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Staphylococcus aureus Penicillin-Binding Protein 4 and Intrinsic β-Lactam Resistance

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Increased levels of production of penicillin-binding protein PBP 4 correlated with in vitro acquired intrinsic β-lactam resistance in a mutant derived from a susceptible strain of *Staphylococcus aureus*, strain SG511 Berlin. Truncation of the PBP 4 C-terminal membrane anchor abolished the PBP 4 content of cell membrane preparations as well as the resistance phenotype. A single nucleotide change and a 90-nucleotide deletion, comprising a 14-nucleotide inverted repeat in the noncoding *pbp4* gene promoter proximal region, were the only sequence differences between the resistant mutant and the susceptible parent. These mutations were thought to be responsible for the observed overproduction of PBP 4 in the intrinsically β-lactam-resistant mutant. The *pbp4* gene was flanked upstream by the open reading frame *abcA*, coding for an ATP-binding cassette transporter-like protein showing similarities to eukaryotic multidrug transporters and downstream by a glycerol 3-phosphate cytidyltransferase (*tagD*)-like open reading frame presumably involved in teichoic acid synthesis. The *abcA-pbp4-tagD* gene cluster was located in the *Sma*I-D fragment in the *S. aureus* 8325 chromosome in close proximity to the RNA polymerase gene *rpoB*.

Resistance to β-lactam antibiotics in *Staphylococcus aureus* involves, in one way or another, penicillin-interactive proteins, whether they are β-lactamase, β-lactam-sensing signal transducing elements, or penicillin-binding proteins (PBPs). PBPs are integral membrane proteins that catalyze the transpeptidation and carboxypeptidation of bacterial cell wall peptidoglycan. They are members of the closely related group of active-site serine D,D-peptidases, which are characterized by three homology boxes (21, 27) consisting of the conserved motifs SXXK, SXN, and K(H)T(S)G that are responsible for the catalytic activities of the proteins. Susceptible *S. aureus* isolates possess three high-molecular-weight PBPs (PBP 1, 85,000; PBP 2, 81,000; and PBP 3, 75,000) and one low-molecular-weight PBP (PBP 4, 45,000) (18). PBPs 2 and 3 can sometimes be resolved into two components by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (11, 55). In contrast to the *Escherichia coli* high-molecular-weight PBPs, which are bifunctional and which show transglycosylase and transpeptidase activities, the high-molecular-weight staphylococcal PBPs were postulated to have solely transpeptidase activity, whereas the transglycosylase activity resides on a separate protein (40). The role of the low-molecular-weight PBP 4 is unclear. The antibacterial effect of β-lactams is mediated primarily by inhibition of the high-molecular-weight PBPs, which have high affinities for β-lactam antibiotics (13, 42, 53). Whereas PBP 1 alone or PBP 1 in combination with PBP 2 or PBP 3 seems to be essential for the survival of the cells (5), the low-molecular-weight PBP 4, which has low binding affinity, is thought to be nonessential (13, 42, 53). No genetic analysis of mutants postulated to be lacking PBP 4 have been done yet, however, and of the other *S. aureus* PBPs, only the sequences of staphylococcal PBP 1 (A. Wada, 1994, accession number D28879) and PBP 2 (37) are known.

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In clinical methicillin-resistant *S. aureus* isolates, intrinsic methicillin resistance is due to the production of a unique, additional, low-affinity PBP, PBP 2a, which is encoded by *mecA* (for a review, see reference 7). However, some *S. aureus* isolates with borderline resistance to methicillin lack *mecA* and therefore do not produce PBP 2a. In those strains, an increased level of resistance was attributed either to modification of the penicillin-binding capacity of the normal PBPs (50), to overproduction of a β-lactamase in a specific genetic background (3), or to a methicillinase (34). The former isolates resemble first-step methicillin-resistant (Mc^r) mutants that can be obtained from susceptible *S. aureus* isolates by stepwise selection for growth on increasing concentrations of β-lactams. A gain in the level of resistance is correlated in those strains with overproduction and/or alterations in the affinities of the normal set of PBPs (9, 17). Often, more than one PBP is modified. Hackbarth et al. (23) showed in in vitro-generated mutants that point mutations in the *pbp2* gene had occurred; this mutation affected the penicillin-binding kinetics. Whereas naturally competent organisms like pneumococci and streptococci can become resistant by acquiring from their naturally resistant relatives cassettes of their low-affinity PBPs, *S. aureus* isolates which are not naturally competent can obtain foreign genes by mating and natural transduction. Alternatively, antibiotic pressure, in the absence of an external donor organism, can trigger chromosomal mutations leading to higher levels of resistance. The in vitro Mc^r mutants described here can therefore serve as a model for the situation observed in nature, and they offer the advantage that they can be compared with the isogenic susceptible parent strain, which is not possible with clinical isolates.

In the study described in this report we investigated the role of PBP 4 in methicillin resistance by cloning and sequencing the region comprising the *pbp4* open reading frame of an in vitro resistant mutant that overproduced PBP 4 and compared it with that of the susceptible parent strain.

