, Mass.) filters by capillary action with the same buffer overnight. The filters were then baked at 80°C for 2 h.

DNA sequencing and computer analysis. Nucleotide sequences were determined by dideoxy chain termination (26) with the M13-based sequencing strategy and a Sequenase sequencing kit (United States Biochemical). DNA sequences were analyzed with the DNA Inspector IIe program (Textco, Inc., West Lebanon, N.H.) and the Genetics Computer Group program (University of Wisconsin Genetics Computer Group, Madison). Amino acid homologies were searched for in the NBRF, Swissprot, EMBLPIR, and MIPSX protein data banks. Amino acid sequence alignments were performed with the GAP program (University of Wisconsin Genetics Computer Group). The nucleotide sequence in Fig. 2 has been submitted to EMBL/GenBank under accession number X76490.

GS assay. All assays were carried out on mid-exponentially growing cells harvested at an OD_{580} of 1.0. GS activity was measured with hexadecyltrimethylammonium bromide (CTAB)-permeabilized cells on the basis of the formation of γ-glutamylhydroxamate by the Mn^{2+}-dependent reverse transferase reaction (37) described for *Bacillus subtilis* (9, 10, 28). Before cells were harvested, CTAB was added to cultures to a final concentration of 100 mg/liter, and the cultures were shaken for 5 min. The cells were then harvested, washed with 10 ml of 50 mM imidazole (pH 7.0)–0.5 mM EDTA–5 mM dithiothreitol (IED), and resuspended in 200 μl of the same buffer. Before this suspension was added to the transferase reaction mixture, it was diluted 1/5 with IED plus 0.4 M K_2SO_4; 50 μl was then added to 450 μl of the transferase assay mixture (25 mM imidazole chloride [pH 7.5], 25 mM Tris-HCl [pH 7.5], 50 mM NH_2OH, 40 mM glutamine, 2 mM ADP, 0.33 mM $MnCl_2$, 0.4 M K_2SO_4, 20 mM sodium arsenate). The transferase reaction was run at 37°C for 15 min, at which time 1 ml of stop solution (0.37 M $FeCl_3$, 0.67 N HCl, 0.2 M trichloroacetic acid) was added and the assay mixture was placed on ice for 5 min. The cells were removed by centrifugation, and the OD_{540} was measured. One unit of GS activity was defined as the ADP-dependent formation of 1 nmol of γ-glutamylhydroxamate per min. All assay mixture components were prepared as separate stock solutions and mixed before the assay. ADP stock (20 mM) was made with 10 mM imidazole (pH 7.5) (kept at −20°C). Glutamine stock (200 mM, in sterile deionized H_2O) was prepared fresh for each experiment. The pH of the NH_2OH and sodium arsenate stock solutions (each at 1 M) was adjusted to 7.5. Protein concentrations were determined with a Bio-Rad protein assay kit as recommended by the manufacturer.

RESULTS

Genetic mapping of Ω2005(*femC*::Tn*551*). *femC* is known to reside on the *Sma*I-A fragment of *S. aureus*, along with the *femAB* operon, also needed for the expression of methicillin resistance (3). Cotransductional crosses were performed with strain ISP851, a mutant carrying several auxotrophy markers, including *thrB*, located on the *Sma*I-A fragment (23). A 35% cotransduction frequency of Ω2005 with Thr^+ was found when erythromycin resistance (Em^r) was selected for with strain BB589 as the donor and strain ISP851 as the recipient.

Isolation and characterization of highly resistant Ω2005 mutants. Tn*551* insertional inactivation of *femC* reduces methicillin resistance, but a few cells can still survive at high concentrations (>100 mg/liter) (3). To analyze the nature of this resistance in *femC* mutants, two highly resistant colonies of BB589 were picked from LB plates containing 256 mg (BB895) or 512 mg (BB905) of methicillin per liter. Both of these strains had retained Em^r. To determine whether any genetic changes had occurred at the Ω2005 insertion site in BB895 and BB905, Ω2005 was transduced from these two strains back into parent strain BB270, with selection for Em^r. One hundred percent of the Em^r transductants regained the original lower-resistance *femC* phenotype, suggesting that highly resistant colonies harbored mutations elsewhere on the chromosome.

