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A Phosphoglucomutase-Like Gene Essential for the Optimal Expression of Methicillin Resistance in *Staphylococcus aureus*: Molecular Cloning and DNA Sequencing

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ABSTRACT

We describe here the cloning and sequencing of a new auxiliary gene identified by Tn*551* insertional mutagenesis of the highly and homogeneously methicillin-resistant *Staphylococcus aureus* strain COL. The insertionally inactivated mutant RUSA315 had intact *mecA* and normal amounts of PBP2A, but drastically reduced antibiotic resistance (drop in methicillin MIC from 1600 to 1.5 μg ml^{-1}), a unique heterogeneous phenotype, and a compositional change in the cell wall characterized by the complete disappearance of the unsubstituted disaccharide pentapeptide from the peptidoglycan. Cloning in *E. coli* followed by sequencing located the Tn*551* insert Ω720 in an open reading frame of 451 codons, provisionally called *femR*315, defining a polypeptide with a deduced amino acid sequence that showed over 26% sequence identity and 57% overall sequence similarity with the phosphoglucomutase (PGM) gene of *E. coli*. The Tn*551* insertion site of a previously described mutant 12F (*femD*) also lies in the same gene as *femR315*. The wild-type form of *femR315* subcloned in a shuttle vector fully restored expression of high level (parental) methicillin resistance in mutant RUSA315. The exact biochemical function of *femR*315 is not known. However, enzymes similar to PGM catalyze the isomerization of hexose and hexosamine phosphates leading to the formation of glucosamine-1-P, which is an obligate precursor in the biosynthesis of UDP-*N*-acetylglucosamine (UDP-NAGA). We propose that the suppression of methicillin resistance in RUSA315 is related to some functional or quantitative abnormality of UDP-NAGA metabolism.

INTRODUCTION

EXPRESSION OF HIGH LEVEL METHICILLIN resistance in *Staphylococcus aureus* requires both normal expression of a chromosomal gene[18,38] called *mecA* and a number of so-called auxiliary or fem genes (factor essential for the expression of methicillin resistance).[4,40] *MecA*[21,39] is harbored within a larger block of "foreign" (nonstaphylococcal) DNA[2]; it encodes for the 78-kDa penicillin-binding protein (PBP) 2A,[15,28,41] which has very low affinity for β-lactam antibiotics and which is believed to function as a surrogate peptidoglycan transpeptidase (PBP2A) to perform cell wall synthesis in the presence of high concentrations of antibiotics in the environment.[8,13] Of the six auxiliary genes (*femA*, *femB*, *femC*, *femD*, *femE*, and *femF*) described earlier,[3,10] at least three were shown to be native *Staphylococcus aureus* chromosomal determinants.[10,16,20] Only models exist for the explanation of how these auxiliary genes contribute to the expression of methicillin resistance: genetic and biochemical studies suggest that several auxiliary genes are involved with steps in the biosynthesis of peptidoglycan precursors.[10]

Recently, a new transposon library constructed in the background of the highly and homogeneously methicillin-resistant *Staphylococcus aureus* (MRSA) strain COL yielded 70 independent insertional mutants with reduced levels of antibiotic resistance, out of which only two were inserts in *mecA* while the rest were scattered over 7 of the 16 *Sma*I fragments of the COL chromosome.[11] Preliminary studies suggest that this library includes at least 10 to 12 new genetic determinants, each of which is needed for optimal expression of methicillin resistance.[11]

RUSA315 (Ω720) is one of the insertional mutants from the new library.[11] In this study, we describe cloning and sequencing the Tn*551* insertional region in RUSA315, the genetic re-

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lation between *femD* mutant RUSA12F and RUSA315, and the consequence of Ω720 insertional inactivation for the expression of methicillin resistance.

EXPERIMENTAL PROCEDURES

Bacterial strains, phage and plasmids

The bacterial strains, phage, and plasmids used in this study are described in Table 1.

Media and growth conditions

Staphylococcus aureus and MRSA mutants were grown as described before.[24] Luria–Bertani (LB) medium was used to propagate *Escherichia coli* DH5α, and ampicillin was added at the concentration of 100μg ml^{-1} for selection and maintenance of the plasmids listed in Table 1. *Escherichia coli* XL1-blue MRA and MRA(P2) were the host cells for Lambda DASH®II phage. They were cultured as recommended by the supplier (Stratagene Cloning Systems, La Jolla, CA).

Susceptibility testing and population analysis

One colony of bacterial strain was inoculated into 5 ml of tryptic soy broth (TSB, Difco Laboratories) and incubated at 37°C with 200 rpm rotation for overnight. Culture density (OD_{600}) was adjusted to give a viable titer of 10^8 to 10^9 cfu/ml. Sterile swabs were dipped into the bacterial suspension and applied to tryptic soy agar (TSA, Difco Laboratories) plates without antibiotics, and 6-mm-diameter disks containing antibiotics were applied to the plates with a disk dispenser (BBL, Beckton-Dickinson and Company). The antibiotics used were as follows: 1 mg of methicillin, 10 U of penicillin, 30 μg of cefazolin, 20 μg of ampicillin/sulbactam, 15μg of erythromycin, 10 μg of gentamicin, 30 μg of tetracycline, 30 μg chloramphenicol, 5 μg of rifampin, and 5μg of ciprofloxacin (BBL, Becton-Dickinson and Company). Diameters of inhibition zones were read after incubation at 37°C for 24 h. To perform population

TABLE 1. STRAINS, PHAGES, AND PLASMIDS USED IN THIS STUDY[a]