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TABLE 1. Relevant bacterial strains[a]

Strain	Genetic background or relevant genotype	Phenotype	Reference
S. aureus			
SG511	SG511	Mc^s	Strain collection of the Robert Koch-Institute, Berlin
PV1	SG511, multiple mutations	Mc^r	Pen^r mutant derived in vitro from SG511 for growth on increasing concentrations of penicillin (24)
RN2906	NCTC 8325-4(pRN3208) (rep^{ts})	Em^r Cd^r	39
UT-1	PV1(pRN3208) (rep^{ts})	Mc^r Em^r Cd^r	This study; by transduction of plasmid pRN3208 of RN2906 via phage 80α in strain PV1, selection for Cd^r (24)
UT-6-2	UT-1, Ω2007(*pbp4*::Tn*551*)	Mc^s Em^r	This study; insertional inactivation of Mc^r UT-1
UT-39-1	PV1, Ω2007(*pbp4*::Tn*551*)	Mc^s Em^r	This study; by transduction of Tn*551* from UT-6-2 via phage 80α in strain PV1, selection for Em^r
UT-77-1	SG511, Ω2007(*pbp4*::Tn*551*)	Mc^s Em^r	This study; by transduction of Tn*551* from UT-6-2 via phage 80α in strain SG511, selection for Em^r
BB270	NCTC 8325 *mec*	Mc^r	(8)
E. coli			
DH10B	F^- *araD139* Δ(*ara leu*)*7697*, *lacX74*, *galU galK mcr* Δ(*mrr-hsdRMS-mcrBC*) *rpsL deoR* ϕ80d*lacZ* ΔM15 *endA1 nupG recA1*		Strain for high-efficiency cloning of large fragments (30)
UT-85	DH10B(pUT-5)	Str^r Kan^r Tc^r	This study; by transformation of DH10B with pUT-5
UT-122	DH10B(pUT-41)	Amp^r	This study; by transformation of DH10B with pUT-41
UT-127	DH10B(pUT-46)	Tc^r	This study; by transformation of DH10B with pUT-46

[a] Abbreviations: Amp^r, ampicillin resistant; Cd^r, cadmium resistant; Em^r, erythromycin resistant; Kan^r, kanamycin resistant; Mc^s, methicillin susceptible; Mc^r, methicillin resistant; Str^r, streptomycin resistant; Pen^r, penicillin resistant; Tc^r, tetracycline resistant; rep^{ts}, replication temperature sensitive.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The strains and plasmids used in the study are listed in Tables 1 and 2. They were grown in Luria-Bertani (LB) medium (10 g of tryptone [Difco] per liter, 5 g of yeast extract [Difco] per liter, 5 g of NaCl per liter). The growth temperature was 37°C unless otherwise noted. The in vitro β-lactam-resistant mutant PV1 was selected over five steps by growth on increasing concentrations of penicillin (0.15, 0.3, 0.7, 2.0, and 6.0 μg/ml). Transductions were performed with phage 80α as described earlier (31). Transductants were selected on 20 μg of erythromycin per ml and were screened for the inability to grow on 5 μg of methicillin per ml. MICs were determined by the broth microdilution method as described by the National Committee for Clinical Laboratory Standards (38).

Tn*551*-directed mutagenesis. The temperature-sensitive plasmid pRN3208 carrying Tn*551* was transduced into the in vitro-generated Mc^r strain PV1 by selecting for resistance to 0.1 mM $CdCl_2$ at 30°C, yielding strain UT-1. Insertional inactivation of methicillin resistance in UT-1 was done by selection for growth in the presence of erythromycin at the nonpermissive temperature (6). The colonies obtained were then screened for insertional inactivation of the methicillin resistance (24) by replica plating.

DNA manipulations. The molecular biological techniques used for nucleic acid manipulations, gel electrophoresis, blotting of DNA, and hybridization procedures were mainly those of Maniatis et al. (32). Pulsed-field gel electrophoresis of *Sma*I-digested chromosomal DNA was carried out essentially as described earlier (24), the *Sma*I-digested fragments were separated with a CHEF DR II electrophoresis cell (Bio-Rad, Richmond, Calif.). Restriction enzymes were obtained from Boehringer Mannheim and were used as recommended by the supplier. The probe for hybridization with PBP 4 was an internal 0.8-kb fragment of *pbp4* starting at the *Acc*I site (Fig. 1). For probing with Tn*551*, the 6.5-kb *Hpa*I-1 fragment, carrying the 5.2-kb Tn*551*, was used (22).

Cloning of the PBP 4 structural gene. By using Tn*551* as a probe, a *Hin*dIII fragment containing the right junction of Tn*551* and part of the inactivated gene of strain UT-39-1 was cloned and was subsequently used to identify and clone the wild-type allele of the original susceptible strain SG511 Berlin and the corresponding allele of Mc^r mutant PV1 into different vectors. The SG511 allele was cloned as a 3.8-kb *Pst*I-*Xba*I insert in pTZ18U yielding pUT-41, the PV1 *pbp4* gene was cloned as a 5.6-kb *Hin*dIII insert in pAW8 yielding pUT-46, and the PV1 *pbp4* gene was cloned as a 8-kb *Sau*3A insert in deletion factory (Gibco) vector pΔ1 yielding pUT-5. Figure 1 shows the localization of the cloned fragments on the genetic map. pUT-5 was used according to the Gibco protocol of the deletion factory system to produce deletions for subsequent sequencing.

The DNA sequences were determined with custom 15-mer oligonucleotides (Microsynth, Balgach, Switzerland) that primed along the sequence and with terminal fragments of pUT-41 and pUT-45 by using the universal primer (Gibco). Denatured double-stranded plasmid DNA was sequenced in both directions by the dideoxynucleotide chain termination method (44) by using the Sequenase 2.0 kit (U.S. Biochemicals) and [α-^{35}S]dATP from Amersham Corp. (Buckinghampshire, England). The nucleotide sequences and the deduced protein sequences were analyzed with Genetics Computer Group software (16) on a VAX-VMS computer. The hydrophobicity profile of the deduced protein was predicted with the GCG program Pepplot, which uses the algorithm of Kyte and Doolittle. The DNA and protein sequences of the D,D-serine-peptidase family proteins were from the European Molecular Biology Laboratory and the Swiss-Prot databases. Multiple alignments of the deduced peptide sequences were carried out with the GCG program Pileup.