Analysis of the cloned *femC* region. When chromosomal DNAs from *femC* mutant BB589 and parent strain BB270 were digested with *Eco*RI and probed with the 0.3-kb *Ava*I-*Dra*I *femC* fragment (Fig. 1), a 3.6-kb band present in BB270 was replaced by an 8.0-kb band in BB589 (*femC*::Tn*551*). This was due to insertion of the 5.2-kb transposon Tn*551* in the *femC* region (data not shown).

A *Hin*dIII fragment of chromosomal DNA from strain BB589 was cloned, creating pBBB104 (see Materials and Methods). A portion of the *Hin*dIII insert of plasmid pBBB104 up to the Tn*551* insertion site and a 1,924-nucleotide *Sau*3A fragment containing the wild-type *femC* allele cloned from strain BB270 in plasmid pBBB105 were sequenced (Fig. 2). Upstream of the site of the Ω2005 insertion, we found a partially cloned open reading frame (ORF), ORF1, which codes for 243 amino acid (aa) residues. The deduced sequence of this protein was strongly similar in a 243-aa overlap with the

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      Sau3A
    1 GATCAAAGACCTTCAATTCCGCTAGATGAAATTGAAAAGGTAATTACTAGGTTGAAAAACGTGCATCCTAATATTTTAATATTTGTGGATAACTGTTATG
      ORF1
 +101 GGGAATTTGTTGAAAGACGTGAACCTATAGAATGTGGTGCCGATTTAATAGCAGGATCATTAATTAAAAACCCTGGCGGTGGTTTAGCTAAGATTGGTGG
                                                                                          AvaIII
 +201 ATACATTGCTGGTAGAAAAGATTTAATTGAACGATGTGGTTATAGATTGACAGCACCTGGTATTGGTAAAGAAGCGGGTGCATCATTAAATGCATTGCTT
 +301 GAAATGTATCAAGGTTTCTTTTTAGCACCACACGTTGTCAGTCAGAGTCTTAAAGGTGCATTGTTTACTAGTTTATTTTTAGAAAAAATGAATATGAACA
              ScaI
 +401 CAACGCCGAAGTACTACGAAAAACGAACTGATTTAATTCAAACAGTTAAATTTGAAACGAAAGAACAAATGATTTCATTTTGTCAAAGTATTCAACACGC
                                                                               PvuII                ClaI
 +501 ATCCCCAATTAATGCACATTTTAGTCCAGAACCTAGTTATATGCCTGGTTACGAAGATGATGTTATTATGGCAGCTGGTACGTTTATTCAAGGTTCATCG
               PstI    AvaII
 +601 ATTGAATTATCTGCAGATGGACCTATTCGTCCTCCTTATGAAGCATATGTTCAAGGAGGATTAACATATGAACACGTTAAAATTGCTGTGACAAGAGCTG
                                   end       ------>                    <------
 +701 TTAATCAGTTGAAAGAACAAGGACTTATA[TAA]tagattaaatttcaccctaaatgataatgtgttttgggtgaaatttttgcggagatttatcagatta
        DraI                                                                                HindIII
 +801 tttttaaaaattgaaaaaatatttagctgtagattgttttatgtcgttttagaagtcgaaatcgcagttatatcaatgttttaagctttatgttagaaa
                                                                          S.D.      EcoRV
 +901 acctgacatattttttgaaatcctaaaaaaattatgataagttattaacaagttcaaaagtagaggagaggaaca[ATG]ATATCGAATGATGCAATCAGACG
                                                                                   glnR
                                              HindII
+1001 AAATATGGCTGTCTTCTCTATGAGTGTAGTAAGTAAGTTAACGGATTTAACGCCAAGGCAAATACGTTACTATGAAACACATGAACTCATCAAACCTGAA
                               ▲
                             Ω2005
+1101 AGAACAGAAGGTCAAAAACGTCTGTTCTCACTCAATGATTTGGAAAGATTACTAGAAATTAAATCATTATTAGAAAAAGGATTTAATATCAAAGGGATTA
+1201 AACAAATCATTTATGACTCACAAGAGCATTTAACAACAGATGAACAAGAGATAAGAAAAAAGATGATTGTAGATGCCACGCAAAAGCCTATTGGAGAAAC
                                               end     S.D.    DraI