Strain/phage/plasmid	*Relevant characteristics*	*Origin or reference*
Strain		
Escherichia coli		
DH5α	recA endA1 gyrA96 thi-1 hsdR17 supE44relA1 ϕ80 dlac ZΔ M15	BRL
XL1-Blue MRA	Δ(MCRA) 183Δ(MCRCB-HSD SMR-mrr)173endA1 supE44 thi-1 gyrA96 relA1 lac	Stratagene
XL1-Blue MRA(P2)	XL1-Blue MRA (P2 lysogen)	Stratagene
Staphylococcus aureus		
RN4220	Restriction$^-$	Novick, R.
COL	Homogeneous Mc^r	RU collection
RUSA315	COL Ω720(*femR315*::Tn*551*) Em^r heterogeneous Mc^r	de Lencastre and Tomasz[11]
RUSA12F	COL Ω558(*femD*::Tn*551*) *Em^r* heterogeneous Mc^r	Kornblum *et al.*[17] Berger-Bächi *et al.*, 1992 de Lencastre *et al.*[10]
SWET3	Restriction$^-$ Amp^r Cm^r(RN4220/pGCSW-3)	This study
SWTD3	COL Ω720(*femR315*::Tn*551*) Em^r Mc^r Cm^r(RUSA315/pGCSW-3)	This study
SWTD5	COL Ω558(*femD*::Tn*551*) Em^r Mc^r Cm^r(RUSA12F/pGCSW-3)	This study
Bacteriophage		
Lambda DASH®II	λsbhλ1° b189 KH54 chiC srIλ4° nin5 shndIIIλ6° srlλ5° red$^+$ gam$^+$	Stratagene
λDII/R315	Lambda DASH®II/15.5 kb *Eco*RI fragment from RUSA315(*femR315*::Tn*551*)	This study
λDII/COL-R315	Lambda DASH®II/10.3 kb *Eco*RI fragment from COL(*femR315* wild-type allele)	This study
Plasmid		
pGEM-3Z	Subcloning vector Amp^r	Promega Corp.
pRT1	pGEM-1/4.0-kb *Xba*I–*Hpa*I fragment of Tn*551*	Matthews and Tomasz[22]
pSW-4	pGEM-3Z/5.0-kb *Pst*I fragment from λDII/COL-R315(*femR315* wild-type allele)	This study
pSW-4A	pGEM-3Z/2.2-kb *Pst*I–*Eco*RV fragment from pSW-4(*femR315* wild-type allele)	This study
pSW-8	pGEM-3Z/2.7-kb *Kpn*I–*Bam*HI fragment from λDII/R315(Tn*551*J_L::*femR315* flanking)	This study
pGC2	Shuttle vector Amp^r Cm^r	Matthews, Peter.
pGCSW-3	pGC2/2.2-kb *Pst*I–*Eco*RI fragment from pSW-4A(*femR315* wild-type allele)	This study

[a]Mc^r, methicillin resistance; Em^r, erythromycin resistance; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

analysis, aliquots of overnight cultures were spread onto TSA plates containing increasing concentrations of methicillin. CFU were determined after 48 h of incubation at 37°C.[9]

DNA methods

All routine DNA manipulations were essentially performed as in Sambrook *et al.*[35] and Ausubel *et al.*[1] Restriction enzymes, calf intestine alkaline phosphatase, and T4 DNA ligase were purchased from New England Biolabs, Inc. and used as recommended by the manufacturer. Southern analysis was performed with ECL™ random prime labeling and detection systems purchased from Amersham Life Science and according to the recommendation of the manufacturer.

Transformation of S. aureus by electroporation

S. aureus RN4220 (r^-) was used as the primary recipient for recombinant pGC2 shuttle plasmids. The cells were harvested in mid-exponential growth (OD 578 = 0.5–0.55), washed with one-half volume of electroporation buffer (2.5 mM sodium phosphate buffer, pH 7.4, 272 mM sucrose, 1 mM $MgCl_2$), and then resuspended in 1/300 volume of electroporation buffer. Forty microliters of the cell suspension was incubated with 0.1 μg plasmid DNA for 30 min at room temperature. A pulse of 25 μF, 2kV, and 200 Ω was delivered by the Gene Pulser (Bio-Rad). The cells were then transferred to 960 μl of LB and incubated at 37°C for 1 h before plating aliquots on selective plates containing 10 μg/ml chloramphenicol. The shuttle plasmids were subsequently transduced from the electrotransformant by phage 80α to the appropriate recipient strain, as described.[24]

DNA sequence analysis

Double-stranded DNA sequencing was accomplished by the dideoxy chain termination method[35] with templates of DNA fragments cloned in pGEM-3Z. The oligonucleotide primers were synthesized and purified by Genosys Biotechnologies, Inc. Sequenase 2.0 (United States Biochemicals) was employed for chain elongation, and [^{35}S]dATP-labeled samples were run in 8 M urea/6% polyacrylaminde gels. Nucleotide and derived amino acid sequences were analyzed with the Wisconsin Genetic Computer Group (GCG) software.

PCR

PCRs were carried out to detect the relation between the insertion site Ω558 in RUSA12F and Ω720 in RUSA315. Three oligonucleotide primers were ORF451N specific for the DNA sequence of FemR315 N-terminal (5′-GGAAAATATTTTG-GTACAG-3′), Tn*551*JROUT specific for a partial DNA sequence of Tn*551* right junction (5′-TATTATCTATTCC-TAAACAC-3′), and Tn*551*JLOUT specific for a partial DNA sequence of Tn*551* left junction (5′-GATGTCACCGT-CAAGTTA-3′). The chromosomal DNAs of RUSA315 and RUSA12F were prepared as templates. PCR amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) by using PCR reagent kit (Perkin-Elmer Cetus) according to the manufacturer. Thirty cycles were used for each reaction, with

