

Role of Penicillin-Binding Protein 2 (PBP2) in the Antibiotic Susceptibility and Cell Wall Cross-Linking of *Staphylococcus aureus*: Evidence for the Cooperative Functioning of PBP2, PBP4, and PBP2A

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Ceftizoxime, a beta-lactam antibiotic with high selective affinity for penicillin-binding protein 2 (PBP2) of *Staphylococcus aureus*, was used to select a spontaneous resistant mutant of *S. aureus* strain 27s. The stable resistant mutant ZOX3 had an increased ceftizoxime MIC and a decreased affinity of its PBP2 for ceftizoxime and produced peptidoglycan in which the proportion of highly cross-linked muropeptides was reduced. The *pbpB* gene of ZOX3 carried a single C-to-T nucleotide substitution at nucleotide 1373, causing replacement of a proline with a leucine at amino acid residue 458 of the transpeptidase domain of the protein, close to the SFN conserved motif. Experimental proof that this point mutation was responsible for the drug-resistant phenotype, and also for the decreased PBP2 affinity and reduced cell wall cross-linking, was provided by allelic replacement experiments and site-directed mutagenesis. Disruption of *pbpD*, the structural gene of PBP4, in either the parental strain or the mutant caused a large decrease in the highly cross-linked muropeptide components of the cell wall and in the mutant caused a massive accumulation of muropeptide monomers as well. Disruption of *pbpD* also caused increased sensitivity to ceftizoxime in both the parental cells and the ZOX3 mutant, while introduction of the plasmid-borne *mecA* gene, the genetic determinant of the beta-lactam resistance protein PBP2A, had the opposite effects. The findings provide evidence for the cooperative functioning of two native *S. aureus* transpeptidases (PBP2 and PBP4) and an acquired transpeptidase (PBP2A) in staphylococcal cell wall biosynthesis and susceptibility to antimicrobial agents.

Although several observations have documented the importance of penicillin-binding protein 2 (PBP2) in the physiology of *Staphylococcus aureus*, the precise nature of its role(s) in cell wall synthesis and drug resistance is not well understood. PBP2 is the only bifunctional penicillin-binding protein in *S. aureus* (3, 8), and the transpeptidase (TPase) domain of the protein was found to be essential for the growth and survival of the bacteria (14, 17). In methicillin-resistant *S. aureus* (MRSA) strains the essential function of PBP2 may be replaced by PBP2A, the protein product of the resistance gene *mecA*, which functions as a surrogate transpeptidase (17). Thus, MRSA strains can grow without PBP2. Nevertheless, this protein becomes essential again once MRSA strains are challenged to grow in the presence of beta-lactam antibiotics. In this case, it is the transglycosylase (TGase) activity of PBP2 that appears to become critical for growth and cell wall biosynthesis (14). PBP2 also plays an important role in the vancomycin-intermediate *S. aureus*-type glycopeptide resistance (21), and PBP2 is essential for the expression of high-level vancomycin resistance from the *vanA* gene complex in vancomycin-resistant *S. aureus* (19). Direct biochemical evidence for the TGase activity of PBP2 is also available: *S. aureus* in which

the active site of the transglycosylase domain of PBP2 was inactivated by a point mutation produced glycan strands in which the chain length was reduced (14). However, evidence for the TPase activity of PBP2 is based primarily on sequence homology with established transpeptidases.

In the study described here, we used a beta-lactam antibiotic, ceftizoxime, with highly selective inhibitory activity against the TPase domain of PBP2 and a spontaneous ceftizoxime-resistant mutant as experimental tools in combination with genetic, physiological, and biochemical approaches for the dissection of the role of PBP2 in staphylococcal cell wall synthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. *S. aureus* was grown in tryptic soy broth (Difco, Detroit, Mich.) at 37°C with aeration. Growth was monitored by measuring optical density (at 620 nm) with an LKB spectrophotometer (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). Antibiotic selection was applied when it was necessary for maintenance of plasmids or chromosomal inserts. The concentrations of antibiotics used as selective agents were as follows: erythromycin (Sigma, St. Louis, Mo.), 10 µg/ml; chloramphenicol (Sigma), 20 µg/ml. All of the *S. aureus* strains that contained derivatives of plasmid pSPT181 (pSTSW-2C, pMGP19, and pMC1) were cultured only at 30°C to prevent the loss of the plasmid, which relied on a temperature-sensitive origin of replication for its maintenance in the cell (24). *Escherichia coli* strains were propagated in Luria-Bertani medium (Difco) with addition of ampicillin (Sigma) at 100 µg/ml when appropriate.

Population analysis profiles (PAPs). Antibiotic susceptibilities of cultures were determined by population analysis, as described before (22). Briefly, the bacterial cells were grown overnight at 37°C, and then four different dilutions of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>S. aureus</i>		
RN4220	Nitrosoguanidine-induced mutant of RN450 (NCTC 8325-4) that is able to accept <i>E. coli</i> DNA	10
27s	Antibiotic-sensitive derivative of RN450	R. Novick
27s- Δ <i>pbpD</i>	27s with <i>pbpD</i> insertionally inactivated; Ery ^r	This study
27s- <i>mecA</i>		This study
27s- Δ <i>pbpD-mecA</i>		This study
RN4220pPBP4-J	RN4220 with insertionally inactivated <i>pbpD</i> ; Met ^r Ery ^r	M. G. Pinho
ZOX3	27s derivative with ceftizoxime MIC of 6 μ g/ml	This study
ZOX3- Δ <i>pbpD</i>	ZOX3 with <i>pbpD</i> insertionally inactivated; Ery ^r	This study
ZOX3- <i>mecA</i>		This study
ZOX3- Δ <i>pbpD-mecA</i>		This study
ZOX12	27s derivative with ceftizoxime MIC of 25 μ g/ml	This study
RN4220-pIB01-3	RN4220 with plasmid pIB01 integrated into chromosome	I. Boncca
ZOX3- <i>pbpB</i> _{27s}	ZOX3 with plasmid pIB01 integrated into chromosome	This study
RN-ZOX3-13.9	RN4220 with plasmid pZOX3-13 integrated into chromosome	This study
27s- <i>pbpB</i> _{ZOX3}	27s with plasmid pZOX3-13 integrated into chromosome	This study
RN-pMC1-1	RN4220 containing pMC1	This study
COLpPBP2iC-2	COL with <i>spac</i> controlled <i>pbpB</i> in the chromosome	17
27s- <i>pbpB</i> _{spac}	27s derivative into which <i>spac</i> -controlled <i>pbpB</i> was transduced from COLpPBP2iC-2	This study
27s- <i>pbpB</i> _{spac} (pMC1)	27s- <i>pbpB</i> _{spac} containing plasmid pMC1	This study
27s- <i>pbpB</i> _{spac} (pMGP19)	27s- <i>pbpB</i> _{spac} containing plasmid pMGP19	This study
<i>E. coli</i>		
DH 5 α ^a	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>phoA supE44 thi-1 gyrA96 relA1</i> λ	Invitrogen
XL1 Blue ^a	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Δ M15 Tn10 (Tet ^r)]	Stratagene
Plasmids		
pSP64E	pSP64 with <i>erm</i> marker from Tn551	15
pMGP19	Plasmid pSPT181 with <i>cat</i> gene and 3.2-kb insert comprising <i>prfA</i> and <i>pbpB</i> ORFs	16
pSTSW-2C	Plasmid pSPT181C with 3,737-bp PCR product of <i>S. aureus mecA</i> region	24
pZOX3-13	pSP64E with <i>pbpB</i> fragment cloned in the <i>AvaI</i> site	This study
pMC1	pMGP19 with C \rightarrow T point mutation at position 1337 of <i>pbpB</i> ORF	This study

^a Genotypes as published by manufacturer.

the bacterial culture (10^0 , 10^{-2} , 10^{-4} , and 10^{-6}) were plated on control plates without antibiotic and on plates which contained a series of twofold dilutions of the appropriate antibiotic. Occasionally, when it was necessary for maintenance of plasmids or chromosomal inserts, a selective agent (erythromycin or chloramphenicol) was incorporated into the medium at a constant concentration. The plates were incubated at 37°C for 48 h, and colonies were counted. Methicillin and ceftizoxime powders were purchased from U.S.P.C. Inc. (Rockville, Md.)