The presence of a deletion in the promoter proximal region was verified in the original strains by PCR with the upstream primer (nucleotides [nt] 681 to 695) 5′-CTA CAA TTC GTC CAG-3′ and the downstream primer (nt 1031 to 1055) 5′-TGT ACG AAG AGC AAA CTT ACT CAAA-3′, which bound to the deleted region. Each PCR mixture contained 500 ng of chromosomal DNA, 10 pmol of each primer, 10 mM (each) the four deoxynucleoside triphosphates, and 0.5 U of *Taq* superscript polymerase in 50 μl under the buffer conditions recommended by the supplier. Thirty cycles of PCR were carried out in a Perkin-Elmer Cetus DNA thermal cycler. After a 10-min hot start at 82°C, each cycle included 1 min of denaturation at 94°C, 30 s of annealing at 55°C, and 30 s of

TABLE 2. Plasmids used in the study

Plasmid	Vector (cloning site)	Insert (cloning site, size)	Reference
	pAW8		A. Wada, *S. aureus* ori-pAMα1 *E. coli ori*-ColE1 Tc^r shuttle vector
	pΔ1		Deletion factory (Gibco BRL)
	pTZ18U		Universal cloning vector (Gibco BRL)
pUT-5	pΔ1 (*Bam*HI)	PV1 (*Sau*3A; 8 kb)	This study
pUT-41	pTZ18U (*Pst*I-*Xba*I)	SG511 (*Pst*I-*Xba*I; 3.8 kb)	This study
pUT-46	pAW8 (*Hin*dIII)	PV1 (*Hin*dIII; 5.6 kb)	This study

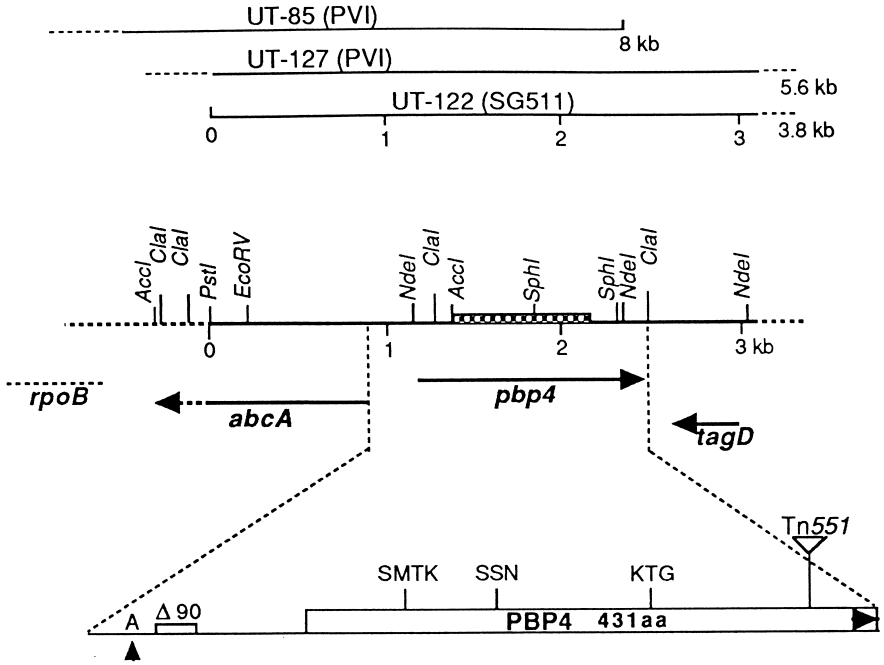

FIG. 1. Restriction map of the *pbp4* region of *S. aureus* SG511. The clones used for sequencing are shown at the top. The solid line of the restriction map indicates the sequenced region. The detailed view shows the restriction sites and the relative positions of the ORFs of the genes *rpoB* (DNA-directed RNA polymerase beta chain), *abcA* (ATP-binding cassette transporter), *pbp4* (penicillin-binding protein 4), and *tagD* (glycerol 3-phosphate cytidyltransferase), with arrows indicating the direction of transcription, when it was known. A dotted bar symbolizes the 0.8-kb internal *pbp4* probe used for hybridizations. The lower part shows an enlargement of the *pbp4* region. The active-site motifs are marked, as is the integration site of the transposon. The 90 nt deleted from Mc^r strain PV1 is indicated by Δ90, and the adenosine insertion in PV1 is indicated (A).

extension at 72°C, with an additional 10 min of extension at 72°C added to the last cycle.

PBPs. Cell membranes from exponentially growing cells were prepared by differential centrifugation as described earlier (9). A total of 10 μg of protein per lane was labelled for 10 min at 30°C in a total volume of 10 μl with 10 μg of [^{3}H]benzylpenicillin (ca. 1 TBq/mmol [Merck]) per ml (final concentration). PBPs were separated by SDS-PAGE on 10% acrylamide–3.75% bisacrylamide minigels (miniprotean; Bio-Rad) at 150 V/h and were visualized by fluorography after 3 days of exposure at −70°C (13).

Nucleotide sequence accession numbers. The *abcA-pbp4-taqD* sequence of SG511 was deposited in GenBank under accession number X87104; that of PV1 was deposited in GenBank under accession number X87105.

