ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1996, p. 2121–2125 Vol. 40, No. 9
0066-4804/96/$04.00+0

Penicillin-Binding Protein 4 Overproduction Increases β-Lactam Resistance in *Staphylococcus aureus*

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Received 3 April 1996/Returned for modification 14 May 1996/Accepted 6 July 1996

The *Staphylococcus aureus* mutant strain PVI selected in vitro for methicillin resistance overexpressed penicillin-binding protein (PBP) 4. In the wild-type parent strain the *pbp4* gene was separated by 419 nucleotides from a divergently transcribed *abcA* locus coding for an ATP-binding cassette transporter. The mutant PVI was shown to have a deletion in the *pbp4-abcA* promoter region that affected *pbp4* transcription but not expression of *abcA*. Introduction of the *pbp4* gene plus the mutant promoter region into different genetic backgrounds revealed that PBP 4 overproduction was sufficient to increase in vitro-acquired methicillin resistance independently of other chromosomal genes. The role of the AbcA transporter in methicillin resistance remained unknown.

Besides strains of *Staphylococcus aureus* with classic methicillin resistance, which depends on an additional low-affinity penicillin-binding protein (PBP), PBP 2′, strains with borderline resistance to methicillin but lacking the methicillin resistance determinant *mec* are increasingly being isolated. In moderately resistant clinical isolates of *S. aureus* (MODSA strains), reduced affinities of the strains' own PBPs to penicillin have been proposed as a mechanism of β-lactam resistance (18), in which PBPs 2 and 4 seem to be involved (4). Similarly, resistance acquired experimentally by stepwise selection for growth on increasing concentrations of β-lactam is due to multiple unlinked mutations which lead to structural modifications that lower the affinities to penicillin of some PBPs and/or to PBP overproduction (2, 6, 9), as well as to alterations in membrane protein profiles (3). Point mutations in the PBP 2-encoding gene altering the binding kinetics were shown to be associated with non-*mec*-associated resistance in experimental as well as in clinical strains (8). In a further experimental strain lineage, we showed that PBP 4 had been recruited in methicillin resistance (11). Because the mutant had been selected in multiple steps, overproduction of PBP 4 was postulated not to be the only mutational event (9). The question remained open if other factors besides PBP 4 were involved and needed for the increased resistance of strain PVI. We therefore investigated the role of PBP 4 overproduction in methicillin resistance in different genetic backgrounds.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Tables 1 and 2. They were grown in Luria-Bertani medium (10 g of tryptone per liter [Difco], 5 g of yeast extract per liter [Difco], 5 g of NaCl per liter). The growth temperature was 37°C unless otherwise noted. Transductants and transformants were selected on 10 μg of tetracycline per ml. MICs were determined by the E test (AB Biodisk, Solna, Sweden). Relative differences in resistance were confirmed on antibiotic-gradient plates (7).

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. (14). Restriction enzymes were obtained from Boehringer (Mannheim, Germany) and used as recommended by the supplier.

The *abcA* locus of the NCTC8325 background was cloned from a genomic library of strain BB938 on a 5.1-kb *Sau*3A insert into the shuttle vector pAW8, yielding pUT6 (Fig. 1).

The probe for identification of *pbp4* was a *pbp4*-internal 1.2-kb fragment, amplified by PCR with an upstream primer extending from nucleotides (nt) 2503 to 2517 and a downstream primer extending from nt 3794 to 3808 of the sequence of GenBank accession number X91786 as described previously (10). For probing the *abcA* transporter, an 870-bp PCR product was amplified with an upstream primer extending from nt 765 to 779 and a downstream primer extending from nt 1623 to 1637 of X91786.

DNA sequences were determined by the dideoxynucleotide chain termination method (17) with custom 15-mer oligonucleotides (Microsynth, Balgach, Switzerland) with a Sequenase 2.0 kit (U.S. Biochemical) and α^{35}-S-dATP from Amersham (Little Chalfont, Buckinghamshire, England). For sequence analysis the GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, Wis.) was used on a VAX-VMS computer.