+1301 TTTGCCAATAAATCGTGGTGATTTATCCCGATTTATTAAA[TAA]aatttggaggattttaaa[ATG]CCAAAACGTACTTTCACTAAAGACGACATTCGTAAA
                                                                      glnA                               ScaI
+1401 TTTGCAGAAGAGGAAAATGTAAGATATTTAAGATTACAATTCACTGATATTTTAGGAACAATTAAAAATGTTGAAGTGCCTGTAAGCCAATTAGAAAAAG
+1501 TACTTGATAACGAAATGATGTTTGACGGTTCTTCTATCGAAGGTTTCGTACGTATCGAAGAATCAGATATGTACTTACATCCAGATTTAGATACTTGGGT
                                                                                               BamHI
+1601 AATCTTCCCATGGACTGCTGGACAAGGTAAAGTTGCACGTTTAATTTGTGATGTATATAAAACAGATGGAACACCATTTGAAGGGGATCCTCGTGCAAAC
                                                                                 EcoRI
+1701 TTAAAACGTGTATTAAAAGAAATGGAAGATTTAGGCTTCACAGACTTTAACCTAGGGCCTGAACCAGAATTCTTCTTGTTTAAGTTGGATGAAAAAGGGG
+1801 AACCAACTTTAGAACTTAATGATGATGGTGGATATTTCGATTTAGCACCTACAGATTTAGGTGAAAACTGTCGTCGTGATATTGTTTTAGAATTAGAGGA
                                                      HindII          DraI
+1901 TATGGGCTTCGATATTGAAGCTAGTCACCATGAAGTTGCCCCTGGTCAACATGAAATTGACTTTAAATATGCAGATGCTGTTACAGCATGTGATAATATC
            DraI
+2001 CAAACATTTAAATTGGTTGTTAAAACAATCGCACGTAAACATAATTTACACGCAACATTTATGCCTAAACCATTATTCGGTGTGAATGGTAGCGGTATGC
                                      AvaIII
+2101 ACTTTAACGTTTCATTATTCAAAGGTAAAGAAAATGCATTCTTTGATCCAAATACTGAAATGGGCTTAACGGAAACTGCATATCAATTTACAGCAGGTGT
+2201 GCTTAAAAATGCACGCGGATTTACTGCTGTATGTAACCCGTTAGTAAACTCATATAAACGTTTAGTACCTGGTTATGAAGCACCATGTTATATTGCATGG
                                                                                              PvuII
+2301 AGTGGTAAAAACCGTTCACCATTAATCCGTGTACCATCTTCAAGAGGATTATCTACTCGTATCGAAGTACGTTCAGTAGATCCAGCTGCAAACCCATACA
                                                                                  HindII
+2401 TGGCGTTAGCTGCAATCTTAGAAGCTGGACTAGATGGTATTAAAAATAAATTAAAAGTTCCAGAACCAGTTAACCAAAATATTTACGAAATGAACCGTGA
                                                                                                HindIII
+2501 AGAACGTGAAGCAGTAGGCATTCAAGACTTACCTTCAACACTTTATACTGCATTAAAAGCAATGCGTGAAAATGAAGTTATTAAAAAAGCTT
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FIG. 2. Nucleotide sequence of the *femC* region. The sequence starts with the first *Sau*3A site of the cloned fragment in pBBB105 and stops with the last *Hin*dIII site of the cloned fragment in pBBB104 (2,592 nucleotides total) (Fig. 1). Noncoding regions are shown in lowercase letters. Initiation and stop codons are boxed. Putative ribosome binding sites (S.D.) are overlined with solid bars. Inverted repeats are overlined with arrows. The Tn*551* insertion site Ω2005 in *glnR* is indicated by the triangle between nucleotides 1024 and 1025. Restriction enzyme sites are indicated.

sequence of a protein produced by ORF306 in *Lactobacillus delbrueckii* subsp. *bulgaricus* (Fig. 3a). An 80-aa protein-encoding partially cloned ORF in *Bacillus cereus* was highly similar to ORF1 (Fig. 3a). Both of these homologous ORFs are found upstream of the *glnA* genes of their respective organisms (14, 21). An inverted repeat 11 bp in length was found immediately downstream of the ORF1 stop codon (Fig. 2). A similar structure was found following the stop codon of the partially cloned *B. cereus* ORF (21).