Peptidoglycan purification and analysis by HPLC. Peptidoglycan was purified from *S. aureus* cells by methods described previously (2). One-liter cultures of bacteria were incubated at 37°C, collected by centrifugation, and extracted with hot sodium dodecyl sulfate. The crude cell walls were washed with distilled water, mechanically ruptured with glass beads, and purified by stepwise treatments with DNase, RNase, protease, 8 M LiCl, and acetone and, finally, washing with distilled water. The lyophilized pellet of purified cell walls was next extracted with 49% hydrofluoric acid at 4°C for 48 h to remove wall teichoic acid. The insoluble peptidoglycan was washed extensively with distilled water, followed by enzymatic hydrolysis to generate mucopeptides which were separated by reversed-phase high-pressure liquid chromatography (HPLC) as described previously (2), except that the step involving treatment with phosphatase was omitted.

Membrane purification and PBP affinity assays. Membranes were prepared from cells grown to the late exponential stage as described previously (20). Proteins (80 μ g per sample) for affinity assays were incubated with increasing concentrations of ceftizoxime (ranging from 0.1 to 20 μ g/ml) at 30°C for 10 min and then labeled with 10 μ g of benzyl-[¹⁴C]penicillin potassium salt (155 μ Ci per mg) (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) per ml for 10 min at 30°C. The reaction was stopped by the addition of an excess of nonlabeled benzylpenicillin. Labeled PBPs were resolved by the technique of Laemmli (7) and visualized by fluorography, as described before.

DNA techniques. The plasmid constructs used in this study are listed in Table 1. DNA manipulations were performed according to standard procedures (18). The restriction enzymes were purchased from New England Biolabs (Beverly, Mass.). Digestions were conducted as recommended by the manufacturer. DNA ligations were done with a Rapid DNA Ligation kit (Roche Diagnostic, Indianapolis, Ind.) according to the manufacturer's instructions. DNA sequencing was performed in The Rockefeller University DNA Sequencing Resource Center by the BigDye terminator cycle sequencing method. Reaction mixtures were thermocycled in an ABI GeneAmp PCR system 9600/9700. Purified extension products were electrophoresed on either an ABI Prism 3700 DNA analyzer or a SpectraMedix 9610 Aurora DNA sequencer. PCRs were done with a DNA thermal cycler 480 (Perkin-Elmer) by standard methodology with either AmpliTaq polymerase (Applied Biosystems, Foster City, Calif.) for routine amplifications or PfuTurbo DNA polymerase (Stratagene, La Jolla, Calif.) for cloning when high-fidelity amplifications were necessary. Electroporation of *S. aureus* recipient strain RN4220 was done as described previously (6). Transduction with phage 80 α was described previously (12).

Allelic replacement of *pbpB*. The allelic replacement of the wild-type *pbpB* gene with the ZOX3 mutant version of the gene was achieved by use of an insertion-duplication strategy. A fragment of the *pbpB* gene not including the promoter and membrane anchor was PCR amplified by using the primers F9-AvaI (5'-GGTTCCTCCGGGGTGCCTCAACATTAACAC-3') and R9-AvaI (5'-TATCCCGGGTTCGTAGGTGGATTCTT-3'). The PCR product was cloned into *AvaI* site of plasmid pSP64E in such a way that the orientations of the *pbpB* and plasmid *ermB* open reading frames (ORFs) were the same. The integration plasmid pZOX3-13 was used to transform RN4220 by electroporation. Transformants were selected on erythromycin. Integration of pZOX3-13 into the chromosome created a structure in the chromosome in which the vector se-

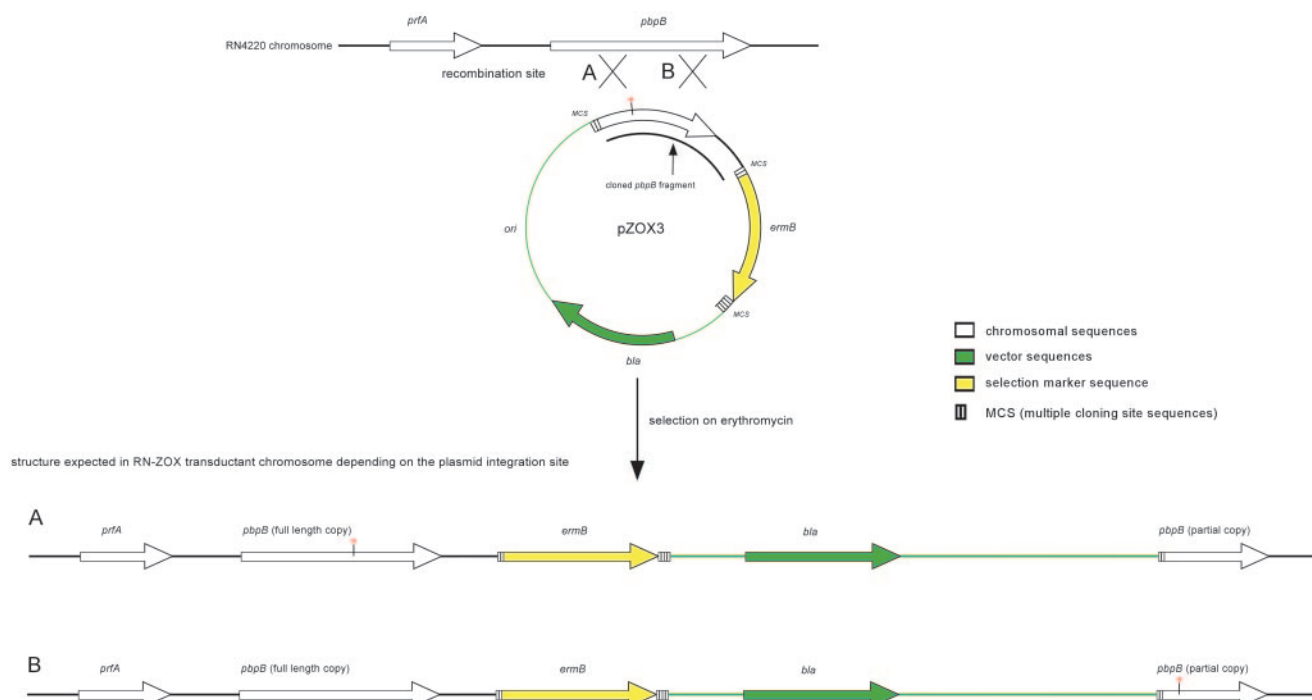


FIG. 1. Strategy for the allelic replacement of *pbpB*. A fragment of *pbpB* from the ZOX3 mutant, not including the promoter and membrane anchor, was cloned into plasmid pSP64E to generate plasmid pZOX3, which was electroporated into the standard recipient strain RN4220, and transformants were selected on erythromycin (10 μ g/ml). The plasmid integrated into the chromosome of RN4220, creating a structure in which vector sequences were flanked by a 5' full-length functional copy of *pbpB* and a 3' partial nonfunctional copy of this gene. There were two types of transformants expected, depending on the integration sites: strains with the mutation in the functional copy of *pbpB* (A) and strains with the mutation in the nonfunctional copy of the gene (B). One of the type A strains was used for transduction into the 27s background.

quences were flanked by two copies of *pbpB* sequence: a full-length, functional 5' copy which included all of the transcription signals and a partial, nonfunctional 3' copy (Fig. 1). The existence of this structure in the transformants was confirmed by PCR. Depending on the site of the integration, one or the other copy of *pbpB* contained the characteristic point mutation. The sequences of *pbpB* of transformants were analyzed by sequencing, and one of the transformants, named RN-ZOX3-13.9, which contained the ZOX3 mutation in the functional *pbpB* copy, was selected. The structure obtained was then transduced into strain 27s. Transductants were selected on erythromycin. The *pbpB* copies of the transductants were analyzed by sequencing. One of the transductants, named 27s-*pbpB*_{ZOX3}, which contained the ZOX3 mutation in the functional *pbpB* copy, was selected for further characterization.