Complementation studies. The *pbp4* open reading frame (ORF), with the complete sequence intervening between *abcA* and *pbp4* and providing the promoter, was amplified by PCR from mutant strain PVI with the upstream primer extending from nt 1900 to 1914 and the downstream primer extending from nt 4107 to 4121 of X91786 and cloned into the *Sma*I site of pAW8, yielding plasmid pUT53 (Fig. 1). The PVI *abcA* gene, truncated C terminally to the last 26 amino acids (aa), was PCR amplified with the upstream primer extending from nt 394 to 408 and the downstream primer extending from nt 2599 to 2613 of X91786 and cloned into the *Sma*I site of pAW8, yielding plasmid pUT54 (Fig. 1). The plasmids pUT53 and pUT54 were first electroporated into restriction-negative *S. aureus* RN4220 and subsequently transduced by phage 85, as described previously (11), into *S. aureus* strains of different genetic backgrounds, selecting for tetracycline resistance. Spontaneous curing of the transductants from the plasmids was done by growing the transductants overnight in drug-free medium and screening them for loss of their plasmid.

Transcription of *pbp4* and *abcA*. Total RNA of exponentially growing cells, harvested at an optical density at 578 nm of 0.8, was prepared with an RNeasy kit from Qiagen (Basel, Switzerland). For Northern (RNA) blotting 10-μg samples of RNA were separated on agarose gels treated with 0.05% diethylpyrocarbonate, soaked in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and transferred by alkaline blotting with 2× SSC–0.5 M NaOH to a nylon membrane (Dupont NEN). The transcripts were probed with either the 1.2-kb *pbp4* PCR fragment or the 870-bp *abcA* probe. An internal 0.8-kb *Eco*RI-*Hin*dIII fragment of the glutamine synthetase gene *glnA* (7) was used as a calibrator and as an internal control for gene expression. The relative differences in the amounts of transcripts were determined with a PhosphorImager (Applied Biosystems).

PBPs. Cell membranes from exponentially growing cells were prepared by differential centrifugation as described previously (2). Thirty micrograms of protein per lane labelled with 10 μg (final concentration) of [^{3}H]benzylpenicillin (ca. 0.5 TBq/mmol; Amersham) per ml was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography after 5 days of exposure at −70°C.

Nucleotide sequence accession number. The *abcA* sequence of NCTC8325 is available from GenBank under accession number X91786.

RESULTS

***S. aureus abcA* gene.** In the susceptible strain SG511, the PBP 4-encoding structural gene was found to be separated by

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TABLE 1. Relevant bacterial strains

S. aureus strain	Genetic background, relevant genotype	Relevant phenotype[a]	Origin and/or method of derivation (reference)
SG511	SG511	Mc^s	Strain collection of the Robert Koch-Institute, Berlin, Germany
PVI	SG511 (multiple mutations)	Mc^r	In vitro-selected Mc^r mutant derived from SG511 (11)
RN4220	NCTC8325-4	Restriction negative	Restriction-negative mutant of RN450 transformable by electroporation (13)
BB255	NCTC8325	Mc^s	(3)
BB938	NCTC8325	Mc^s	Teicoplanin-resistant transformant derived from BB255 (1a)
UT39-1	PVI, Ω2007(*pbp4*::Tn*551*)	Mc^s Em^r	(9)
UT77-1	SG511, Ω2007(*pbp4*::Tn*551*)	Mc^s Em^r	(9)
UT158	RN4220(pUT53)	Tc^r Mc^r	This study, by electroporation of pUT53 into RN4220
UT162	RN4220(pUT54)	Tc^r Mc^s	This study, by electroporation of pUT54 into RN4220
UT168	BB255(pUT53)	Tc^r Mc^r	This study, by transduction of pUT53 from UT158 into BB255
UT170	BB255(pUT54)	Tc^r Mc^s	This study, by transduction of pUT54 from UT162 into BB255
UT179	UT39-1(pUT53)	Em^r Mc^r Tc^r	This study, by transduction of pUT53 from UT158 into UT39-1
UT184	UT39-1(pUT54)	Em^r Mc^s Tc^r	This study, by transduction of pUT54 from UT162 into UT39-1
UT189	SG511(pUT53)	Mc^r Tc^r	This study, by transduction of pUT53 from UT179 into SG511
UT192	SG511(pUT54)	Mc^s Tc^r	This study, by transduction of pUT54 from UT162 into SG511
UT199	UT77-1(pUT53)	Em^r Mc^r Tc^r	This study, by transduction of pUT53 from UT79 into UT77-1
UT202	UT77-1(pUT54)	Em^r Mc^s Tc^r	This study, by transduction of pUT54 from UT162 into UT77-1