RESULTS

Construction and characterization of PBP 4 mutants. The methicillin-resistant mutant strain PV1 was constructed by selection of susceptible *S. aureus* SG511 Berlin strains by growth on increasing concentrations of penicillin and was obtained after the fifth step. The MIC of methicillin rose from 1 μg/ml for strain SG511 to 8 μg/ml for mutant PV1. The increase in the level of resistance was correlated with changes in the amount and/or affinity of the low-molecular-weight PBP 4, which appeared as a band strongly labelled by [^{3}H]benzylpenicillin in membrane preparations separated by SDS-PAGE (Fig. 2, lane c). The subsequent Tn*551*-mediated insertional inactivation of methicillin resistance in PV1 yielded two genetically different types of susceptible strains. In one of the susceptible mutants, FemB was inactivated, resulting in the production of a shortened, triglycine side chain of the peptidoglycan stem peptide and no effect on PBP 4 production (24). In the second type of mutant, represented here by strain UT-6-2 [Ω2007(*pbp4*::Tn*551*)], no PBP 4 was visible in membrane preparations (Fig. 2, lane d). The MIC of methicillin for the inactivated strain UT-6-2 dropped from 8 to 2.5 μg/ml. Transductional studies showed 100% cotransduction of the Mc^s phenotype with the insertion Ω2007(*pbp4*::Tn*551*) when it was crossed back into original Mc^r strain PV1. We therefore speculated that the transposon had integrated into the *pbp4* gene itself or into a region controlling or interacting with its expression.

Sequencing and identification of PBP 4. A 3,233-nt contiguous fragment of SG511 was sequenced (Fig. 3). Two open reading frames (ORFs) and a partial ORF were identified on this sequence. The middle ORF started at the position at 1,239 nt with ATG and was terminated by the stop codon TAA at

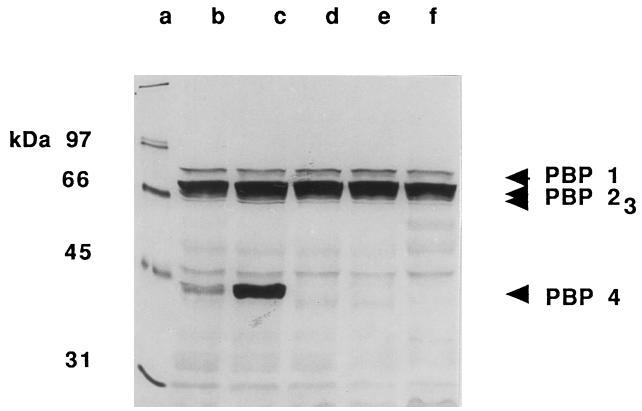

FIG. 2. Fluorography of the ^{3}H-labelled PBPs of different Mc^s and Mc^r *S. aureus* strains. The positions of the single PBPs are indicated by arrows. Lanes: a, molecular mass marker; b, wild-type strain SG511; c, PV1, in vitro-selected Mc^r mutant of SG511; d, Mc^s UT-6-2, derived from PV1 by Ω2007(*pbp4*::Tn*551*) insertional inactivation; e, UT-39-1, backcross of Ω2007 in PV1; f, UT-77-1, backcross of Ω2007 in SG511.