The Ω2005 insertion occurred in an ORF (between nucleotides 1024 and 1025) coding for a protein highly similar to the GlnR proteins of *B. subtilis* and *B. cereus* (Fig. 3b). Downstream of this ORF, separated by 18 nucleotides (Fig. 2), another ORF was discovered. In a 410-aa overlap, the encoded protein, at the amino acid level, is 77.7 and 76.0% identical to the GlnA proteins of *B. subtilis* and *B. cereus*, respectively. Because of these homologies, we termed the *S. aureus* ORFs *glnR* and *glnA*. Both ORFs are preceded by potential ribosome

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a)

L. d. b.      ORF306   1 QPHIVVIQRSRGYDTRQSYTVDQIKKMTAFVKKVSPESLVFVDNCYGEFS 100
                                      I I.I..:I:I.I:.. :I.I I: I:IIIIIIIII
S. aureus     ORF1     1              DQRPSIPLDEIEKVITRLKNVHPNILIFVDNCYGEFV  37

L. d. b.      ORF306 101 EKHEPTEYGVDFTAGSLIKNAGGGIAQTGGYIVGKEELVENAAIRLTAPG 150
                         I::II.I:I.I:.IIIIIII:III:I..IIII.I:.:I:I..:.IIIIII
S. aureus     ORF1    38 ERREPIECGADLIAGSLIKNPGGGLAKIGGYIAGRKDLIERCGYRLTAPG  87

L. d. b.      ORF306 151 IGKEEGATLTNMHEFYEGFFLAPHTTGEAIKGMIFSAALLEKMGCEVTPK 200
                         IIII.II.I..: I:I:IIIIIII..::.:II :I.. :IIII. :.III
S. aureus     ORF1    88 IGKEAGASLNALLEMYQGFFLAPHVVSQSLKGALFTSLFLEKMNMNTTPK 137

L. d. b.      ORF306 201 WHEPRTDLIQTIIFNVPEKMINFTKEVQKNSPIDSFVEPIPSDMPGYEDK 250
                         :.I.IIIIIII: I:..I.II.I...:I..III:. ..I II IIIIII.
S. aureus     ORF1   138 YYEKRTDLIQTVKFETKEQMISFCQSIQHASPINAHFSPEPSYMPGYEDD 187
                                                      IIIII.II.I :.IIIIIIII
B. cereus     ORF?     1                              ASPINSHFTPYANYMPGYEDD  21

L. d. b.      ORF306 251 VIMAAGNFVSGSTMEFSADGPIRPPYALYMQCGLTYAHDRIAVTNAVNHL 300
                         IIIIII.I: II.:I:IIIIIIIIII. I:I.IIII.I :IIII.III:I
S. aureus     ORF1   188 VIMAAGTFIQGSSIELSADGPIRPPYEAYVQGGLTYEHVKIAVTRAVNQL 237
                         IIIIIIIIIII.IIIIIIIIIIIIII IIIIIIIII.IIIII:..I::.I
B. cereus     ORF?    22 VIMAAGTFIQGASIELSADGPIRPPYVAYVQGGLTYSHVKIAICSAIDAL  72

L. d. b.      ORF306 301 FFKENG                                             306
                           II.I
S. aureus     ORF1   238 --KEQGLI                                           243
                            I.:I:
B. cereus     ORF?    73   IEKELLTIS                                         80

b)

B. subtilis   GlnR     1   MSDNIRRSMPLFPIGIVMQLTELSARQIRYYEENGLIFPARSEGNRRL  48
                            .I.III.I::I.:::I .II:I.:IIIIIII.::II I.I.II.:II
S. aureus     GlnR     1 MISNDAIRRNMAVFSMSVVSKLTDLTPRQIRYYETHELIKPERTEGQKRL  50
                             .. II. ::I.:::I .II:I.:IIIIIII.I:I:.I.II.I.:II
B. cereus     GlnR     1    MKEDRRSAPLFPIGIVMDLTQLSARQIRYYEEHNLVSPTRTKGNRRL  47

B. subtilis   GlnR    49 FSFHDVDKLLEIKHLIEQGVNMAGIKQILAKAEAEPEQKQNEKTKKPMKH  98
                         II::I:::IIIII I:I.I.I: IIIII: ..:                .
S. aureus     GlnR    51 FSLNDLERLLEIKSLLEKGFNIKGIKQIIYDSQ----------------E  84
                         II:II:::IIIII.II:.I:I: IIII::. .:                :
B. cereus     GlnR    48 FSFNDVDKLLEIKDLLDQGLNMAGIKQVLLMKE----------------N  81