[a] Abbreviations: Em^r, erythromycin resistant; Mc^s, methicillin susceptible; Mc^r, methicillin resistant; Tc^r, tetracycline resistant.

only 419 nt from a divergently transcribed ORF, *abcA*, whose deduced partial amino acid sequence showed strong similarities to ATP-binding cassette (ABC) transporters and eukaryotic multidrug resistance proteins (9). The *abcA* gene of the experimentally derived methicillin-resistant mutant strain PVI, which is known to overproduce PBP 4, was identical to that of SG511 over the 1,451 nt that were sequenced. To compare the *abcA* gene from SG511 with that from the better characterized NCTC8325 background, the complete *abcA* gene from BB938 (a NCTC8325 derivative) was cloned and sequenced. The 1,724-nt *abcA* coding region (represented in Fig. 2) starting at nt 478 with ATG was preceded by a putative ribosome binding site (AAGAGGT) at nt 465 and was terminated by the stop codon TAA at nt 2203. The stop codon was followed by an 88-nt-long region containing a 13-nt imperfect repeat (ΔG = 12.0 kcal/mol [50.2 kJ/mol]). This region coded for a deduced protein of 575 aa with a calculated mass of 64.8 kDa.

The *abcA* sequence of BB938 revealed 98% identity to the *abcA* allele of *S. aureus* RN4220-4 (GenBank accession number U29478 [5]), varying mainly at the 3′ end of the sequence, although the two strains are both isogenic derivatives of NTCT8325. Ten amino acid substitutions, making up nonconservative exchanges, could be detected. The *abcA* sequence of PVI, which was determined up to nt 1646, differed from that of BB938 in only 14 nt scattered along the gene, as shown in Fig. 2; from those positions, only two nucleotide exchanges affected the deduced amino acid sequence, namely, Thr-376 to Ala and Asp-530 to His. This close sequence similarity was rather unexpected, since the SG511 derivative PVI was genetically unrelated to both NCTC8325 derivatives BB938 and RN4220-4.

When *S. aureus* AbcA was aligned to other ABC-transporter-like protein sequences from GenBank, the closest similarities were found to be with *Staphylococcus epidermidis* PepT (Z49865) encoded within the lantibiotic *pep5* gene cluster (56% identical residues within an overlap of 593 amino acids). *S. aureus* AbcA also showed similarities to the eukaryotic Xemdr (U17608), the multidrug resistance protein of *Xenopus laevis* (37% identity), and to Ywja (P45861), the *Bacillus subtilis* hypothetical transporter in the *acdA* 5′ region (33% identity). We found lower but still significant degrees of similarity to HlyB (P08716), the hemolysin secretion ATP-binding pro-

TABLE 2. Relevant plasmids

Plasmid	Vector (cloning site)	Insert characteristics (size, restriction fragment, relevant genetic marker)	Source, description
	pAW8		A. Wada, *S. aureus ori*-pAMα1–*E. coli ori*-colE1 Tc^r shuttle vector
pUT6	pAW8 (*Bam*HI)	5.1-kb partial *Sau*3A insert from BB938, *abcA-pbp4*	This study
pUT53	pAW8 (*Sma*I)	2.2-kb blunt PCR product from PVI, *pbp4*	This study, amplicon of *pbp4*
pUT54	pAW8 (*Sma*I)	2.2-kb blunt PCR product from PVI, truncated *abcA*	This study, amplicon of truncated *abcA*

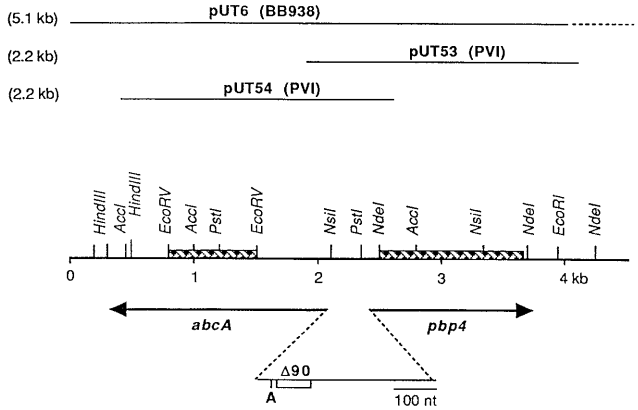

FIG. 1. Restriction map of the *pbp4-abcA* ORF and promoter region of *S. aureus*. Clones used for the sequencing of *abcA* and for *trans* complementation studies are shown at the top. The detailed diagram below shows restriction sites and the relative positions of the ORFs of the genes *abcA* (encoding ABC-transporter-like protein) and *pbp4*, with arrows indicating the directions of transcription. Dotted lines mark the internal *abcA* and *pbp4* probes used for hybridizations. The enlarged diagram of the promoter region indicates the relative positions of the 90-bp deletion and the additional adenosine (A) in PVI.