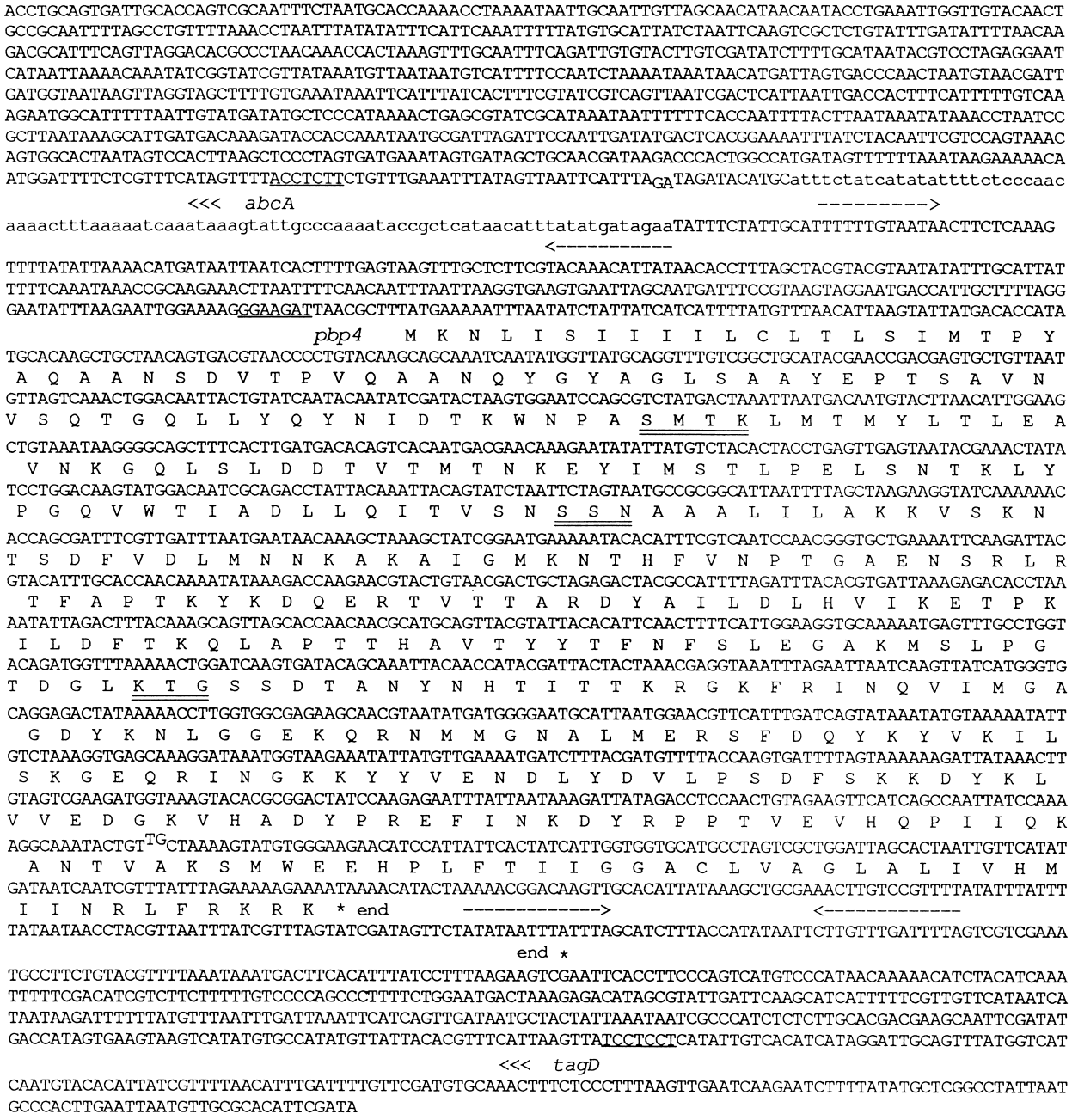

FIG. 3. Nucleotide sequence of the *pbp4* region from wild-type *S. aureus* SG511. Two ORFs and one partly sequenced ORF are named in the order of their appearance: *abcA*, *pbp4*, and *tagD*. The deduced amino acid sequence of *pbp4* is given as a one-letter code. *abcA* and *tagD* are indicated by their corresponding ATG start codons, and the direction of transcription is indicated by <<<. Putative RBSs are underlined. The three active-site motifs of PBP 4 are double underlined. Stop codons are marked by asterisks followed by the word "end." Sequences that have the potential to form inverted repeats are underlined with arrows. The 90 nt deleted from Mc^r strain PV1 are indicated with lowercase letters; the insertion of an adenosine in PV1 occurred between the GA indicated as a subscript. The insertion site of Ω2007(Tn*551*) is located between the TG indicated as a superscript.

2,532 nt. The ORF was preceded by a putative ribosome-binding site (RBS) (GGAGAT) at the position at 1,223 nt (46). The stop codon was followed by a region of dyad symmetry ($\Delta G = -18.3$ kcal/mol) which resembled a rho-independent transcription terminator; this was followed by a T-rich region (43). The deduced 431-amino-acid (aa) protein exhibited a high degree of aa sequence similarity (65 to 80%), with class A low-molecular-weight PBPs and carboxypeptidases (CPases) belonging to the superfamily of penicillin-susceptible and penicillin-interacting enzymes (21, 28). Its deduced molecular mass of 48 kDa was in good agreement with the size of PBP 4 calculated by SDS-PAGE, and it was therefore named *S. aureus* PBP 4. When sequences were aligned by using the Pileup program (16) (see Fig. 4), the best homologies were found to be with *Bacillus subtilis* PBP 5 (35% identical residues within 282-aa overlaps) and *Bacillus stearothermophilus* CPase (31% identical residues within 336-aa overlaps). *S. aureus* PBP 4 also showed similarities to *B. subtilis* PBP 5* (30%), *B. subtilis*