Allelic replacement of the mutated *pbpB* copy in strain ZOX3 with the wild-type sequence of strain 27s was performed in similar manner. In these experiments the source of the wild-type *pbpB* gene was strain RN4220pIB01-3. This strain was constructed by the integration of plasmid pIB01 into the RN4220 chromosome. Plasmid pIB01 is a derivative of pJF751; it carries a fragment of the *pbpB* gene without the promoter and membrane anchor from strain 27s. Sequences of both *pbpB* copies in this strain are wild type (unpublished data). Lysates of RN4220pIB01-3 were used to transduce the ZOX3 mutant. Transductants were selected on chloramphenicol. One of them, named ZOX3-*pbpB*_{27s}, in which the functional copy of *pbpB* was confirmed to have the wild-type sequence by sequencing, was selected for further characterization.

Site directed mutagenesis of plasmid-borne *pbpB* and introduction of the mutated gene in the background of *spac*-controlled chromosomal *pbpB*. In order to obtain plasmid pMC1 carrying a mutated version of the *pbpB* gene whose sequence is identical to the one present on the chromosome of the ZOX3 strain, we mutagenized plasmid pMGP19 (Table 1). pMGP19 contains the wild-type *pbpB* ORF together with sequences controlling its expression. The single nucleotide substitution of C to T at position 1373 of the *pbpB* open reading frame was introduced into pMGP19 by use of the QuikChange site-directed mutagenesis kit (Stratagene) according to manufacturer's instructions, using the primers ZOX3-MutF (5'-CGACAAAGTTTCAATATCCTAGCTTTAAAGCTTGGCAATCAG-3') and ZOX3-MutR (having the complementary sequence). The resulting plasmid, pMC1, was subsequently introduced into RN4220 by electroporation.

Transformants were selected on chloramphenicol, and one of them was named RN-pMC1-1. The chromosomally located *spac*-controlled *pbpB* gene from strain COLpPBP2iC-2 was transferred into the 27s background by transduction followed by selection on erythromycin. The resulting strain was named 27s-*pbpB*_{spac}. Next, plasmid pMC1 was introduced into 27s-*pbpB*_{spac} by transduction with strain RN-pMC1-1 as a donor and selection of transductants on plates containing both chloramphenicol and erythromycin. The resulting strain was named 27s-*pbpB*_{spac}(pMC1).

In order to obtain a control strain carrying a plasmid with the wild-type *pbpB* sequence, the pMGP19 plasmid was introduced into strain 27s-*pbpB*_{spac} by electroporation and transformants were selected on plates containing both chloramphenicol and erythromycin. The resulting strain was named 27s-*pbpB*_{spac}(pMGP19). Strains were grown in medium containing 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) (17).

Inactivation of *pbpD*. An internal 0.67-kb DNA fragment of *pbpD* was amplified from the chromosome of strain COL by high-fidelity PCR with primers PBP4P22A (5'-TATCCCGGTATGACACCATATGCACAAG-3') and PBP4P23B (5'-GCAGGATCCGTGTAATACGTAACCTGCTAG-3'). After digestion with *Ava*I and *Bam*HI, the fragment was cloned into plasmid pSP64E and was electroporated into strain RN4220 (13). The inactivated *pbpD* was transduced into the 27s and ZOX3 backgrounds to generate strains 27s- Δ *pbpD* and ZOX3- Δ *pbpD*, respectively. Erythromycin was used as a selective agent in these transductions.

Introduction of plasmid-borne *mecA* into strains 27s and ZOX3. Plasmid pSTSW-2C (Table 1), carrying a copy of *mecA* gene including its transcription signals, was transduced into strains 27s, 27s- Δ *pbpD*, ZOX3, and ZOX3- Δ *pbpD*. Transductants were selected on plates containing chloramphenicol or chloramphenicol and erythromycin (in the case of strains carrying the *pbpD* deletion).

RESULTS

Selection of mutant ZOX3. The relevant properties of strains and plasmids used in this study are shown in Table 1. Mutant ZOX3 was selected by plating an overnight culture of