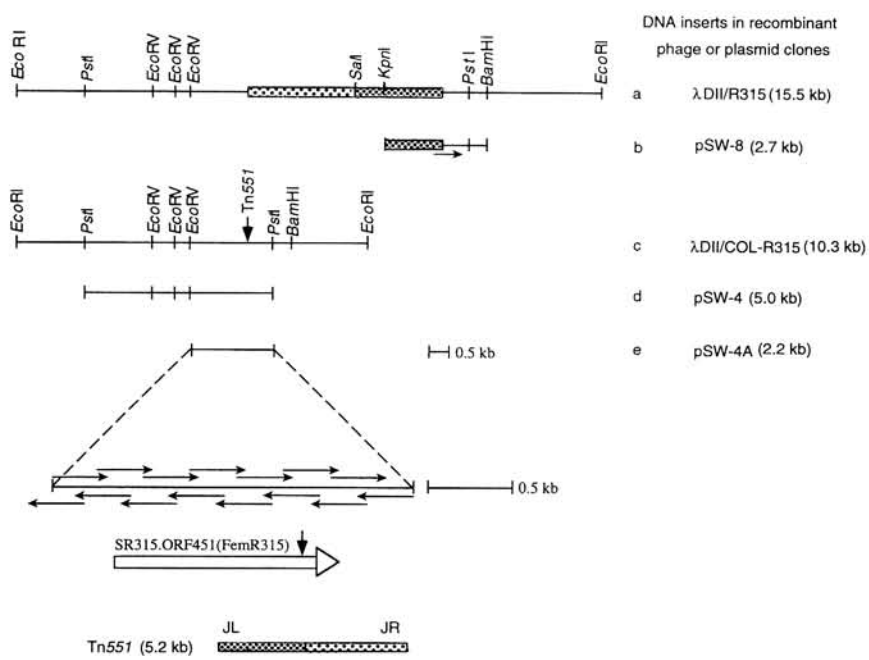

FIG. 1. Restriction map, genetic organization, and sequencing strategy of the *femR315* region. (a) Restriction map of 15.5-kb DNA insert in 1DII/R315, transposon Tn*551* is shown as box; (b) 2.7-kb DNA insert in pSW-8, box is the Tn*551* left junction and the arrow is the direction of sequencing; (c) Restriction map of 10.3-kb DNA insert in 1DII/COL-R315, Tn*551* insertion site of Ω720 is shown; (d) 5.0-kb DNA insert in pSW-4; (e) 2.2-kb DNA insert in pSW-4A, the small arrows indicate the sequencing strategy, and the open reading frame of *femR315* is shown as a box with a triangle to indicate the orientation of this ORF.

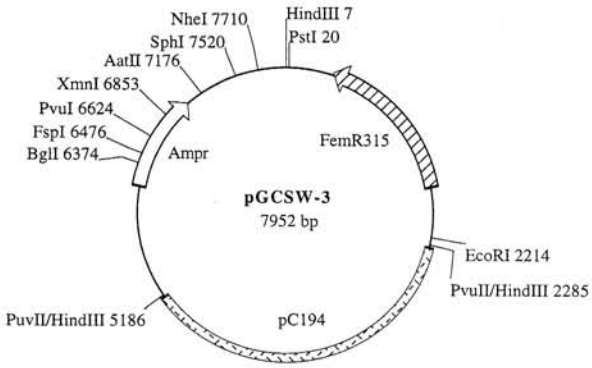

FIG. 2. Physical map of plasmid pGCSW-3. The shuttle plasmid pGC2 was constructed by inserting the plasmid pC194 into the *Pvu*II site of pGEM-1.[22] The 2.2-kb insert fragment of pSW-4A was subcloned into *Eco*RI/*Pst*I sites of pGC2.

the following temperature profiles: 94°C, 5 min; 30× [92°C, 1 min; 40°C, 1 min; 72°C, 1 min], 4°C, hold.

Procedures used for sequence and structure database searching to find the relation to PGM were described by Sali *et al.*[33,34]

RESULTS

Cloning of Ω720 (femR315:Tn551)

The insertion site Ω720 of transposon Tn*551*, which generated the insertional mutant RUSA315, is located on a 10.3-kb *Eco*RI DNA fragment of the COL chromosome.[11] The approximately 15.5-kb *Eco*RI fragment (which includes transposon Tn*551*) was isolated from strain RUSA315 and was ligated with the lambda DASH®II/*Eco*RI phage vector. An internal DNA fragment of Tn*551* was purified from plasmid pRT1 and labeled with ECL™ random prime labeling and detection system as probe to screen the *Eco*RI sublibrary of RUSA315 in the λ phage vector. The recombinant λ phage was named λDII/R315, and physical mapping of the DNA insert was obtained by digestion with various restriction endonucleases and Southern hybridization (Fig. 1a). The *Pst*I, *Eco*RV, and *Bam*HI recognition sites on the flanking sequence of Tn*551* were mapped with respect to the unique *Sal*I restriction site of Tn*551*. This physical mapping showed that the Tn*551* insertion site Ω720 in mutant RUSA315 is located in a 2.2-kb *Eco*RV–*Pst*I segment which was included in the 5.0-kb *Pst*I fragment, and that the end of Tn*551* left junction is about 1.2 kb from the *Bam*HI restriction site. The 2.7-kb *Kpn*I–*Bam*HI fragment, which includes 1.5 kb of the left junction of Tn*551* plus 1.2 kb of flanking sequence, was isolated from the DNA of λDII/R315 and ligated to *Kpn*I/*Bam*HI-digested pGEM-3Z to form the recombinant plasmid pSW-8 (Fig. 1b).