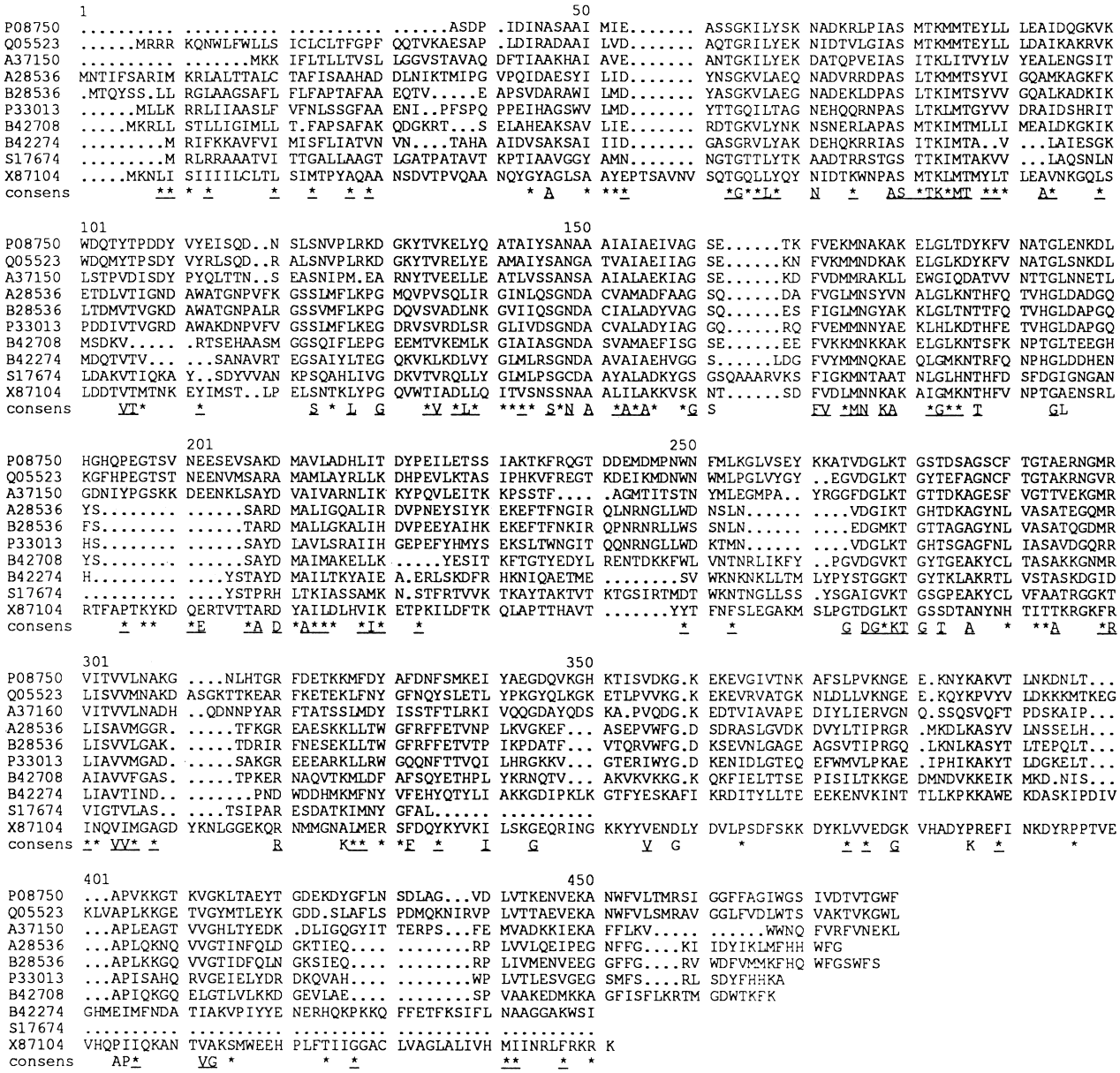

FIG. 4. Alignment of *S. aureus* PBP 4 with several class A low-molecular-mass PBPs. The level of sequence conservation at each position is indicated under the aligned sequences. The aa conserved in 70% of the proteins are exemplified by a letter; the presence of chemically similar aa in 70% of the proteins is indicated with asterisks. The homologies to *S. aureus* PBP 4 are underlined. Chemically similar groups of aa were considered to be (A,G), (S,T), (N,Q), (D,E), (H,K,R), (F,W,Y), and (F,I,L,M,V). Initial sequence similarities were detected by using the Fasta program. Complete and partial PBP sequences were extracted from the databases and were aligned by using the Pileup program (20). The DNA sequence accession numbers symbolize the following proteins: B42274, *B. subtilis* PBP5*; S17674, *Streptomyces* sp. strain K15 CPase; A28536, *E. coli* PBP 5; B28536, *E. coli* PBP 6; P33013, *E. coli* Phse protein; P33013, *B. subtilis* PBP 5; Q05523, *B. stearothermophilus* CPase; B42708, *B. subtilis* SpoVIIA; A37105, *S. pneumoniae* PBP 3; X87104, *S. aureus* PBP 4.

SpoIIA PBP (39%), and *E. coli* PBP 6 (31%), but they were restricted to shorter aa overlaps. The degrees of identity are rather low but are in good agreement with the reported low percentage of homology found for PBPs on the primary structure level (2).

As for all PBPs and β-lactamases, the highly conserved motifs (19, 27, 47) were found in *S. aureus* PBP 4. The SXXK tetrad, containing the active-site serine, which covalently binds β-lactam antibiotics, was located close to the N terminus at the position 76SMTK79. The aa triad SXN, which may act as a proton shuttle, resided at aa position 146SSN148. The C-terminal K(H)T(S)G triad was found at aa position 256KTG261 and was followed by a 172-aa-long extension. In *S. aureus* PBP 4 the conserved domains showed spacings similar to those in the other low-molecular-weight PBPs (19) and exhibited strong homologies within the motif-surrounding regions. The alignments also revealed other regions with a degree of high consensus; these regions were not defined as special boxes, but nevertheless, they showed conserved aa demonstrating common structural elements (Fig. 4).

PBPs are localized in the cytoplasmic membrane, pointing outward to the peptidoglycan. For *S. aureus* PBP 4, an N-terminal signal peptide mediating the translocation of the PBP through the cytoplasmic membrane could be predicted with

the algorithm of von Heijne (52). The characteristic features for signal peptides were found to be a positive charge within the first 50 aa (pI = 10), a highly hydrophobic region within the first 22 aa, and then a few charged aa (T18, threonine; Y20, tyrosine; Q23, glutamic acid) and a signal peptide cleavage site at aa position 24.

For the low-molecular-weight PBPs, the membrane anchor is located at the C terminus (21). As deduced from the hydrophobicity plot (12) (data not shown), the last 33 aa could be predicted to be responsible for anchoring *S. aureus* PBP 4 in the cytoplasmic membrane. The integration site of Tn*551*, localized 39 aa before the C terminus at nt 2,414 in strain UT-39-1, resulted in the truncation of the putative membrane anchor. This was supported by the missing PBP 4 band in membrane preparations of UT-39-1 (Fig. 2).

Homologies of the other ORFs. Downstream of the *pbp4* gene an ORF coding for a putative 132-aa protein was identified; it had 83% similarity and 68% identity to the *B. subtilis tagD* gene (glycerol 3-phosphate cytidyltransferase), which is involved in teichoic acid synthesis (36). The *tagD* gene was transcribed in the direction opposite that of *pbp4* and started with the ATG start codon at the position at 3,049 nt, extending to the stop codon TAA at the position at 2,654 nt. The *tagD* ORF was preceded by a putative RBS (AGGAGGA) at 3,057 nt; the stop codon was followed after 52 nt by the putative rho-independent transcriptional terminator of *pbp4*. Promoter distal from *pbp4*, a divergently transcribed and only partially sequenced ORF was identified. Its deduced aa sequence showed 26% homologies to eukaryotic multidrug resistance proteins (accession numbers P21439, P08183, and P21449) and was 100% identical to *S. aureus abcA*, a putative transporter-like protein (accession number Sau29478) (14). The *abcA* ORF started with the ATG start codon at 819 nt; this was preceded by a putative RBS (AAGAGGT) at 826 nt.