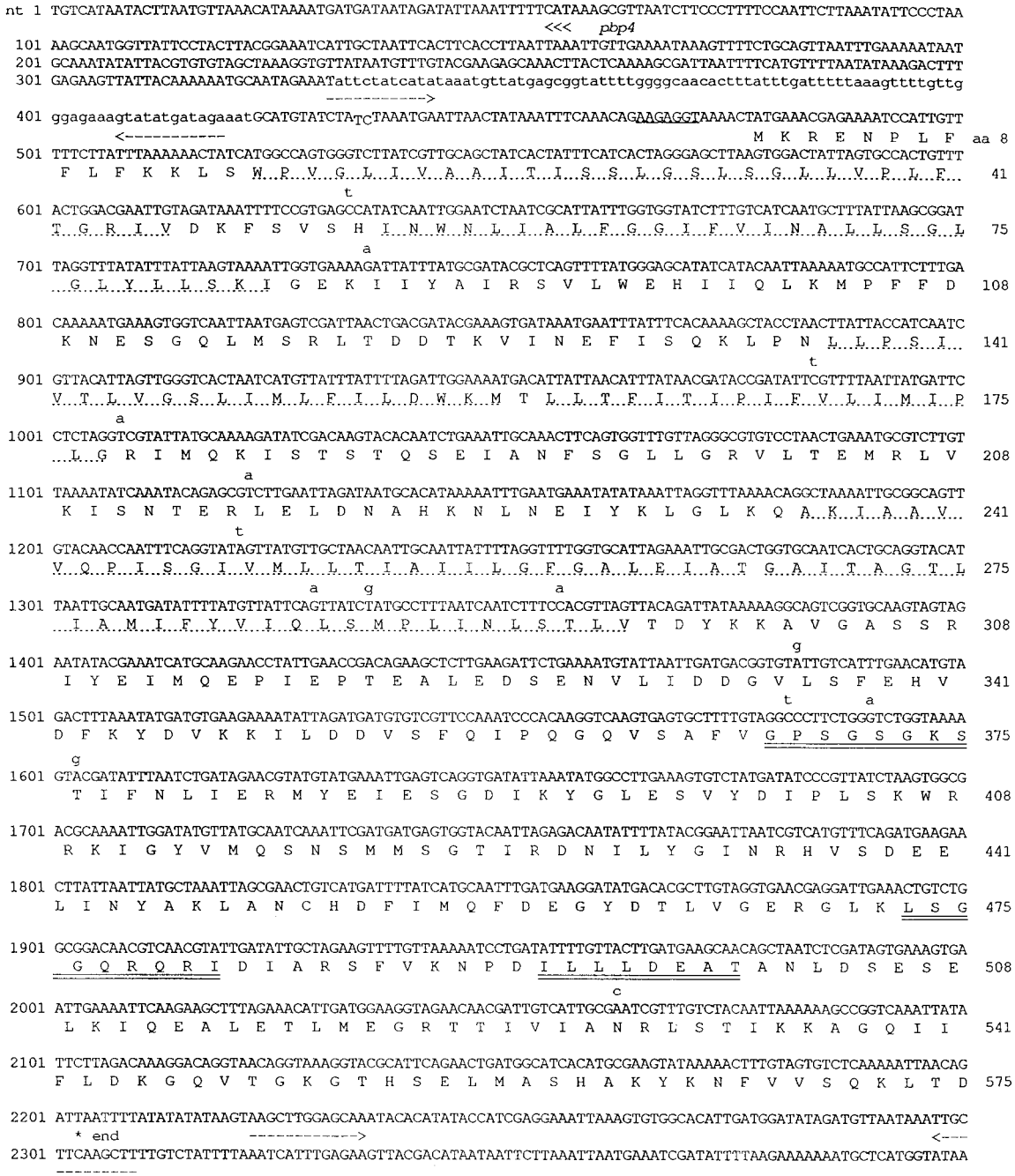

FIG. 2. Nucleotide sequence of the *abcA* region from *S. aureus* NCTC8325. The deduced amino acid sequence of *abcA* starting at the ATG start codon is shown in the one-letter code. The putative ribosome binding site is underlined, and the stop codon is marked by an asterisk followed by the word "end." Sequences that have the potential to form inverted repeats are underlined with dashed arrows. The TMS are underlined with dots, and the Walker motifs I and II and the glutamine- and glycine-rich regions are double underlined. Nucleotides of the SG511 *abcA* allele that differ from the presented NCTC8325 sequence are indicated above the corresponding nucleotide position in lowercase letters. *pbp4* is indicated by its corresponding ATG start codon, and the direction of transcription is marked by <<<. The 90 nt deleted from strain PVI are indicated with lowercase letters; the insertion of an adenosine in PVI occurred between the subscripted T and C. The numbering of nucleotides is indicated on the left, and the numbering of amino acids is indicated on the right. The *abcA* sequence is presented here for reasons of clarity as the inverse sequence of X91786 (nt 116 to 2516).

tein of *Escherichia coli*; ValA (L17003), the *Francisella novicida* virulence operon-associated transporter; the *E. coli* probable transport ATP-binding protein MsbA (P27299); and the *E. coli* multidrug resistance-like ATP-binding protein Mdl (P30751). Like other members of the family of ABC-transporter-like proteins, *S. aureus* AbcA showed the two characteristic motifs for ATP-binding proteins (Walker motifs I and II [19]), which were predicted for aa 368 to 375 and 493 to 500, and a glutamine- and glycine-rich motif resembling the consensus sequence L/FSGGQQ/R/KQR at aa 473 to 481, which is well conserved in ATP-binding components of ABC transporters (1). Whereas the ATP-binding components exhibit high de-

TABLE 3. Expression of the genes *pbp4* and *abcA*

Strain	% Expression of[a]:	
	abcA	*pbp4*
BB255	78	59
SG511	100	100
PVI	131	267
UT39-1	90	15
UT77-1	61	14

[a] The amounts of *abcA* and *pbp4* transcripts were normalized with respect to that of *glnA* after transcription. The values for *abcA* and *pbp4* expression in SG511 were then arbitrarily set at 100% and compared with those for wild-type BB255, PBP 4-overexpressing mutant PVI and its inactivated derivative UT39-1, and UT77-1, which contains an inactivated *pbp4* gene in a SG511 background.