Cloning of the RUSA315 wild-type allele

An *Eco*RI sublibrary of the highly methicillin-resistant parental strain COL was constructed in λ phage by ligating the approximately 10-kb *Eco*RI DNA fragment of COL chromosome with λDASH®II/*Eco*RI phage vector. The recombinant λ phage carrying the RUSA315 wild-type allele was identified by screening this *Eco*RI library with the ECL-labeled 2.7-kb *Kpn*I–*Bam*HI fragment of pSW-8 and was named λDII/COL-R315. The 5.0-kb *Pst*I fragment of λDII/COL-R315 (Fig. 1c) was isolated and ligated with *Pst*I-digested pGEM-3Z to give a recombinant plasmid pSW-4 (Fig. 1d). Next, the plasmid pSW-4 was digested with *Eco*RV and *Sma*I, and then the 4.9-kb fragment of pSW-4 was purified and self-ligated by using the *Sma*I and *Eco*RV restriction sites of this fragment in order to generate plasmid pSW-4A (Fig. 1e). Plasmid pSW-4A contains the 2.2-kb insert of λDII/COL-R315 covering the Ω720 insertion site: thus the region of *femR315* wild-type allele was cloned. From pSW-4A, the 2.2-kb insert fragment extending from the *Pst*I site to the *Eco*RI site of the polylinker of pGEM-3Z was subcloned into shuttle vector pGC2 yielding recombinant shuttle plasmid pGCSW-3 (Fig. 2).

Complementation analysis with plasmid pGCSW-3

Plasmid pGCSW-3 was transformed into *S. aureus* RN4220 (r^-) by electroporation and the electrotransformant SWET3 was used as donor to introduce pGCSW-3 into recipients RUSA315 and RUSA12F. The transductants SWTD3 and SWTD5 showed restored resistance to methicillin, penicillin, cefazolin, and ampicillin/sulbactam (Fig. 3). Population analysis indicated that this transductant SWTD3 expressed the same level of methicillin resistance as the parental Mc^r strain COL (data not shown).

DNA sequence of the femR315 region

The 2187 basepair (bp) DNA insert depicted in Figure 4 was sequenced through both strands with the strategy of primer walking. The sequencing of pSW-4A DNA insert was initiated with vector-based primer pUC/M13 forward at the *Eco*RV end and pUC/M13 reverse at the *Pst*I end. Twelve more oligonucleotide primers were subsequently synthesized as new sequences were identified to generate enough overlapping region in the process of primer walking. The 2187-bp region was analyzed for open reading frames (ORFs). An ORF of 451 codons

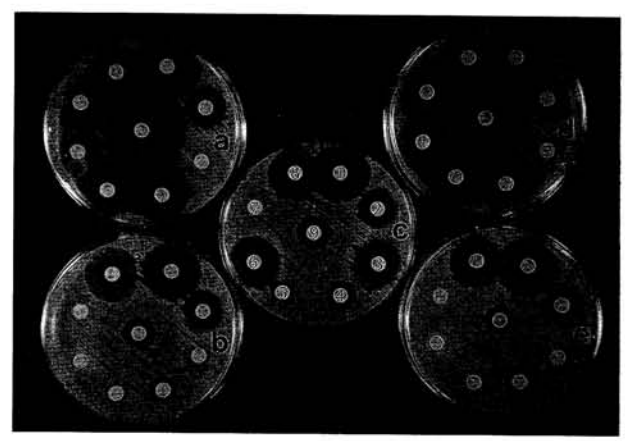

FIG. 3. Antimicrobial susceptibilty of various strains. The standard disk susceptibility procedure was used to test the antimicrobial susceptibility of RUSA315 (plate a), SWTD3 (plate b), COL (plate c), RUSA12F (plate d), and SWTD5 (plate e). Antibiotics: 1, rifampin; 2, gentamicin; 3, erythromycin; 4, ampicillin/sulbactam; 5, penicillin; 6, chloramphenicol; 7, cefazolin; 8, ciprofloxacin; 9, methicillin.