B. subtilis   GlnR    99 D-----LSDDELRQLLKNELMQAGRFQRGNTFR--Q-----GDMSRFFH  135
                         .     I..II .:: I. :::I.. . I:I:.   .     II:III:
S. aureus     GlnR    85 H-----LTTDE-QEIRKKMIVDATQKPIGETLPINR-----GDLSRFI   121
                         :     :..:. .II.I. : .  ...:..I .:II     II:III:
B. cereus     GlnR    82 QTEAVKVKEET-KEISKTELRKILRDELQHTGRFNRTSLRQGDISRFFH  129
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FIG. 3. (a) Amino acid alignment of ORF1 from *S. aureus* with ORF306 from *L. delbrueckii* subsp. *bulgaricus* (*L. d. b.*) and the partially cloned ORF (ORF?) upstream of *glnR* in *B. cereus*. Percent identity and percent similarity between ORF1 of *S. aureus* and ORF306 of *L. delbrueckii* subsp. *bulgaricus* and between ORF1 of *S. aureus* and ORF? of *B. cereus* were 57.4 and 72.3% and 76.6 and 84.4%, respectively. (b) Amino acid alignment of *S. aureus* GlnR (FemC) with the GlnR proteins of *B. subtilis* and *B. cereus*. Percent identity and percent similarity between *S. aureus* GlnR and *B. subtilis* GlnR and between *S. aureus* GlnR and *B. cereus* GlnR were 46.1 and 70.0% and 42.3 and 66.9%, respectively. Amino acid identities are represented by I, and conservative amino acid substitutions are represented by dots and colons. Gaps formed during alignment are represented by broken lines.

binding sites (Fig. 2). As in *B. subtilis* GlnA and *B. cereus* GlnA (21), the N-terminal region of *S. aureus* GlnA has a 26-aa deletion, in comparison with GlnA of *E. coli.*

Transcription of *glnRA* and ORF1. A Northern (RNA) blot of various *femC* mutants and parent strains, probed with an internal 0.8-kb *Eco*RI *glnA* fragment (Fig. 1), is shown in Fig. 4. In BB255 (lane 1), BB270 (lane 3), and BB399 (lane 7), two bands of 1.9 and 1.7 kb were present. The respective Ω2005 mutants BB906 (lane 2), BB589 (lane 4), and BB913 (lane 8), as well as the resistant derivatives of BB589, BB895 (lane 5) and 905 (lane 6), all weakly expressed 7.0- and 7.2-kb transcripts. This result was presumably due to the insertion of 5.2-kb Tn*551* upstream of the region covered by this probe. The same results were obtained when this Northern blot was probed with a 0.3-kb *Ava*I-*Dra*I *glnR* fragment.

The faintly detected transcripts of low molecular weight in Ω2005 mutants could represent degradation products still retaining part or all of the *glnA* region or could have resulted from transcription initiating from within Tn*551*.

When the same Northern blot was probed with a 0.6-kb *Pst*I ORF1 fragment (Fig. 1), a 2.8-kb transcript at the same intensity was present in all strains (data not shown).

GS activity. Because of the transcriptional alterations seen in the Ω2005 mutants, we measured the GS levels in isogenic

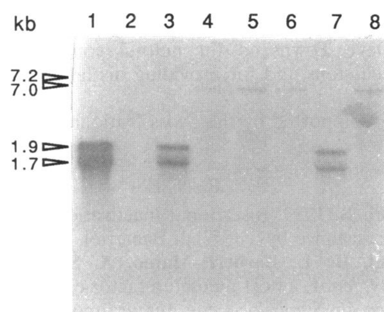

FIG. 4. Northern blot of total cell RNA probed with an internal 0.8-kb *Eco*RI *glnA* fragment from pBBB104 (Fig. 1a). Lanes: 1, BB255; 2, BB906; 3, BB270; 4, BB589; 5, BB895; 6, BB905; 7, BB399; 8, BB913. Ten micrograms of RNA was loaded in each lane, except for lane 2, which contained only 8 μg.

Ω2005 mutants and parent strains. The results of the GS assay are shown in Table 2. In the Ω2005 mutants, including BB895 and BB905, the level of GS was 57 to 65% lower than that in the parent strains.

In strain BB913, the Ω2005 mutant of BB399, the level of GS was also reduced, but the difference between these two strains was not as dramatic (10 to 20% lower level) as the differences between the strains shown in Table 2.