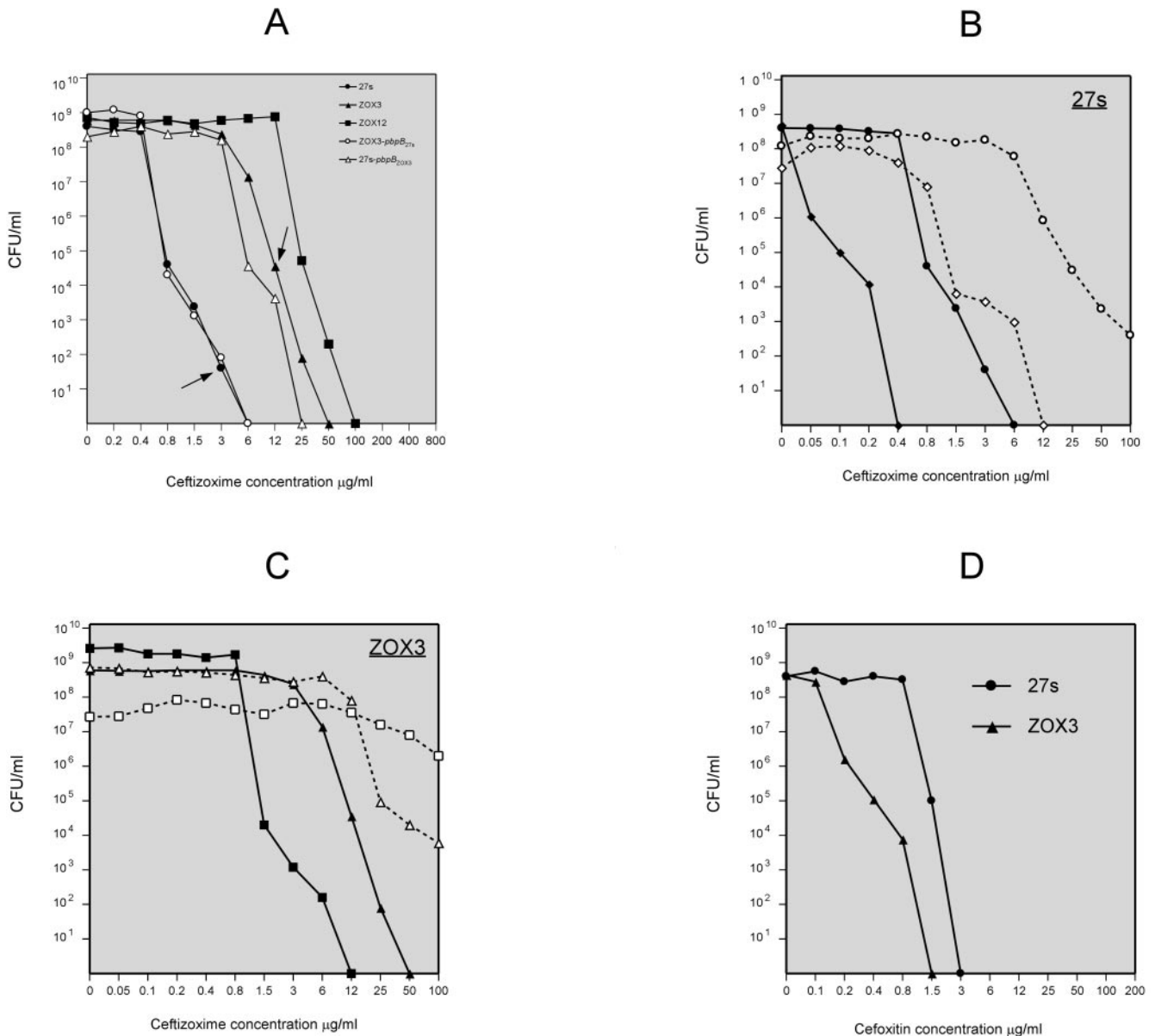


FIG. 2. Antibiotic susceptibility profiles of *S. aureus* strain 27s and ZOX3 (carrying a mutated form of *pbpB*) and mutant derivatives of these strains. Antibiotic susceptibilities of strains were determined in overnight cultures plated on agar containing different concentrations of ceftizoxime and/or cefoxitin and were analyzed for PAPs as described in Materials and Methods. (A) Ceftizoxime susceptibility profiles of parental strain 27s and ceftizoxime-resistant mutants ZOX3 and ZOX12 (solid squares). Susceptibility profiles of transformant derivatives of strains 27s and ZOX3 carrying mutant and/or wild-type forms of *pbpB* (strain 27s-*pbpB*_{ZOX3} [27s with the normal *pbpB* replaced by the mutant gene] and strain ZOX3-*pbpB*_{27s} [ZOX3 in which the mutant *pbpB* was replaced by the wild-type form of the gene]) are also shown. The arrow pointing to the line with solid circles indicates the source of ZOX3. The arrow pointing to the line with solid triangles indicates the source of the ZOX12. (B) Ceftizoxime susceptibility profiles of strain 27s (solid circles), strain 27s with inactivated *pbpD* (solid diamonds), strain 27s carrying the plasmid-borne *mecA* (empty circles), and 27s with inactivated *pbpD* carrying the plasmid-borne *mecA* (empty diamonds). (C) Ceftizoxime susceptibility profiles of strain ZOX3 (solid triangles), ZOX3 with inactivated *pbpD* (solid squares), ZOX3 carrying the plasmid-borne *mecA* (empty triangles), and ZOX3 with inactivated *pbpD* carrying the plasmid-borne *mecA* (empty squares). (D) Cefoxitin susceptibility profiles of strains 27s and ZOX3.

strain 27s on a series of agar plates containing increasing concentrations of the antibiotic ceftizoxime. A spontaneous ceftizoxime-resistant mutant was picked from the plate containing 3 μg of ceftizoxime per ml. The approximate frequency of the mutant was 10⁻⁷. The PAP of the ZOX3 mutant cultures showed elevated resistance to ceftizoxime for the majority of the bacteria, with an MIC of 6 μg/ml, an increase from the

MIC of 0.8 μg/ml for the parental strain 27s (Fig. 2A). A second-stage mutant, ZOX12, with an additional increase in the ceftizoxime MIC to 12 μg/ml was also isolated from the subpopulation of ZOX3 colonies that grew on agar containing 6 μg of the antibiotic per ml. The mutant ZOX12 was not further characterized.

Mutant ZOX3 was stable: daily serial passage by picking and

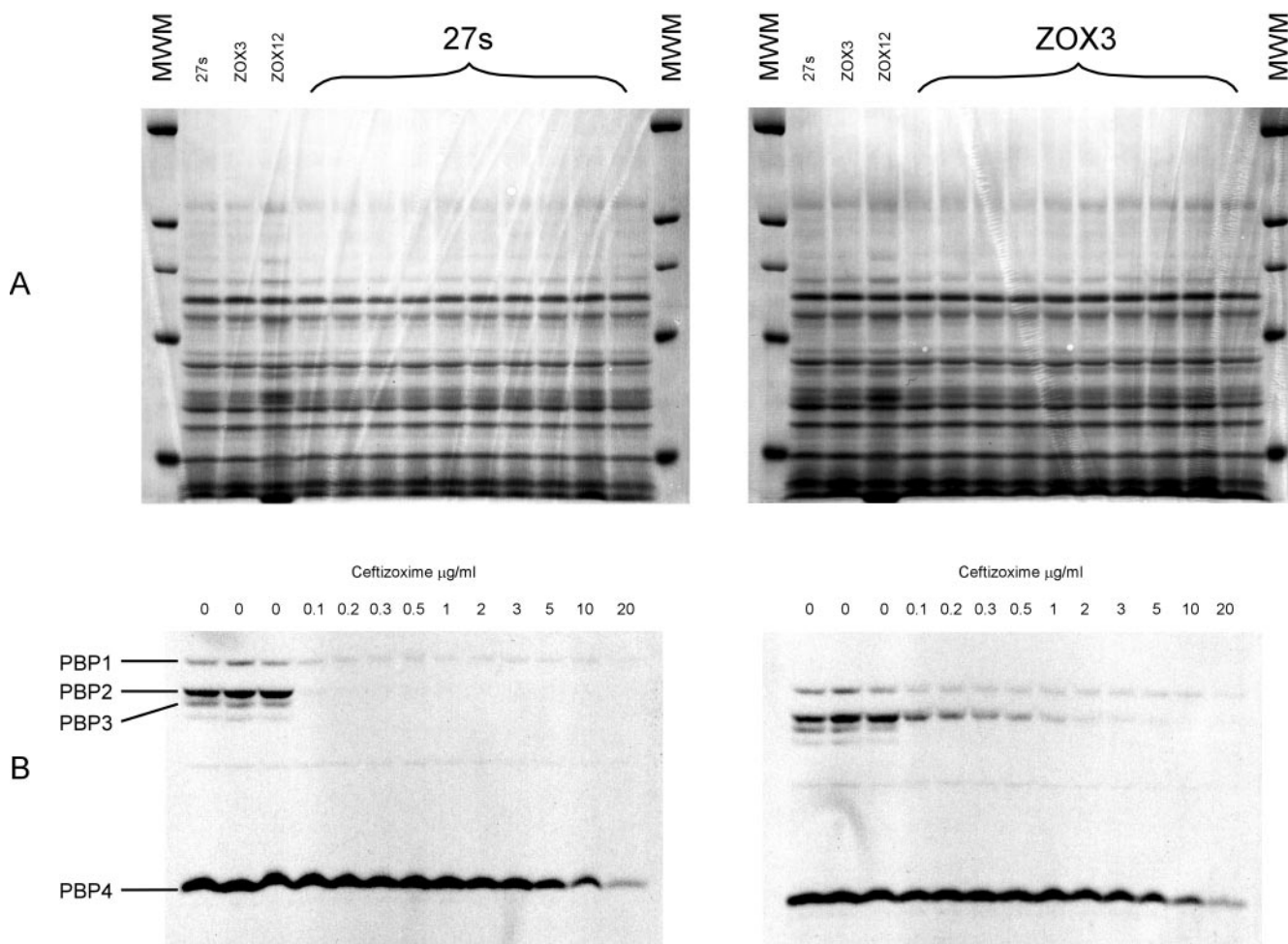


FIG. 3. Ceftizoxime binding capacity of PBPs of susceptible and ceftizoxime-resistant *S. aureus* strains. Fluorographic competition assays were used, with membrane preparations from the susceptible parental strain 27s and its ceftizoxime-resistant mutant ZOX3, to determine the ceftizoxime binding capacities of PBPs as described in Materials and Methods. Panel A shows the protein patterns of sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue. Panel B shows the titration of the ceftizoxime binding capacity of PBPs by using the fluorographic assay.

restreaking colonies on antibiotic-free tryptic soy agar for over 2 weeks did not cause any reduction in antibiotic resistance as determined by the PAP (not shown). The antibiotic resistance of ZOX3 was selective for ceftizoxime: there was no increase in the resistance of ZOX3 to methicillin, oxacillin, and a number of other beta-lactam antibiotics. The MICs of methicillin and oxacillin were actually slightly reduced, from 0.8 μg/ml (the MIC for the parental strain 27s) to 0.4 μg/ml and from 0.1 to 0.05 μg/ml (in ZOX3).