Comparison of wild-type PBP 4 and mutant PBP 4. The *pbp4* DNA sequences of wild-type *S. aureus* SG511 and of mutant PV1 were determined as described in Materials and Methods. Figure 1 shows schematically the differences between the *pbp4* region of PV1 and the *pbp4* region of the wild-type strain SG511. Both sequences differed solely in the noncoding *pbp4* proximal region: the in vitro resistant strain PV1 showed a single adenosine insertion between nt positions 863 and 864 and a 90-nt deletion from nt positions 877 to 952. This deletion removed a structure that was framed by a 14-nt inverted repeat (Fig. 3).

Amplification of chromosomal DNA by PCR with a primer pair surrounding this region showed that the deletion was not a cloning artifact but was indeed present in the PV1 genome. Analysis of another methicillin-resistant *S. aureus* line of clones obtained in vitro from the susceptible SG511 strain and of a clone family selected from strain 8325 in vitro (9), both of which also produced an altered PBP 4, did not contain this deletion, suggesting that in vitro resistance might arise via alternate mechanisms.

Physical mapping of the *pbp4* gene on the *Sma*I map in *S. aureus*. For physical mapping of *pbp4* in *S. aureus*, *Sma*I chromosomal digests were separated by pulsed-field gel electrophoresis. A different banding pattern between the wild-type strain SG511 Berlin and strain 8325 showed that the strains had different genetic backgrounds (24). Southern blots were hybridized with the internal *pbp4* probe, and PBP 4 was mapped to one of the two largest *Sma*I fragments. These fragments run close to each other and are difficult to separate for strain SG511, whereas in strain 8325 *pbp4* was located in the *Sma*I-D fragment (data not shown). By partially sequencing pUT-5, which contains an 8-kb fragment which overlapped with the *pbp4* gene, an ORF was identified. This ORF had 100% sequence identity to the *S. aureus rpoB* gene, which codes for the DNA-directed RNA polymerase beta chain (1). The *pbp4* gene therefore was localized in SG511 within an approximately 10-kb gene cluster containing the *rpoB- -abcA-pbp4-tagD* genes, as shown in Fig. 1.

TABLE 3. MICs of different antibiotics for *S. aureus* strains

Strain[a]	MIC (μg/ml)				
	Methicillin	Imipenem	Cefotaxime	Mecillinam	Cefoxitin
SG511	1	<0.0008	1	128	1
PV1	8	0.03	32	>256	4
UT-6-2	2	0.015	0.25	32	2
UT-39-1	2	0.015	0.25	32	2
UT-77-1	1	<0.0008	0.003	16	1

[a] Strains: SG511, wild-type strain; PV1, in vitro β-lactam-resistant mutant of SG511; UT-6-2, by Ω2007(*pbp4*::Tn*551*) insertionally inactivated mutant of PV1; UT-39-1, backcross of Ω2007(*pbp4*::Tn*551*) in PV1; UT-77-1, backcross of Ω2007(*pbp4*::Tn*551*) in SG511.

Is PBP 4 essential in *S. aureus*? To study the effect of PBP 4 inactivation in different genetic backgrounds, we tried to transduce Ω2007(*pbp4*::Tn*551*) into methicillin-susceptible and methicillin-resistant *S. aureus* strains. The Tn*551*-inactivated strain PV1 was used as a donor, and the various *S. aureus* mutants, stemming either from SG511 or from 8325, were the recipients. Selection was for the erythromycin resistance coded for by the *ermB* gene on Tn*551*. The restriction barrier between strains 8325 and SG511 could be overcome by heat inactivation (as shown earlier for transduction of Ω2006 (*femB*::Tn*551* [24]), but nevertheless, no transductants with Ω2007(*pbp4*::Tn*551*) in 8325 were obtained (transduction frequency, $<10^{-9}$). Even transduction into the restriction-negative *S. aureus* recipient RN4220 yielded no transductants. In contrast, in SG511 backgrounds good transduction frequencies were obtained. A reason for the unsuccessful transductions in 8325 might be a different organization of the chromosomal sequences around the *pbp4* gene. This was supported by a strong restriction site polymorphism between both genetically different strains in the vicinity of *pbp4* (data not shown). When the intrinsically β-lactam-resistant mutant PV1 was inactivated by Ω2007(*pbp4*::Tn*551*), as exemplified by transductant UT-39-1, it showed a decrease in its level of resistance (Table 3) and a loss of PBP 4 overproduction (see PBP profiles in Fig. 2, lane 3). Resistance against different β-lactams differed significantly between insertionally inactivated transductants and recipients. Transduction of Ω2007(*pbp4*::Tn*551*) into susceptible strain SG511 resulted in increased β-lactam susceptibility (Table 3). Compared with the MICs for the insertionally inactivated PV1 strain (UT-39-1), the MICs for the inactivated SG511 strain (UT-77-1) were even lower, suggesting that additional genes must have contributed to resistance in PV1. Since transductants with the truncated PBP 4 are viable, either the truncated protein retained some of its activity or PBP 4 might be dispensible.