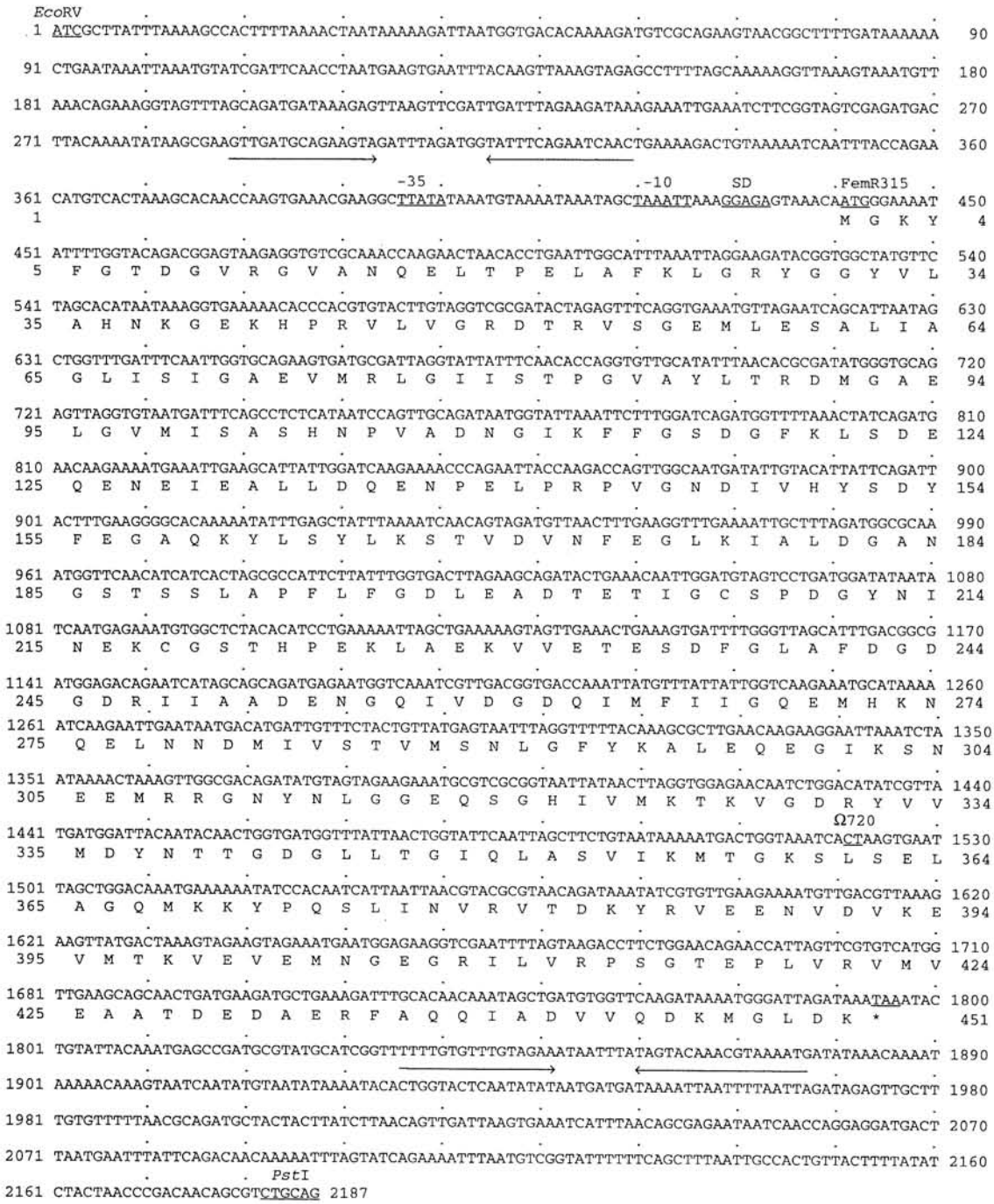

FIG. 4. Nucleotide sequence of the 2187-bp *Eco*RV–*Pst*I fragment containing the *femR315* coding region. Numbering starts at the 1/2 *Eco*RV site and ends at the *Pst*I site (position 2187). The putative start codon is located below the gene designation and the stop codon is designated with an asterisk. Putative Shine–Dalgarno (S.D.) sequence is underlined. The possible candidate for promotor sequences (−35 and −10 regions) is shown. Inverted repeat sequences are indicated by arrows. The insertion site of Tn*551* is shown. Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations.

was found encoded by the strand extending from the *Eco*RV to the *Pst*I site. This ORF, designated as FemR315, begins with the characteristic ATG initiation codon and ends with a TAA termination codon. The FemR315 is preceded by sites similar to the *Escherichia coli* consensus ribosome-binding sequence,[37] which is 7 nucleotides upstream of the FemR315 initiation codon. A putative promotor sequence,[31] TTATA for the −35 region and TAAATT for the −10 region, was found 3 nucleotides upstream of the Shine–Dalgarno sequence. In addition, two palindromic sequences were also identified. The first one is located 68 bp upstream from the putative −35 region of FemR315 and includes the sequence from 290 to 328 bp, which

TABLE 2. AMINO ACID SEQUENCE SIMILARITY OF FEMR315 WITH KNOWN PROTEINS[a]

Organism	*Protein*	*Number of aa*	*Similarity with FEMR315 (%)*
Mycobacterium leprae	UreD	462	65
Helicobacter pylori	UreC	444	61
Escherichia coli	PGM	455	57
Pseudomonas aeruginosa	PMM	462	53
Rabbit muscle	PGM	561	51

[a]aa, amino acid; Ure, urease; PGM, phosphoglucomutase; PMM, phosphomannomutase.

is able to form a structure with a stem of 14 bp and loop of 11 bp. The second palindromic sequence is a structure with a stem of 16 bp and loop of 8 bp located 42 nucleotides downstream from the termination codon of FemR315. No ORF of significant length was found on the reverse complement of the sequence.

To determine the Tn*551* insertion site Ω720 in mutant RUSA315, the DNA insert of pSW-8 was also partially sequenced initiated with an oligonucleotide primer based on the left junction of Tn*551* (Fig. 1b). The sequence generated includes about 50 bp of the Tn*551* left junction and 300 bp of flanking region. By matching the sequence of pSW-8 and that of pSW-4A, the insertion site of Ω720 was determined to be between 1521 and 1522 bp, which is 1077 bp from the initiation codon and 272 bp from the termination codon of FemR315.

Detection of the Tn551 insertion site Ω558 in mutant RUSA12F by PCR

A 1.2-kb amplification product was obtained when PCR was performed by pairing the primer ORF451N with the primer Tn*551*JROUT and using RUSA315 chromosomal DNA as template. When the RUSA 12F chromosomal DNA was employed as template, the primer pair of ORF451N and Tn*551*JROUT yielded no PCR product; however, a 1.15-kb PCR product was amplified by using primer ORF451N and Tn*551*JLOUT. The result indicated that the Tn*551* insertion site of Ω558 in RUSA 12F is located approximately 50 bp upstream from that of Ω720 in RUSA315, but the orientation of transposon Tn*551* in these two mutants is reversed. RUSA315 and mutant 12F[17] represent two different insertional mutants in the same gene formerly called *femD*.

Comparison of the amino acid sequence of FemR315 with known proteins

The deduced amino acid sequence (Fig. 4) of FemR315 was compared with sequences of known polypeptides in both Tblastn and Blastp databank (1994). Using the appropriate search programs,[33,34] the amino acid sequence of FemR315 showed significant homology with proteins of *Mycobacterium leprae* UreD, *Helicobacter pylori* UreC, *Escherichia coli* PGM, and *Pseudomonas aeruginosa* phosphomannomutase (PMM) and rabbit muscle PGM (Table 2).