Change in ceftizoxime affinity of PBP2 in mutant ZOX3. Figure 3 shows the results of a fluorographic competition assay in which the binding capacities (affinities) of PBPs for ceftizoxime in the parental strain 27s and mutant ZOX3 were compared. Membrane preparations containing the same amounts of protein (80 μg) from the two bacterial strains were preincubated with increasing amounts of ceftizoxime (0.1 to 20 μg), after which a single identical amount of radioactive penicillin was added to each test preparation and the concentration of ceftizoxime preventing binding of the radioactive reagent to various PBPs was determined. A concentration of

ceftizoxime as low as 0.1 μg/ml inhibited the binding of radioactive penicillin to PBP2 in the parental strain 27s. A similar degree of inhibition required 1 to 2 μg of the drug per ml in the ZOX3 mutant. No differences in the affinity profiles of the other PBPs were apparent.

Analysis of the *pbpB* gene in mutant ZOX3. The *pbpB* gene was PCR amplified from the parental and resistant strains and sequenced. The two sequences were identical except for a single point mutation detected in the transpeptidase domain of the mutant strain at position 1373 of the *pbpB* ORF, in which a cytosine residue was replaced by a thymine, causing a proline-to-leucine substitution at amino acid residue 458 in the protein. The point mutation was located in the immediate vicinity of the conserved motif SFN (8), which forms a part of the active site of the PBP2 TPase domain (Fig. 4).

Decreased level of peptidoglycan cross-linking in the ZOX3 mutant. Cell wall peptidoglycan was isolated from the parental strain 27s and mutant ZOX3, and the mucopeptide compositions were determined after enzymatic hydrolysis and HPLC separation. Comparison of the HPLC elution profiles of the

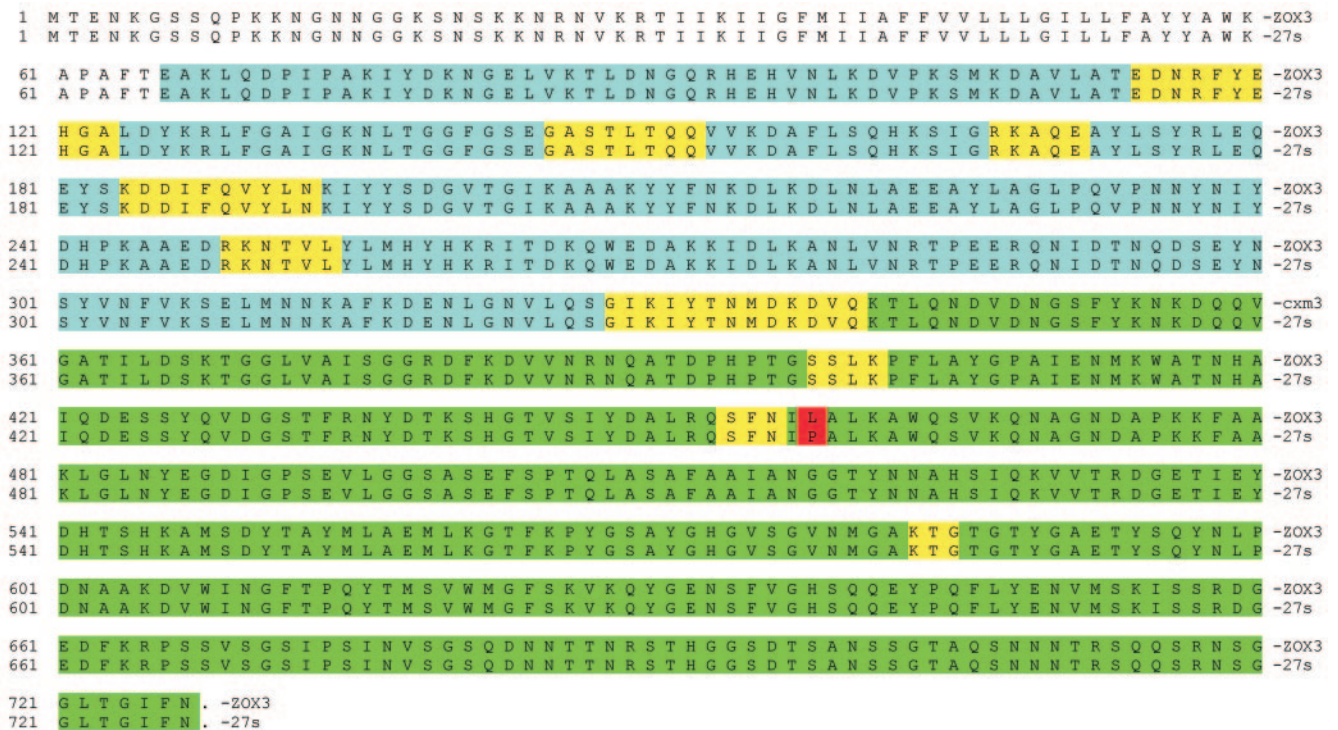


FIG. 4. Localization of the mutation in the *pbpB* gene of ceftizoxime-resistant mutant ZOX3. Deduced amino acid sequences of the PBP2 protein are shown for strains 27s and ZOX3. The TGase domain of the protein is shown in blue, the TPase domain is in green, conserved motifs are in yellow, and the proline (P)-to-leucine (L) mutation site is in red.

two peptidoglycan preparations (Fig. 5A and C; see Fig. 7A) indicates a significant reduction in the highly cross-linked oligomeric mucopeptides in the peptidoglycan of the mutant strain. A similar change in the elution profile of mucopeptides was observed in the parental strain 27s grown in the presence of 1/10 times the MIC of ceftizoxime (Fig. 5B).

Allelic replacement experiments. In order to test whether the point mutation detected in the TPase domain of the resistant mutant was responsible for the antibiotic resistance and for the observed alterations in PBP2 affinity and cell wall composition, allelic replacement experiments were performed. The mutant form of *pbpB* from ZOX3 was replaced by the wild-type *pbpB* sequence to generate transformant ZOX3-*pbpB*_{27s}. In the reciprocal cross, the wild-type *pbpB* gene of the parental strain 27s was replaced by the mutant *pbpB* sequence to generate transformant 27s-*pbpB*_{ZOX3}. Details of the methods used in the allelic replacement experiments are outlined in Fig. 1 (see Materials and Methods).

The PAP data show that ceftizoxime resistance was linked to the type of *pbpB* gene carried by the particular isolate (Fig. 2A); the ceftizoxime MIC for transformants carrying the wild-type *pbpB* was 0.8 $\mu\text{g/ml}$, and transformants carrying the mutated version of *pbpB* showed a ceftizoxime MIC of 6 $\mu\text{g/ml}$. The reduced affinity of PBP2 for ceftizoxime (not shown) and the reduced degree of peptidoglycan cross-linking (Fig. 5D and E; see Fig. 7A) was also reproduced in the reciprocal transformants.

Site-directed mutagenesis of *pbpB* and its expression from a plasmid in the absence of expression of its chromosomal copy. To obtain additional confirmation that the point mutation in

pbpB is indeed responsible for ceftizoxime resistance, the mutated *pbpB* copy (obtained by site-directed mutagenesis of the wild-type sequence) was expressed from a plasmid in strain 27s-*pbpB*_{spac}, in which chromosomal copy of *pbpB* was put under the control of an IPTG-inducible promoter (see Materials and Methods).

Strain 27s-*pbpB*_{spac}(pMC1), containing mutated *pbpB*, and control strain 27s-*pbpB*_{spac}(pMGP19), containing wild-type *pbpB*, could grow in the absence of IPTG, indicating the successful transcription of the *pbpB* genes from the plasmids. The ceftizoxime MIC for the strain carrying the mutated *pbpB* was 4 to 6 $\mu\text{g/ml}$, while the strain carrying the wild-type *pbpB* on the plasmid had a ceftizoxime MIC of 0.38 $\mu\text{g/ml}$.