grees of similarity, the hydrophobic components of ABC transporters show structural similarities (12). As for most of the transporters, six putative transmembrane segments (TMS) that were followed by the ATP-binding motif could be predicted for the hydrophobic component of *S. aureus* AbcA. The TMS, in general extending over 20 hydrophobic aa, were found at aa 16 to 46 (TMS1), aa 54 to 83 (TMS2), aa 137 to 158 (TMS3), aa 160 to 177 (TMS4), and aa 236 to 296 (TMS5 and -6), exhibiting topologies similar to those of other members of ABC transporters.

Gene expression of *pbp4* and *abcA*. Overproduction of PBP 4 in the step-selected mutants derived from SG511 started in the fourth-step mutant and coincided with the appearance of a 90-nt deletion comprising a 12-nt inverted repeat in the noncoding *pbp4*-proximal region and an adenosine insertion 12 nt upstream of the deletion in strain PVI (9). Since the *pbp4* structural gene was separated by only 420 nt from the divergently transcribed *abcA* ORF and the deletion started at nt 55 from the *abcA* gene, we could not rule out the hypothesis that the 90-nt deletion affected not only the promoter region of *pbp4* but also that of the *abcA* gene. The levels of gene expression in exponentially growing cells were quantified from Northern blots and hybridized with an internal *abcA* and a *pbp4* fragment, and the values for the sensitive parent strain SG511 were set at 100%. As an internal control and to calibrate gene expression, hybridization was performed with the 0.8-kb *Eco*RI-*Hin*dIII fragment of the glutamine synthetase gene *glnA* (7), an enzyme central to nitrogen metabolism. The in vitro-resistant strain PVI showed an almost three-times-higher level of transcription of *pbp4* than that of its parent, SG511, whereas the level of *abcA* expression was only negligibly higher. Inactivation of *pbp4* by the Tn*551* insertion reduced *pbp4* transcription to 5% of the value seen with wild-type SG511. The *abcA* gene was expressed in similar amounts in the strains BB255, SG511, and PVI and in the *pbp4*::Tn*551*-inactivated derivatives of SG511 and PVI, presuming that the deletion in the promoter region, even though it was closer to the *abcA* ORF, mainly affected *pbp4* transcription (Table 3).