Growth on defined media with methicillin gradients. The isogenic Ω2005 mutant and parent strains were streaked onto plates of defined media containing gradients of methicillin, with and without the addition of glutamine (Fig. 5). BB255 (rows a) was more resistant than BB906 (rows b) without the addition of glutamine on the 0- to 2-mg/liter methicillin gradient plate. However, when the two strains were streaked onto a similar plate containing 0.6 mM glutamine, they both grew to the same resistance level. This was also true for another strain pair, BB270 (rows c) and BB589 (rows d), on both 0- to 2- and 0- to 120-mg/liter methicillin gradient plates.

With strains BB399 (rows e) and BB913 (rows f), no differences in resistance levels were observed on defined media with and without glutamine. This is because both strains grew in the presence of the highest methicillin concentration (120 mg/liter) under these conditions. However, when an E-test was performed with these two strains, BB913 had a reduced resistance level (4 mg/liter), in comparison with BB399 (24 mg/liter). Therefore, the lowered resistance level due to Ω2005

TABLE 2. GS levels in isogenic *femC* mutants and *S. aureus* parent strains

Strain	*femC*[a]	GS level (mean ± SD)[b]	% Activity[c]
BB255	+	2.20 ± 0.70	100
BB906	−	0.80 ± 0.23	36
BB270	+	2.35 ± 0.89	100
BB589	−	0.82 ± 0.19	35
BB895	−	1.00 ± 0.17	43
BB905	−	1.00 ± 0.09	43

[a] +, without Ω2005; −, with Ω2005.

[b] Determined from three separate experiments with cells grown in LB at 30°C to an OD_{580} of 1.0 and permeabilized with CTAB. GS was assayed by the Mn^{2+}-dependent reverse transferase assay as described in Materials and Methods. Units are expressed as micromoles per minute per milligram of protein.

[c] In comparison with that in the parent strain.

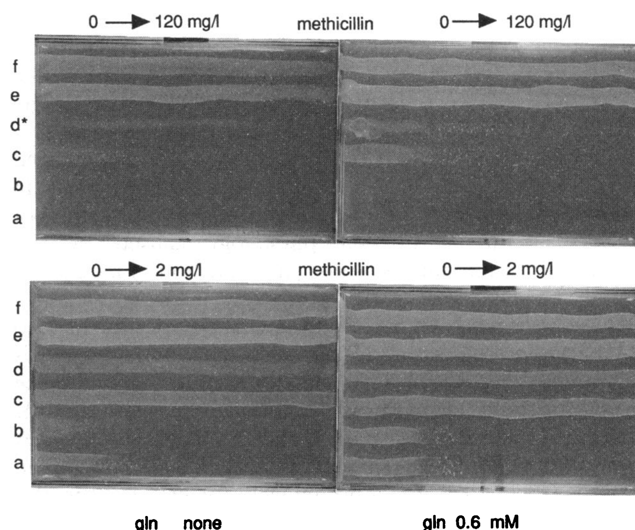

FIG. 5. Growth of different *S. aureus* strains and corresponding mutants derived by insertion of Ω2005(*femC*::Tn*551*) on defined medium plates with and without glutamine (gln) (0.6 mM final concentration) and containing a gradient of methicillin. A suspension of 5 × 10^7 cells of an overnight culture per ml was streaked with a cotton swab onto the plates. Pictures were taken after 48 h of growth at 37°C. Rows: a, BB255; b, BB906; c, BB270; d, BB589; e, BB399; f, BB913. *, what appears to be growth in row d without glutamine is actually an optical artifact caused by the cotton swab scratching the agar surface.

was still present in mutant BB913. The level of growth of BB270, BB399, and BB913 was higher on both methicillin gradient plates when they contained glutamine.

DISCUSSION

The Tn*551* insertion Ω2005 in *glnR* is the reason for the decrease in methicillin resistance levels in Ω2005 mutants of MRSA and methicillin-susceptible *S. aureus*. This insertion has a polar effect on *glnA*, decreasing its transcription. The resultant decrease in glutamine production is what causes the decrease in methicillin resistance, since the addition of glutamine restored the methicillin resistance levels in Ω2005 mutants on defined media. The transcript sizes of 1.7 and 1.9 kb that are both increased by 5.2 kb in Ω2005 mutants when probed with *glnR* or *glnA* suggest that *glnR* and *glnA* are transcribed together. Analogous transcriptional alterations were found in *femA*::Ω2003 mutants (2).