Effect of inactivation of *pbpD* and introduction of *mecA* on ceftizoxime resistance and cell wall composition. The significant change in the degree of cross-linking of the peptidoglycan observed in the PBP2 transpeptidase mutant ZOX3 prompted us to test the effect of inactivation of PBP4 on the antibiotic resistance and cell wall structure of the bacteria, since PBP4 was shown earlier to participate in the cross-linking of the peptidoglycan of *S. aureus* (25). It was also of interest to test the effect of introducing the *mecA* determinant into these genetic backgrounds, since PBP2A, the protein product of *mecA*, is able to substitute for the TPase function of PBP2 (17).

Insertional inactivation of *pbpD* caused a drop in the ceftizoxime MIC for ZOX3 from 6 to 1.5 $\mu\text{g/ml}$, and inactivation of *pbpD* in the parental strain 27s decreased the ceftizoxime MIC from 0.4 to 0.05 $\mu\text{g/ml}$. Introduction of the plasmid-borne *mecA* gene caused the opposite effect: the ceftizoxime MIC for strain 27s increased from 0.8 to 12 $\mu\text{g/ml}$, and that for ZOX3

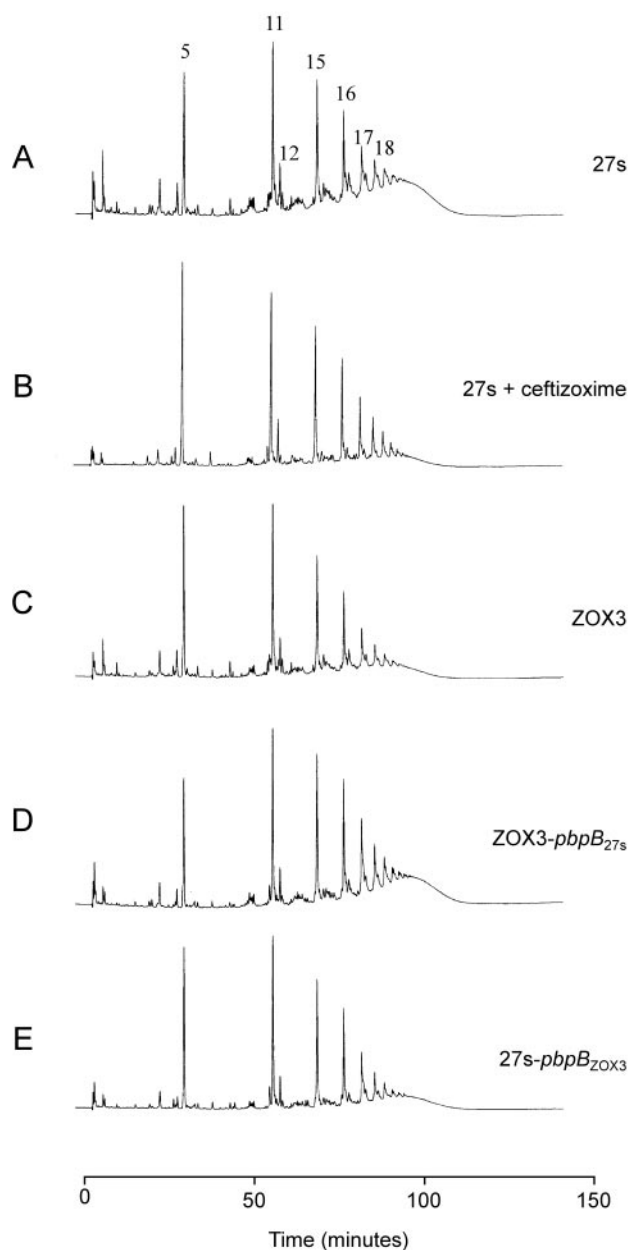


FIG. 5. Differences in the peptidoglycan compositions of the ceftizoxime-resistant *S. aureus* mutant ZOX3 and its parental strain 27s. Peptidoglycans were prepared, and muropeptide patterns were analyzed by HPLC, as described in Materials and Methods. Panel A: parental strain 27s. Panel B: 27s grown in the presence of 1/10 times the MIC of ceftizoxime. Panel C: mutant ZOX3. Panel D: strain ZOX3-*pbpB*_{27s} (mutant ZOX3 in which the mutated *pbpB* was replaced with the wild-type form by allelic replacement). Panel E: 27s *pbpB*_{ZOX3} (parental strain in which the normal *pbpB* was replaced with the mutated form). Numbers above the HPLC peaks identify a structure of the respective muropeptides, as described earlier (2). Peaks 5, 11, 15, 16, and 17 represent the monomeric muropeptide (5) and its di-, tri-, tetra-, and pentameric derivatives. Peak 12 is the unique doubly cross-linked muropeptide dimer (1). The poorly resolved part ("hump") of the HPLC profile eluting with retention times of greater than 100 min (i.e., after peak 18) contain highly cross-linked oligomeric components.

increased from 6 to about 25 $\mu\text{g/ml}$ (Fig. 2B and C). Introduction of *mecA* into strain 27s caused no detectable change in the oxacillin MIC for the majority of cells (data not shown).

Inactivation of *pbpD* in 27s also caused a large reduction in the representation of highly cross-linked muropeptides, i.e., in the material eluting from the HPLC column with retention times of higher than 100 min (Fig. 6). The proportion of this muropeptide fraction dropped from 45 to 22% in strain 27s with the inactivated PBP4 (Fig. 7B). Inactivation of PBP4 in the resistant mutant ZOX3 had an even more pronounced effect: the highly cross-linked muropeptide fraction decreased from 35 to less than 5%, and there was a striking accumulation of monomeric muropeptides (peak 5 in Fig. 6) (from about 7 to 27%) and in the dimeric muropeptides (peak 11 in Fig. 6) (from about 8 to 17%) (Fig. 7B). In contrast to the highly significant increase in ceftizoxime resistance caused by the introduction of *mecA* into either strain 27s or ZOX3, the presence of the plasmid-borne *mecA* gene caused no detectable change in the muropeptide HPLC profiles of either strain 27S or mutant ZOX3 (data not shown).

DISCUSSION

The *S. aureus pbpB* gene encodes a two-domain protein carrying the characteristic motifs of a TPase as well as a TGase (3, 8). Direct biochemical evidence for the TGase activity of PBP2 has been obtained (14), and it was also shown that the TGase domain of PBP2 becomes essential for the growth of MRSA strains in the presence of methicillin in the medium (14).

Much less is known about the nature of the TPase activity, except that in methicillin-susceptible *S. aureus* strains it is essential for growth, and this essential function may be replaced by the activity of PBP2A in MRSA strains which carry the drug resistance determinant *mecA* (17). MRSA conditional mutants in which the transcription of the PBP2 gene was blocked grew and produced a cell wall peptidoglycan with close to a normal composition. However, this peptidoglycan must have been the product of the resistance protein PBP2A that has replaced the functions of the native PBP2 (17). Thus, direct biochemical evidence for the nature of the TPase activity of PBP2 in staphylococcal cell wall synthesis is not available.