A multiple amino acid sequence alignment of FemR315, *Mycobacterium leprae* UreD (MlUreD), *Helicobacter pylori* UreC(HpUreC), *Escherichia coli* PGM(EcoPGM), and *Pseudomonas aeruginosa* PMM (PaPMM) with rabbit muscle PGM (RmPGM) was prepared by Modeller.[33] It was then inspected to better understand the significance of the overall similarities among the amino acid sequences (Fig. 5). While enzymatic properties of *Escherichia coli* PGM and *Pseudomonas aeruginosa* PMM have been described,[25,32,42] RmPGM was chosen as the reference in the multiple amino acid sequence alignment analysis because the biological function has been characterized by kinetic studies[29,42] and because its three-dimensional structure has been determined by X-ray crystallography.[6] Using this analysis, we could identify sequence patterns believed to be critical for the enzymatic activity of RmPGM[6,27] within the amino acid sequences of FemR315, HpUreC, M1UreD, EcoPGM, PaPMM and RmPGM sequences (Fig. 5). Such patterns include the substrate binding and catalysis region (RmPGM positions 115–119, Fig. 5, box a); the metal-binding loop (positions 286–292, Fig. 5, box c), and other three active site flaps (positions 258–262, Fig. 5, box b; 374–377, Fig. 5, box d; 388–392, Fig. 5, box e).

A more detailed comparison of FemR315 and RmPGM in the key regions of amino acid sequences is presented in Figure 6. The amino acid sequence Thr-Ala-Ser-His-Asn (RmPGM positions 114–119, Fig. 6a) is known to be critical for PGM activity; the sequence is within the 21-amino acid-long active site region of PGM.[27] A similar amino acid sequence Ser-Ala-Ser-His-Asn was found in the FemR315 coding sequence (FemR315 positions 100–105, Fig. 6a). The region surrounding the Ser-Ala-Ser-His-Asn sequence in FemR315 was compared with the 21-amino acid-long active site region of RmPGM (Fig. 6a). Considerable degree of homology (62%) was observed between FemR315 and RmPGM in this region (which lies in the N-terminal portion of both proteins). Furthermore, of the matched amino acids comprising this 62% homology, 69% (9 out of 13) were exact matches (Fig. 6a). The sequences of the metal ion binding loop (-Asp^{287}-Gly^{288}-Asp^{289}-Gly^{290}-Asp^{291}-) and its two anchoring residues (Phe^{286} and Arg^{292}) in RmPGM were also identical to a segment in FemR315 (positions 241–247, Fig. 6c). Three other short polypeptide chains (representing flaps of the active-site cleft of rabbit muscle PGM [6]), which include His^{260}, Ser^{377}, and Lys^{388}, respectively, also exhibited close similarities between RmPGM and FemR315 (Fig. 6b, d, and e).

DISCUSSION

Identity of ORF femR315 and femD

The Tn*551* insert Ω720 was shown to reduce the methicillin MIC (μg/ml) of the parental strain from 1600 to 1.5 $\mu g\ ml^{-1}$, and also generated a heterogeneous phenotype. The insertion site of Ω720, together with at least five additional independent inserts (Ω721, Ω722, Ω723, Ω724, and Ω725), was located on the *Sma*I-I fragment, at a site that appears to be a hot spot for Tn*551* insertion, and was mapped to share the same restriction pattern with *Hin*dIII, *Eco*RI, *Eco*RV, and *Pst*I. The RUSA315 cluster maps in the same *Eco*RI, *Eco*RV, and *Pst*I fragments as mutant RUSA12F,[11] but has a different *Hin*dIII restriction pat-