DISCUSSION

By Tn*551* insertional inactivation of in vitro methicillin resistance, a mutant was obtained. In this mutant integration of the transposon was clearly linked with a decrease in methicillin resistance. PBP 4 was no longer detectable in membrane preparations. The integration of the transposon was therefore speculated to reside in the *pbp4* gene itself or in a region controlling or interacting with its expression. It turned out that Tn*551*

had integrated into the structural gene of an ORF that we could identify to be PBP 4. Alignments revealed high degrees of similarity (66 to 80%) of *S. aureus* PBP 4 to other low-molecular-weight PBPs and CPases and grouped PBP 4 in the superfamily of penicillin-susceptible and penicillin-interacting enzymes (21, 28). PBPs are evolutionarily related to class A and C β-lactamases and D,D-CPases but, in contrast to β-lactamases, show a low degree of homology among each other at the level of the primary structure (2), as was also shown for *S. aureus* PBP 4, with levels of identity to the members of the superfamily of D,D-serine-proteases of 30 to 35%. The three-dimensional structure reveals similarities to a greater extent, i.e., the organization of domains, the distribution of secondary structure elements, and the architecture of the active-site cavern. The conservation of common structural elements is reflected in three aa fingerprints constituting the active center. *S. aureus* PBP 4 appeared to be well conserved in regard to the homology boxes within the penicillin-binding domain as well as in the motif-surrounding regions.

In contrast to the high-molecular-weight PBPs, which are anchored in the cytoplasmic membrane via an N-terminal, highly hydrophobic region (20), the membrane anchoring of the low-molecular-weight PBPs is mediated by the C terminus. For *E. coli* PBP 5 the C-terminal 18 aa (26) and for *E. coli* PBP 6 the last 17 aa (51) have been shown to be essential for anchoring; they are predicted to form surface-active amphiphilic α-helices (41) capable of interacting with the membrane. In *S. aureus* PBP 4 the putative membrane anchor consists of a highly hydrophobic C-terminal region which seems to be of great importance. Despite the apparently intact active center, suggesting a functional PBP 4, methicillin resistance clearly decreased after Tn*551* truncation of this putative membrane anchor. Since PBP 4 was no longer detectable in membrane preparations, PBP 4 might have become a soluble protein rather than membrane bound. A similar interruption of the C terminus in *B. subtilis* produced a soluble derivative of PBP 5 (54) and that in *Streptococcus pneumoniae* produced a soluble, enzyme-active variant of PBP 3 (45).

E. coli PBPs 2 and 3 are active only in association with intrinsic membrane proteins, like RodA-PBP2, which is involved in maintaining the rod shape of the cell (35), and FtsW-PBP3, which is involved in cell division (25), respectively. In *B. subtilis*, during sporulation, the PBP profile changes and additional sporulation-specific proteins which interact with PBPs are also produced (48). On the other hand, morphological effects that could not be attributed to the missing membrane localization of a soluble *S. pneumoniae* PBP 3 suggested that PBP 3 retained activity during its transport across the cell wall membrane or that the missing C terminus only played a role in the stabilization of the protein (45). We cannot rule out, in our mutant, a residual interplay of PBP 4 with other membrane proteins that might not be affected by the probable mislocalization of PBP 4; therefore, the question of its essentiality for *S. aureus* is still open.

The specific functions of individual *S. aureus* PBPs have not yet been determined. In contrast to *E. coli*, in which the high-molecular-weight PBPs are the lethal targets for β-lactam action and are important for shaping the cell (PBP 2) and cell division (PBP 3), no PBP seems to be an individual lethal target for *S. aureus*. PBP 1 plays a key role within the staphylococcal PBPs without being the lethal target itself (4, 5, 42), because an inhibition of PBP 1 can be overcome by PBPs 2 and 3 (5). The low-molecular-weight PBPs are the most abundant, but they are not essential for cell growth under laboratory conditions, because mutants of *E. coli* defective in PBP 4, PBP 5, or PBP 6 (15), as well as mutants of *B. subtilis* PBP 5 (49) and *S. aureus* PBP 4 (13, 57), are viable. They all have CPase activities in vitro, but their in vivo functions remain unclear. According to Wyke et al. (57), PBP 4 of *S. aureus* H was postulated be needed for secondary cross-linking, similar to *E. coli* PBP 5, which was postulated to regulate the degree of total cross-linking of the murein layer via the amount of free pentapeptides (33). Secondary transpeptidases form cross-links that are not involved in the primary incorporation of peptidoglycan into the growing cell wall and that are therefore thought to be dispensible. Furthermore, in *S. aureus* PBP 4 is supposed to have different functions in strains with different genetic backgrounds (29). Its inactivation in *S. aureus* H clearly decreased the cell wall cross-linking (56), whereas a similar effect was observed for SG511 only after additional inhibition of PBPs 2 and 3 (5).

On the basis of these facts, questions about the function and the importance of the low-molecular-weight PBPs in cell wall metabolism arise. Do they interact and depend on other factors that are involved in peptidoglycan metabolism? The close proximity of the divergently transcribed *abcA* and *pbp4* ORFs (which start within 300 nt from each other) leads us to speculate that their expression might interfere with each other. The mutations in the putative promoter region of strain PV1 are responsible directly or indirectly for the overproduction of PBP 4; inactivation of the overproduced PBP 4 in the mutant as well as inactivation of PBP 4 in the original wild-type parent led to increased levels of β-lactam susceptibility. This proves that PBP 4 affects in vitro-acquired β-lactam resistance. In the 8325 lineage of in vitro-constructed Mc^r *S. aureus*, Chambers et al. (10) measured a decreased deacylation rate for PBP 4, suggesting mutations in the structural gene. Since we showed that no deletion had apparently occurred in that particular strain lineage, it can be deduced that different ways of acquiring resistance exist.

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