In order to bypass this problem, we chose a strategy which involved the use of the beta-lactam antibiotic ceftizoxime, which, at low concentrations, has extremely selective inhibitory activity against PBP2 of *S. aureus* (11). According to Okonogi et al., the 50% inhibitory concentrations of ceftizoxime for the *S. aureus* PBPs were 2.74 $\mu\text{g/ml}$ for PBP1, 0.0626 $\mu\text{g/ml}$ for PBP2, and 3.61 and 50.8 $\mu\text{g/ml}$ for PBP3 and PBP4, respectively (11). We used this antibiotic as an experimental tool to isolate a stable spontaneous ceftizoxime-resistant mutant, ZOX3, that showed an increased MIC only of ceftizoxime and not of other beta-lactams. The PBP2 of the mutant showed a striking and selective decrease in its binding capacity for ceftizoxime, and the mutant produced a peptidoglycan with an altered composition, the most prominent features of which were (i) a reduction in the percentage of the highly cross-linked muropeptides (from about 45% in the susceptible strain to about 35% in the mutant) and (ii) an increase in the percentage of monomeric (peak 5) and dimeric (peak 11) mu-

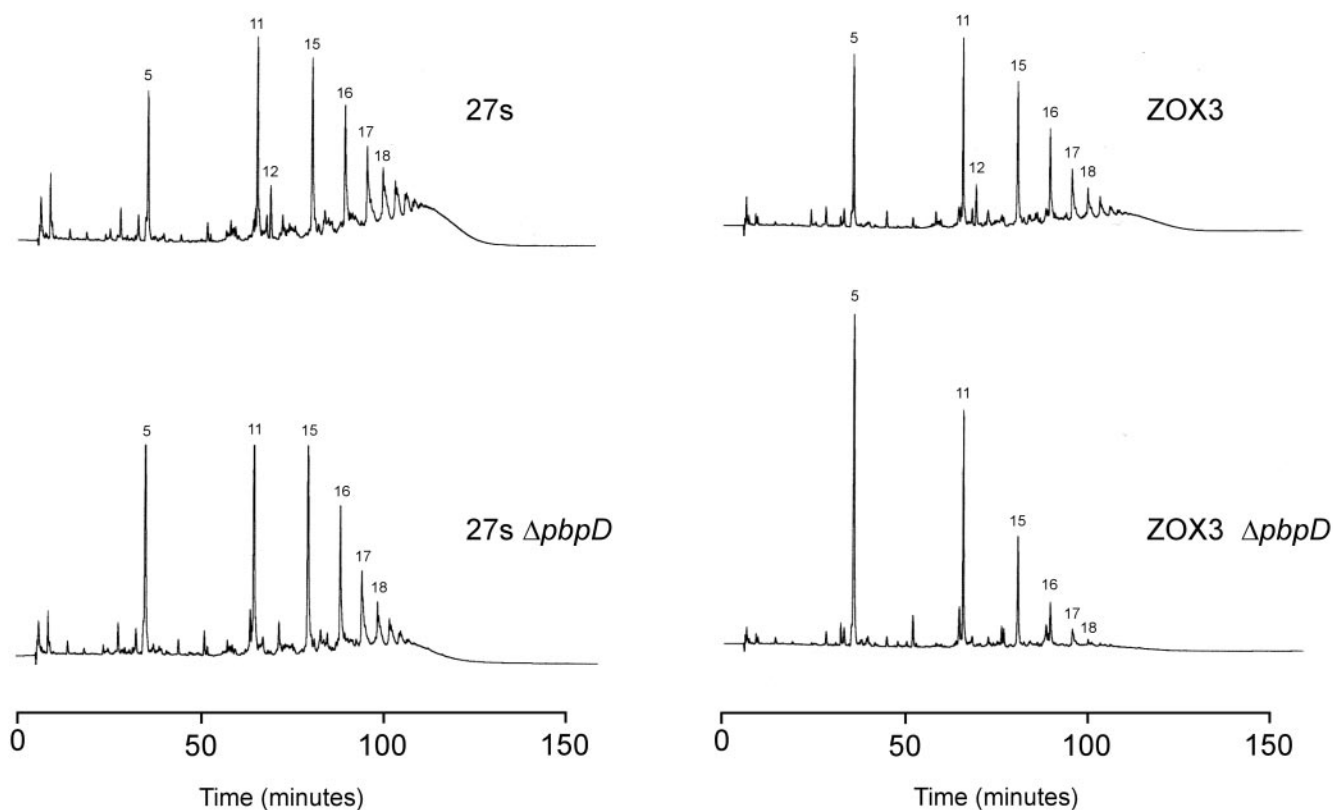


FIG. 6. Effect of inactivation of *pbpD* on the muropeptide compositions of *S. aureus* strains 27s and ZOX3. Strains 27s and ZOX3 and their derivatives with a disrupted *pbpD* gene (strains 27s- Δ *pbpD* and ZOX3- Δ *pbpD*) were grown, and HPLC analysis of peptidoglycan was performed as described in Materials and Methods.

ropeptides. Reciprocal allelic replacement experiments and site-directed mutagenesis clearly showed that the three phenotypes, i.e., the increased ceftizoxime MIC, reduced affinity for PBP2, and altered cell wall composition, were the consequences of the proline-to-leucine point mutation in the structural gene of PBP2. The cell wall alterations appeared in the mutant bacteria growing in antibiotic-free medium, indicating that the replacement of a proline by a leucine residue in the mutant protein affected the normal functioning of PBP2 in the cross-linking of cell wall muropeptides. This interpretation is supported by the finding that parental cells grown in the presence of from 1/10 up to 1/3 the MIC of ceftizoxime also produced peptidoglycan with an altered muropeptide composition similar to that of ZOX3 grown in antibiotic-free medium (Fig. 5).

The reduced amounts of highly cross-linked oligomers in the cell walls of the ZOX3 mutant were surprising, since several previous studies showed that these oligomers are primarily the products of the activity of PBP4, a protein with known "secondary" transpeptidase activity (25). In order to clarify this issue, we examined the muropeptide composition of cell wall prepared from a mutant of strain 27s in which PBP2 was intact but PBP4 was interrupted. Inactivation of *pbpD*, the structural gene of PBP4, had relatively little effect on the relative proportions of monomeric (peak 5) and moderately cross-linked (peaks 11, 15, 16, and 17) muropeptides, but the percentage of

highly cross-linked components was reduced from 45 to about 22%.

Next we analyzed the muropeptide composition of a double mutant carrying both the ZOX3 point mutation and the disrupted PBP4. The peptidoglycan of the double mutant showed a greatly increased proportion of monomeric muropeptides (an increase from 7 to 27%) and an increase in muropeptide dimers (from 9 to 17%). In addition, there was a drastic reduction in the highly cross-linked oligomers (a decrease from 35 to less than 5%) (Fig. 7B).

Peak 12 disappeared completely from the cell walls of bacteria with inactivated *pbpD*, suggesting that PBP4 participated in its biosynthesis. This unusually structured dimeric muropeptide has doubly cross-linked components by two pentaglycine bridges (1). Most interestingly, disruption of *pbpD* also affected ceftizoxime resistance: the ceftizoxime MIC for strain 27s decreased from 0.8 to 0.05 μ g/ml, and that for mutant ZOX3 decreased from 6 to 0.8 μ g/ml, when the *pbpD* gene was inactivated (Fig. 2B C). This finding was surprising, since the affinity titration experiments of Fig. 2 clearly demonstrated the selectivity of inhibition of PBP2 by ceftizoxime. This antibiotic showed no detectable inhibitory activity against PBP4 in the concentration range close to the MIC. Inactivation of *pbpD* had no effect on the methicillin MIC (data not shown).

Our findings provide direct biochemical evidence for the functioning of the TPase domain of PBP2 and also suggest that