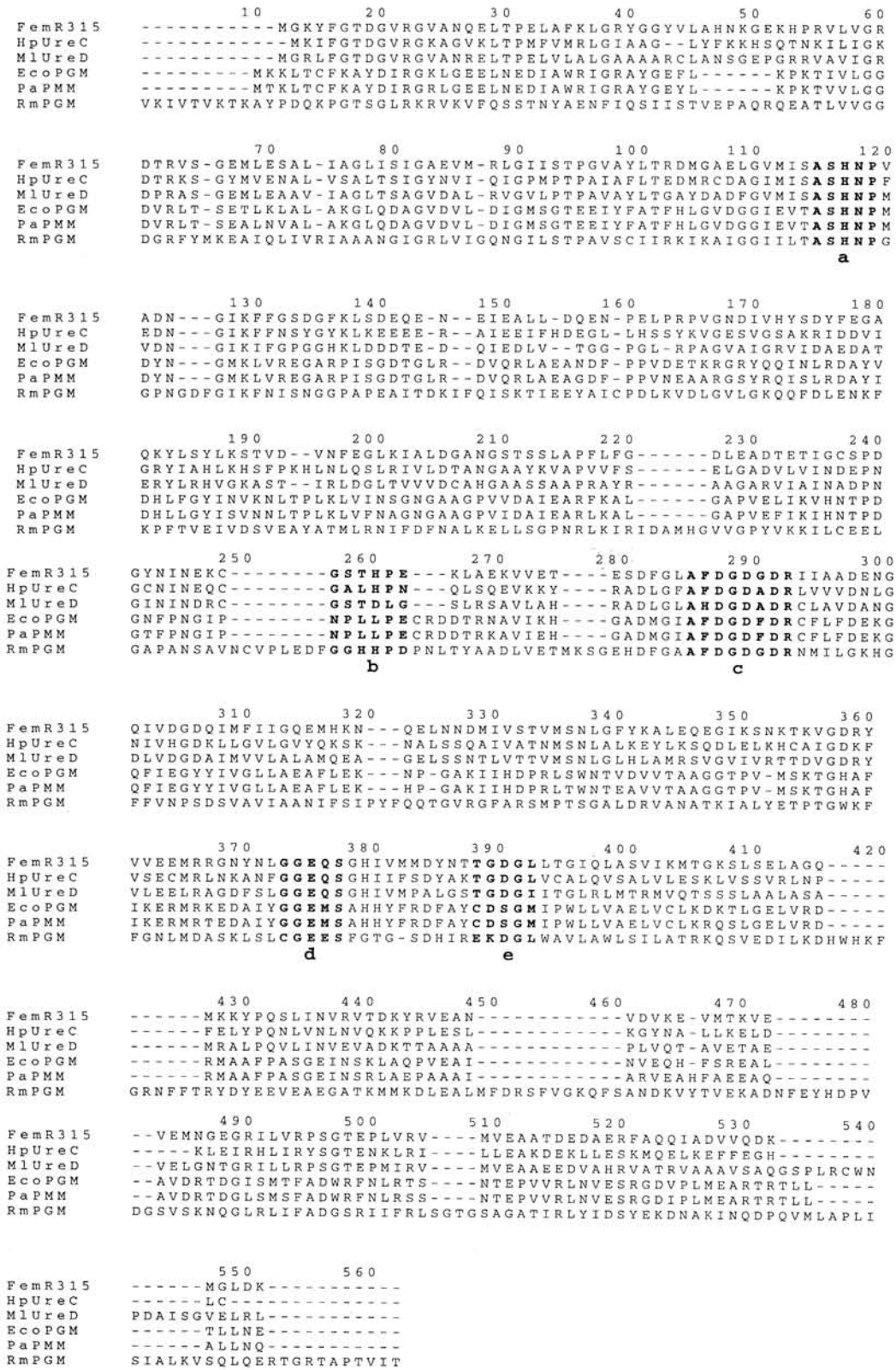

FIG. 5. Multiple amino acid sequence alignment of FemR315 with *Helicobacter pylori* UreC (HpUreC), *Mycobacterium leprae* UreD (MlUreD), *Escherichia coli* PGM (EcoPGM), and *Pseudomonas aeruginosa* PMM (PaPMM). The amino acid stretches in the boxes are known to be critical for PGM activity based on the study on rabbit muscle PGM. These stretches are identical or highly homologous among the sequences shown.

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FemR315      93 AELGVMISASHNPVADNGIKF 113
                |  |:::.|||||.:.||          a
RmPGM       107 AIGGIILTASHNPGGPNGDFG 127

FemR315     216 EKCGSTHPEK 225
                |. |: ||:.              b
RmPGM       254 EDFGGHHPDP 263

FemR315     236 DFGLAFDGDGDRII 249
                ||| ||||||| :          c
RmPGM       281 DFGAAFDGDGDRNM 294

FemR315     320 GNYNLGGEQSGHI 332
                :...|.||:|  .          d
RmPGM       368 SKLSLCGEESFGT 380

FemR315     339 TTGDGLLTGI 348
                 . |||:..:             e
RmPGM       386 REKDGLWAVL 395
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FIG. 6. Comparison of the FemR315 amino acid sequence with rabbit muscle PGM protein sequence in the key regions. (a) The 21-amino acid-long active site region including Ser^{116} stretch. (b) His^{260} flap. (c) Metal binding loop and anchoring residues. (d) Ser^{377} flap. (e) Lys^{388} flap. The bold letters are the amino acid stretches critical for PGM activity. The vertical bars indicate identical amino acids and colons and periods indicate two degrees of similarity of amino acids.

tern from the one of RUSA12F (unpublished observation). The compositional change of peptidoglycan in RUSA315 was the complete disappearance of the unsubstituted disaccharide pentapeptide monomer (Ornelas-Soares *et al.*, unpublished data), which was identical to that in RUSA12F.[7] To facilitate discussion, the putative genetic determinant inactivated by Tn*551* insertion in the *femD*-type mutant RUSA315 was provisionally named as *femR315*.

The insertion site of Ω720 was identified in the new auxiliary mutant *femR315* through cloning and sequencing as an in-frame insertion of Tn*551* that interrupts transcription of this ORF or causes abnormal transcription to yield a gene product with reduced activity. We assume that it is this in-frame mutation of *femR315* that is directly or indirectly responsible for generating the reduced and heterogeneous antibiotic resistance phenotype and the change in peptidoglycan composition of mutant RUSA315.

Introduction of the shuttle plasmid pGCSW-3, which contains the intact *femR315* into RUSA315 and RUSA12F, resulted in the full restoration of high-level resistance in both of these mutants, and these complementation experiments provided the evidence that the cloned pgm-like gene of *femR315* was indeed the genetic element responsible for the phenotype changes in both RUSA315 and RUSA12F. The insertion site of Ω558 was determined by PCR to be in the same ORF as that of Ω720, thereby confirming that the so-called *femD* is actually the identical gene characterized here as *femR315*.

The biochemical nature of femR315: Sequencing similarities to determinants of PGM-like enzymes