Our evidence suggests that a fully functional *glnRA* operon is needed to express the resistance levels observed in MRSA and methicillin-susceptible *S. aureus*. *femC*::Ω2005 mutants are not auxotrophic for glutamine (Fig. 5 and Table 2), indicating that even with *femC*::Ω2005, some GlnA is produced.

Strains BB895 and BB905 harboring Ω2005 grew at high concentrations of methicillin even though they still had altered *glnRA* transcription and reduced GS levels. This evidence suggests that another mutation(s) can circumvent Ω2005, enabling the expression of methicillin resistance despite low glutamine availability.

BB913 did not show as great a reduction in GS levels in comparison with its parent, BB399, as did BB589 and BB906 in comparison with their parents, BB270 and BB255, respectively. BB399 also behaved differently from other *S. aureus* strains in

autolysis studies (11). These results demonstrate that the genetic background and physiology of the studied strains differ.

In bacteria, glutamine biosynthesis occurs solely via the action of GS. The amido group of glutamine is used in the production of histidine, tryptophan, other amino acids, glucosamine 6-phosphate, *p*-aminobenzoate, NAD, purines, and pyrimidines. Glutamine is also used as a building block in protein synthesis (24, 27). Thus, any one of a number of possible alterations in cell physiology caused by the *femC*::Ω2005 mutation may cause the reduction of methicillin resistance levels in *S. aureus*.

It was previously reported that changes detrimental to peptidoglycan synthesis and structure occur when *S. aureus* is grown in the presence of β-lactams (for a review, see reference 18) and that mutations disturbing peptidoglycan synthesis in *S. aureus* also lower resistance to methicillin (6, 13, 19). MRSA strains possess PBP2a, which may be involved in peptidoglycan synthesis (4, 7). PBP2a is a prerequisite for high-level methicillin resistance. These findings indicate that genes necessary for the synthesis of cell wall peptidoglycan are important in determining resistance levels.

The muropeptide building block of *S. aureus* peptidoglycan contains isoglutamine residues (32). The amidation of glutamate residues in peptidoglycan involves the participation of glutamine as a donor of NH_4^+ (29). It has been suggested that in *Gaffkya homari*, these amidated residues are needed for efficient cross-linking (22). The growth of *S. aureus* in the presence of penicillin decreases the number of amidated glutamate residues (33) and the level of cross-linking (18) in peptidoglycan.

femC::Ω2005 could cause alterations in peptidoglycan synthesis and structure by decreasing the synthesis of glucosamine 6-phosphate and isoglutamine, thereby lowering the levels of cross-linking and methicillin resistance in *S. aureus*. In other words, the peptidoglycan in Ω2005 mutants grown without exposure to methicillin might show some of the same characteristics as those seen in wild-type cells grown in the presence of methicillin. This situation is already known to apply to the peptidoglycan of *femA*::Tn*551* and *femB*::Tn*551* mutants, which is hypocross-linked before exposure to methicillin (6, 13).

The number of Tn*551* insertion sites outside the *mec* determinant affecting methicillin resistance may be rather high (8). This evidence demonstrates the complexity of the inherent physiological and genetic requirements for an *S. aureus* cell growing in the presence of methicillin under laboratory conditions.

GS is "one of the oldest existing and functioning genes" (17). Besides the functions of glutamine mentioned above, its production is the focal point of nitrogen assimilation in bacteria (24, 27). We now show the partial genetic characterization of the *glnRA* operon of *S. aureus*. The *S. aureus glnRA* operon is arranged in a fashion similar to that in *B. subtilis* and *B. cereus* (21, 30) and is linked transductionally to the *S. aureus thrB* gene. This fact is of great interest, because these organisms are phylogenetically closely related (36). Given the strong similarities of the *S. aureus*, *B. subtilis*, and *B. cereus* GlnA proteins, it is likely that *S. aureus* GS is a type I GS (17, 27). Strong similarities among *S. aureus* (ORF1), *B. cereus* (ORF?), and *L. delbrueckii* subsp. *bulgaricus* (ORF306) in the ORF upstream of their respective *glnA* genes suggest that all three could encode proteins with similar functions.

Further studies will be needed to find the mechanistic link between methicillin resistance and *glnRA*.

ACKNOWLEDGMENTS

We thank Dave Townsend for helpful conversations and Hal Schreier for discussions and for providing his laboratory's glutamine synthetase assay.

This work was supported by the Swiss National Foundation (grant 32-33529.92).

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