Complementation studies. To evaluate if PBP 4 overproduction was sufficient to increase resistance or if AbcA was involved in some way in PBP 4 production and/or resistance and to determine the influence of the strains' genetic backgrounds on PBP 4 production, *trans* complementation experiments with PBP 4 were performed. For these purposes, either the *pbp4* gene plus the intervening putative promoter region of strain PVI or the putative promoter region plus the major part of *abcA* was cloned onto the shuttle vector pAW8. Plasmid pUT53 carried the complete *pbp4* gene plus its mutant promoter region, whereas pUT54 contained the mutant promoter region plus 96% of the *abcA* gene, termed here *abcA*Δ. These

TABLE 4. MICs of different strains complemented with pUT53(*pbp4*) and pUT54

Strain	Plasmid	MIC (mg/liter)		
		Ampicillin	Cefoxitin	Methicillin
BB255		0.016	3	0.25
UT168	pUT53	0.023	4	1
UT170	pUT54	0.016	3	0.25
SG511		0.023	1	0.75
UT189	pUT53	0.047	2	1.5
UT192	pUT54	0.023	1	0.75
UT77-1		0.016	0.5	0.5
UT199	pUT53	0.064	1.5	2
UT202	pUT54	0.023	0.5	0.5
PVI		0.25	4	6
UT39-1		0.032	2	1
UT179	pUT53	0.25	4	6
UT184	pUT54	0.023	1.5	1

recombinant plasmids were introduced into different genetic backgrounds, namely, susceptible NCTC8325 strain BB255, susceptible strain SG511 and its *pbp4*::Tn*551*-inactivated derivative UT77-1, and UT39-1, the *pbp4*::Tn*551*-inactivated derivative of PVI. The changes in resistance caused by the introduction of pUT53 or pUT54 were monitored by comparing MICs (Table 4) and were verified by examining growth on methicillin-containing gradient plates. The MICs of ampicillin, methicillin, and the PBP 4-specific inhibitor cefoxitin for both SG511 and NCTC8325 strains containing pUT53(*pbp4*), independent of the strains' genetic backgrounds, increased in comparison with those of the susceptible control strains. These data were confirmed by comparing growth of these strains on plates containing an antibiotic gradient (data not shown). The effect of PBP 4 overproduction was especially pronounced against methicillin. All strains were cured of their respective plasmids and regained their original susceptibilities. Whereas *trans* complementation with the *pbp4* gene increased resistance, the introduction of pUT54, which contained the putative promoter region and the major part of *abcA*, had no apparent effect on resistance.

Membrane preparations of the different strains comple-

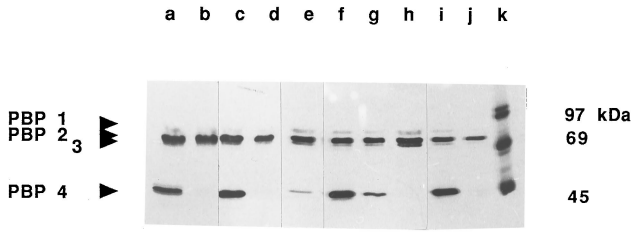

FIG. 3. Fluorography of the ^{3}H-labelled PBPs of different methicillin-susceptible and methicillin-resistant *S. aureus* strains. The positions of the single PBPs are indicated by arrowheads. Lanes: a, PVI, in vitro-selected methicillin-resistant mutant of SG511; b, methicillin-susceptible UT39-1, derived from PVI by Ω2007 (*pbp4*::Tn*551*) insertional inactivation; c, methicillin-resistant UT179 [UT39-1, complemented with pUT53(*pbp4*)]; d, methicillin-susceptible UT184 (UT39-1, complemented with pUT54); e, SG511, wild type; f, methicillin-resistant UT 189 [SG511, complemented with pUT53(*pbp4*)]; g, methicillin-susceptible UT192 (SG511, complemented with pUT54); h, methicillin-susceptible UT77-1, derived from SG511 by Ω2007 insertional inactivation; i, methicillin-resistant UT199 [UT77-1, complemented with pUT53(*pbp4*)]; j, methicillin-susceptible UT202 [UT77-1, complemented with pUT54); k, molecular mass markers.