The biochemical functions of staphylococcal auxiliary genes that have been identified so far are not fully understood. While *femA* and *femB* are clearly involved with the structure of cross-links in the cell wall muropeptides, it is not yet clear whether these are structural genes for enzymes catalyzing the addition of amino acid residues to the chain of cross-linking oligopeptides or genes in some related regulatory function. Similar uncertainties exist with regard to *femC* and another auxiliary gene involved with the addition of the diamino acid residue.[11] The amino acid sequence similarity between the several regions that are important for enzymatic activity of rabbit muscle PGM, *Escherichia coli* PGM, *Pseudomonas aeruginosa* PMM, and the corresponding regions of FemR315 (Fig. 5) suggests that the gene product of *femR315* may perform in *S. aureus* a catalytic function similar to that of PGM or PMM. Both PGM and PMM can catalyze internal transfer of phosphate residues on a hexose molecule from one hydroxyl group to another.[29,42] PGM catalyzes the interconversion of glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P) in various organisms, and the G1P serves as a substrate for the UDP-glucose pyrophosphorylase-catalyzed condensation with UTP to form UDP-glucose, which is a common nucleotide sugar involved in synthesis of glycoproteins. In the peptidoglycan biosynthesis of *Staphylococcus aureus*, the first step in the formation of nucleotide-linked cell wall precursors is the reaction of *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) with UTP, catalyzed by UDP-*N*-acetylglucosamine pyrophosphorylase, to yield UDP-*N*-acetylglucosamine (UDP-GlcNAc), a reaction analogous to the reactions that lead to the production of UDP-glucose and other UDP-linked sugars. UDP-GlcNAc is not only the precursor of UDP-*N*-acetylmuramic acid, but is also the source of the GlcNAc residue as it is transferred to undecaprenyl-PP-*N*-acetyl-muramyl pentapeptide. If the gene product of *femR315* is assumed to have a PGM-like enzyme activity, it may catalyze the conversion of GlcNAc-6-P to GlcNAc-1-P, the latter being a key intermediate in cell wall biosynthesis since it is the substrate of the UDP-*N*-acetylglucosamine pyrophosphorylase catalyzed reaction yielding UDP NAGA.[14] While a specific bacterial isomerase of this type has not yet been described, PGMs are known to be capable of catalyzing interconversion of 1- and 6-phosphate isomers of many α-D-hexoses as well as those of GlcNAc.[26] A partial defect in a PGM-like enzyme may affect the methicillin-resistant phenotype and cell wall composition in the following manner. The absence of the unsubstituted disaccharide pentapeptide monomer from the peptidoglycan of mutant RUSA315 may be the consequence of a slow-down in the production of the muramyl pentapeptide due to the partial block of GlcNAc-1-P synthesis, which, in turn, would limit availability of UDP NAGA for peptidoglycan synthesis. It has been proposed that *in vivo*, bactoprenyl-linked cell wall precursors may compete with the methicillin molecule for some site on PBP2A and an abnormality of chemical structure in the wall precursors (as in the case of *femA*, *femB*, and *femC* mutants) tilts the balance of this competition in favor of the antibiotic resulting in reduced MIC value.[11] In the case of RUSA315, a quantitative reduction in the pool size of the wall precursors may favor interaction of PBP2A with the antibiotic, causing decrease in the MIC value.

The femR315 gene product

At this time it is not clear whether the gene product of *femR315* is PGM or an enzyme specific to the isomerization of

GlcNAc. The GlcNAc phosphates are much less reactive with PGM than glucose phosphates.[26] However, if the gene product of *femR315* were the PGM of *Staphylococcus aureus*, then one may expect that the insertional mutant RUSA315 would exhibit pleiotrophic properties related to the perturbation of any one or several of the metabolic events catalyzed by this enzyme in the cell.

Alternatively, *femR315* may be the structural gene of an enzyme the catalytic activity of which would be specific for isomerization of GlcNAc phosphates. A complete inhibition of this enzyme in RUSA315 may then necessitate the takeover of the function of this enzyme by phosphoglucomutase, an enzyme with relatively poor efficacy in the catalysis of the isomerization of hexosamine phosphates. The resulting slow-down of this enzymatic step may then lead to the defective phenotype of RUSA315. One has to emphasize that these proposals are based on DNA sequence comparison. An unambiguous assignment of function to the *femR315* gene will require isolation of its gene product and identification of its enzymatic function(s).

Interpretation of sequence similarities to UreD and UreC

The amino acid sequence of FemR315 also exhibited a high degree of similarity with UreD of *Mycobacterium leprae* and UreC of *Helicobacter pylori* (Table 2). *Mycobacterium leprae* UreD is one of the ORFs in a hypothetical urease operon on the chromosome of *Mycobacterium leprae*.[30] The function of the gene product encoded by this operon has not been investigated. *Helicobacter pylori* UreC is one of the accessory factors in urease gene cluster, and was supposed to play a regulatory role for the expression of urease because deletion of both UreC and UreD regions resulted in the increase of urease activity.[5, 19] However, the effect of *Helicobacter pylori* UreC gene itself on urease activity has not been investigated. Urease, which catalyzes the hydrolysis of urea to ammonia and carbon dioxide, is found in many species of plants, bacteria and fungi.[23] Many organisms produce urease to generate ammonia as a source of nitrogen.[12] So far, the effect of urease activity on antibiotic resistance has not been reported, and we could not associate the elevated urease activity to the decreased methicillin resistance. On the other hand, if *femR315* was considered to be a UreC-like gene, an urease operon or gene cluster should be found in the vicinity of the *femR315* region because mutiple genes would be necessary for expression of urease; no such cluster could be detected within a region of about 1 kb upstream and 4 kb downstream of the *femR315* (data not shown). Furthermore, our multiple sequence alignment analysis showed both *Mycobacterium leprae* UreD and *Helicobacter pylori* UreC share the similar critical amino acid stretches for PGM or PMM activity with rabbit muscle PGM, *Escherichia coli* PGM, as well as *Pseudomonas aeruginosa* PMM. The function of *Mycobacterium leprae* UreD and *Helicobacter pylori* UreC should be reconsidered.

Cloning and sequencing of the *femR315* region made it possible to discuss the genetic events at a molecular level and the biochemical consequence in peptidoglycan synthesis caused by the inactivation of the *femR315* allele. Expression of the *femR315* gene and studies on the enzymatic function of the *femR315* gene product are in progress to test our assumptions and further understand the role of the *femR315* gene in high-level methicillin resistance in *Staphylococcus aureus*.

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