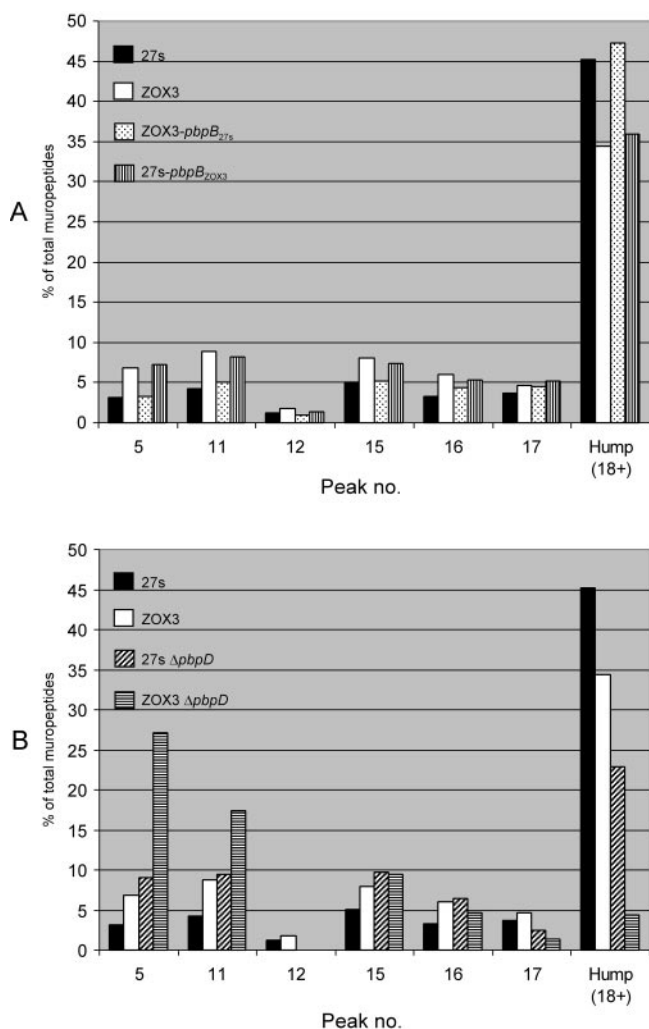


FIG. 7. Quantitative differences in the peptidoglycan compositions of strains 27s and ZOX3 and their derivatives. The bars show the relative amounts of mucopeptide species calculated from UV absorbances of the different mucopeptide components.

the two transpeptidases, PBP2 and PBP4, act in concert in the cross-linking of cell wall building blocks during the biosynthesis of the staphylococcal cell wall. While PBP4 cannot replace the essential TPase activity of PBP2, the hypersensitivity of PBP4 deletion mutants to ceftizoxime and the compositional changes in the cell walls of double mutants suggest that these two proteins function cooperatively to produce a staphylococcal peptidoglycan that carries the minimal numbers and types of cross-links essential for the survival and growth of the bacteria. Such cooperative functioning is also consistent with the observation that mutant ZOX3, in which PBP2 is suboptimal in function, becomes hypersensitive to cefoxitin, a specific inhibitor of PBP4 (9) (Fig. 2D).

Examination of the HPLC profiles in Fig. 6 and 7 suggests that the primary TPase function of PBP2 may be in producing mucopeptide dimers (peptide 11) and trimers, tetramers, and pentamers (peptides 15 through 17) (with progressively decreasing efficiencies) and that the primary function of PBP4 appears to be in the production of mucopeptides with a high degree of cross-linking.

The modest but significant reduction in the highly cross-linked mucopeptide fraction (from 45 to 35%) in the ZOX3 mutant may indicate the direct participation of PBP2 in the production of these oligomers. Alternatively, it may be the consequence of an indirect effect in which a suboptimal functioning of the mutant PBP2 affects the functioning of PBP4. One specific mechanism for this could be if the transpeptidase activity of PBP2 were to produce the substrates for PBP4, i.e., if the primary TPase activity of PBP2 were to be the production of dimers, trimers, and tetrameric mucopeptides which then would be used by PBP4 to produce the more highly cross-linked oligomers by the addition of monomeric donor peptides to the more highly cross-linked acceptors. Basic features of this model are shown in Fig. 8.

The accumulation of large amounts of un-cross-linked monomeric mucopeptides in the double mutant is consistent with this proposal: it would be the result of the suboptimal TPase activity of the mutant form of PBP2, combined with the lack of PBP4 activity, which would stop the “consumption” of mucopeptide monomers for the production of highly cross-linked

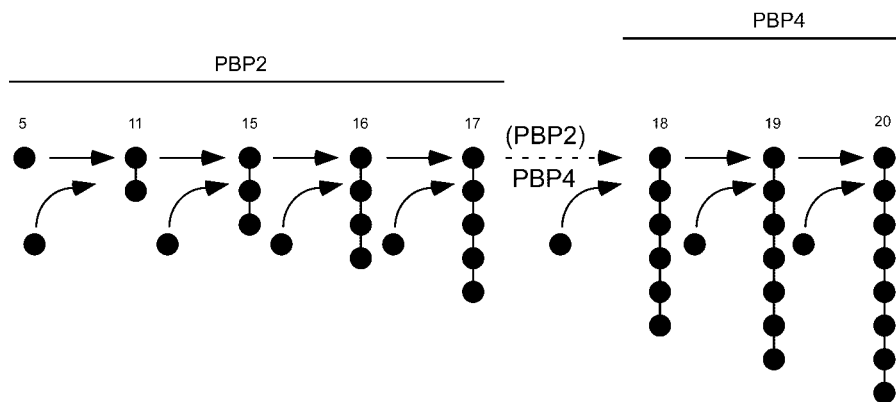


FIG. 8. Model for the cooperative functioning of PBPs in cross-linking the mucopeptides in the *S. aureus* cell wall. Mucopeptide monomers and multimers are identified by their peak numbers in the HPLC profile and by the bead diagram symbolizing the number of monomeric units cross-linked. Monomeric mucopeptides are indicated by solid circles. The primary cross-linking functions of PBP2 (and PBP2A) are suggested to involve cross-linking of up to pentameric components, and cross-linking of higher multimers is catalyzed primarily by PBP4.

wall components. Thus, under these conditions, the major contribution of PBP2 to cell wall composition would be through its TGase activity, producing a peptidoglycan enriched for uncross-linked muropeptide monomers.

We introduced the *mecA* gene on a plasmid into the parental strain 27s and also into the mutant ZOX3. In each case the ceftizoxime MIC for the bacteria increased significantly. There was no effect on the methicillin MIC for the majority of the cells. This observation indicates that the *mecA* gene product PBP2A can effectively compensate for the loss of antibiotic resistance and at least partially replace the transpeptidase activity of PBP2, when the latter is inhibited by ceftizoxime or has a decreased efficiency of functioning as in the ZOX3 mutant. However, attempts to directly document such an effect have failed so far: strain 27s or mutant ZOX3 carrying the plasmid-borne *mecA* gene showed little if any increase in the proportion of muropeptide dimers and trimers (data not shown). Thus, the mechanism of the proposed "assistance" by PBP2A to the TPase activity of PBP2 remains to be determined.

Our studies exploited a combination of a selectively acting beta-lactam antibiotic and a specific mutant for the dissection of the functioning of PBP2 in staphylococcal cell wall synthesis. The cooperative functioning of PBP2 and PBP4 documented in our study is consistent with the notion that proteins involved with the complex task of bacterial cell wall assembly act as a coordinated multienzyme complex (4). How the activities of the other PBPs (the "essential" PBP1 [23] and the "nonessential" PBP3 [15]) of *S. aureus* interact in this system remains to be explored.

The significant effect of *mecA* on the ceftizoxime-resistant phenotype, in contrast to the lack of effect on peptidoglycan composition (or resistance to other beta-lactams such as oxacillin), requires comment. Introduction of *mecA* on a plasmid into the background of either 27s, ZOX3, or their *pbpD*-inactivated derivatives caused highly significant increases in the ceftizoxime MIC for the bacteria. In the *mecA* derivative of 27s, the ceftizoxime MIC increased from 0.8 up to 25 µg/ml; in strain 27s carrying the inactivated *pbpD* gene, the introduction of *mecA* caused an increase of the ceftizoxime MIC from 0.05 up to 0.8 to 1.5 µg/ml (Fig. 2B). Introduction of *mecA* into mutant ZOX3 or its *pbpD*-inactivated derivative also caused major increases in the ceftizoxime resistance level (Fig. 2C). Yet these major "corrections" of resistance level were specific for ceftizoxime; there was no change in the oxacillin MIC for the majority of bacteria. Also, the introduction of *mecA* did not cause any significant alteration in muropeptide composition as detectable by our HPLC system. These observations suggest that *mecA* (or PBP2A) interacts directly with the PBP2-PBP4 transpeptidation system which is targeted by the selective action of ceftizoxime. The biochemical mechanism by which *mecA* and/or its protein product PBP2A provides such a highly selective protection of staphylococci remains to be explained.

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