mented with pUT53 and pUT54 were labelled with [^{3}H]benzylpenicillin and separated by SDS-PAGE (Fig. 3). The increase in resistance due to introduction of the *pbp4* gene correlated with a strong labelling of PBP 4 in PBP profiles. On the other hand, strains complemented with the promoter region plus part of the *abcA* gene revealed no changes in PBP 4 production and no gain in resistance. Further differences in the labelling of the other PBPs could not be detected after introduction of either plasmid.

DISCUSSION

On the basis of the identities of the *pbp4* genes of SG511 and its step-selected, PBP 4-overproducing, methicillin-resistant mutant PVI, and corroborated by the increase in β-lactam resistance obtained by complementing different strains in *trans* with the PBP 4-overproducing plasmid pUT53, overexpression of PBP 4 can be proposed here as a mechanism contributing to a small but reproducible increase in β-lactam resistance. Increased PBP 4 production has been shown previously to be linked to greater cross-linking of the peptidoglycan (10), suggesting for PBP 4 a role as a secondary transpeptidase.

Unexpectedly, the deletion located closer to the *abcA* putative promoter region apparently stimulated *pbp4* but not *abcA* transcription. The role of the closely linked divergently transcribed *abcA* gene in PBP 4 overproduction is still unknown. Our results suggest no direct relationship between *abcA* and *pbp4* regulation, since the same amounts of *abcA* transcripts could be detected independently of *pbp4* overproduction.

In contrast, Domanski and Bayles (5) showed an increased resistance to the PBP 4-specific inhibitor cefoxitin and increased levels of *pbp4* transcripts in an *abcA* knockout mutant, postulating that *S. aureus* AbcA regulates PBP 4 production and thus controls the level of cross-linking in the staphylococcal cell wall. Their observation might be due to additional changes in the *abcA-pbp4* promoter region that led presumably to PBP 4 overproduction in the *abcA* knockout mutant, whereas in our mutant the upstream deletion in the putative promoter region seemed to trigger PBP 4 overproduction.

The function of *S. aureus* AbcA has not been elucidated yet. Several bacterial exporters have been found to contain accessory membrane-bound proteins, not unlike *abcA* and *pbp4*, which are separated by only 419 nt. The lantibiotic Pep5-associated transporter PepT in *S. epidermidis*, to which AbcA showed the highest similarity, is likewise located upstream of the structural gene cluster on the complementary strand, but it is not essential for Pep5 production (16). The ABC transporters form a large family of membrane transporter systems found in both prokaryotic and eukaryotic cells that function in the import or export of a wide range of products, such as proteins, peptides, polysaccharides, vitamins, and drugs utilizing ATP as the source of energy (12). In addition, the function of an ABC transporter may be connected to the regulation of cellular functions and signal transduction. ABC transporters have a complex structure consisting of a transmembrane component(s) (TMS) channeling the solute to the cytoplasm and an ATP-binding component(s) providing energy to the system. *S. aureus* AbcA seems to belong to the structural group of *hlyB*-like genes, in which ATP and TMS domains are transcribed as one gene. A possible role for *S. aureus* AbcA is to transport either cell wall precursors, which are cross-linked by the action of PBP 4, or D-alanine, the product of the cross-linking reaction, into the cytoplasm. Similarly, in *Citrobacter freundii*, cell wall muropeptides are transported into the cytoplasm by the specific transport protein AmpG (15).

Whereas the role of AbcA is still unknown, PBP 4 overproduction clearly contributes, although in a small way, to increased β-lactam resistance. These results were obtained with experimentally constructed strains. It seems likely that similar mechanisms may operate in nature, presumably in certain low-level-resistant MODSA strains. The occurrence and relevance of PBP 4 overproduction in clinical isolates will have to be investigated.

ACKNOWLEDGMENTS

This work was supported in part by Swiss National Science Foundation grant 3100-042182.90/1. Uta Henze received a fellowship from the Bonizzi-Theler Stifung.

We thank A. Wada for plasmid pAW8.

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