JOURNAL OF BACTERIOLOGY, Apr. 1998, p. 1831–1840 Vol. 180, No. 7
0021-9193/98/$04.00+0

Acquisition of Five High-M_r Penicillin-Binding Protein Variants during Transfer of High-Level β-Lactam Resistance from *Streptococcus mitis* to *Streptococcus pneumoniae*

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Received 29 October 1997/Accepted 2 February 1998

Penicillin-resistant isolates of *Streptococcus pneumoniae* generally contain mosaic genes encoding the low-affinity penicillin-binding proteins (PBPs) PBP2x, PBP2b, and PBP1a. We now present evidence that PBP2a and PBP1b also appear to be low-affinity variants and are encoded by distinct alleles in β-lactam-resistant transformants of *S. pneumoniae* obtained with chromosomal donor DNA from a *Streptococcus mitis* isolate. Different lineages of β-lactam-resistant pneumococcal transformants were analyzed, and transformants with low-affinity variants of all high-molecular-mass PBPs, PBP2x, -2a, -2b, -1a, and -1b, were isolated. The MICs of benzylpenicillin, oxacillin, and cefotaxime for these transformants were up to 40, 100, and 50 μg/ml, respectively, close to the MICs for the *S. mitis* donor strain. Recruitment of low-affinity PBPs was accompanied by a decrease in cross-linked muropeptides as revealed by high-performance liquid chromatography of muramidase-digested cell walls, but no qualitative changes in muropeptide chemistry were detected. The growth rates of all transformants were identical to that of the parental *S. pneumoniae* strain. The results stress the potential for the acquisition by *S. pneumoniae* of high-level β-lactam resistance by interspecies gene transfer.

Since the first reports of the phenomenon in the 1970s, penicillin-resistant isolates of *Streptococcus pneumoniae* have been reported with increasing frequency worldwide (3, 25, 43). New bacterial clones emerge via intra- and interspecies gene transfer involving the penicillin resistance determinants, the penicillin-binding proteins (PBPs) (20, 48). The gene pool which is accessible to the pneumococcus includes a variety of commensal streptococci, similar to the situation which exists for another naturally transformable bacterial group, the neisseriae (1, 32, 35, 49).

Penicillin resistance in clinical isolates of *S. pneumoniae* is due to altered PBPs with a reduced affinity to penicillin, i.e., a higher antibiotic concentration is required to inhibit their in vivo function. Such low-affinity PBP variants are encoded by mosaic genes in which sequence blocks are replaced by homologous genes that diverge approximately 20% from the DNA sequence of susceptible isolates (12, 31, 34). PBP2b and PBP2x constitute primary resistance determinants and confer low-level β-lactam resistance (15, 19, 29). PBP2b does not interact with expanded-spectrum cephalosporins (23) and thus is not involved in resistance to this group of β-lactams (9, 36). PBP1a is required for high-level β-lactam resistance (15, 36, 41). Although the role of these three PBPs in β-lactam resistance has been clearly established, it is not certain whether the other two high-M_r PBPs, PBP2a and -1b, also participate in this process. Some penicillin-resistant clinical isolates contain a low-affinity PBP2a (31), and PBP2a is also affected in cefotaxime-resistant laboratory mutants (27). Since the DNA sequences of these genes were not previously available, investigations on the molecular level have been lacking.

Interspecies gene transfer of a variety of antibiotic resistance markers has been documented between different streptococcal species, all of which are naturally transformable (5, 39). Identical or closely related DNA sequences of PBP genes occur in penicillin-resistant *S. pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguis* (10, 14, 37, 38, 40), and transformation of penicillin resistance from one *Streptococcus* sp. to another accompanied by changes in PBPs has been reported on several occasions (6, 8, 47). *S. oralis* and *S. mitis* have been implicated as potential origins for PBP2x and/or PBP2b, which act as resistance determinants in *S. pneumoniae* (11, 47).

In 1994, two *S. mitis* strains, B6 and B7, were isolated in Germany that had unusually high resistance levels to a variety of β-lactam antibiotics, i.e., the MICs of cefotaxime and benzylpenicillin was >32 μg/ml (unpublished data). The strains have identical properties and probably belong to one clonal group. A combination of MICs as high as those for the *S. mitis* isolates has not been reported for *S. pneumoniae* but would be disastrous from a therapeutic standpoint. In the study described here we have investigated whether and to what degree resistance to cefotaxime, benzylpenicillin, and oxacillin could be transferred into a penicillin-susceptible laboratory *S. pneumoniae* strain by successive transformations using chromosomal DNA from *S. mitis* B6, and we have analyzed alterations in PBPs and the murein of resistant transformants. High-level β-lactam-resistant transformants were obtained that showed successive alterations in up to five PBPs. Transfer of *pbp2a* and *pbp1b* alleles into pneumococci during selection with cefotaxime could be documented for the first time.

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TABLE 1. Susceptibilities to β-lactam antibiotics of *S. pneumoniae* transformants obtained with *S. mitis* B6 DNA

Strain or transformant[a]	Selective antibiotic[b] (μg/ml)	PBP changed[c]	MIC (μg/ml)			
			Cefotaxime	Oxacillin	Benzylpenicillin	Piperacillin
S. pneumoniae R6			0.02	0.08	0.02	0.04
S. mitis B6			50	120	50	64
$R6_T$-C	C (0.2)	2x	0.2–0.25	0.2–0.5	0.03	<0.2
$R6_{T3}$-C	C (0.2)	2x	0.2–0.25	0.2–0.5	0.03	<0.2
$R6_T$-CC	C (2)	2x, 2a	1.5–2	0.5	0.02	<0.2
$R6_{T2}$-CC	C (2)	2x, 2a	1.5–2	0.5	0.02	<0.2
$R6_T$-CCC	C (6)	2x, 2a, 1a	12–16	1–2	0.05–0.06	<0.2
$R6_{T5}$-CCC	C (6)	2x, 2a, 1a, 1b	12–16	0.5–1	0.05	<0.2
$R6_T$-CCCO	O (200)	2x, 2a, 1a, 1b, 2b	25–30	60–80	1–3	2–4
$R6_T$-$CCCO_{2b}$	O (20)	2x, 2a, 1a, 1b, 2b	25–35	60	1–2	2–4
$R6_T$-CCCB	B (10)	2x, 2a, 1a, 1b, 2b	50	80–100	40	16
$R6_T$-CCP	P (0.1)	2x, 2a, 2b	1–2	4–16	0.05–0.12	0.5–4
$R6_T$-P	P (0.1)	2b	0.02	0.1	0.02	0.1–0.15

[a] Transformants were obtained with chromosomal DNA or in the case of $R6_T$-$CCCO_{2b}$ with the cloned PBP2b gene of *S. mitis* B6. Between three and eight transformants were tested. Boldface indicates the transformants used as recipients in successive transformations. Thus, $R6_{T5}$-CCC is a third-step transformant obtained after three successive selections with increasing concentrations of cefotaxime and is the parental strain of (i) $R6_T$-CCCO transformants obtained via oxacillin selection with chromosomal *S. mitis* B6 DNA, (ii) $R6_T$-$CCCO_{2b}$ (oxacillin selection by using cloned PBP2b of *S. mitis* B6 as donor), and (iii) $R6_T$-CCCB (transformation with chromosomal DNA and selection with benzylpenicillin).

[b] Antibiotics used for selection are listed in the order of the transformation steps used. The concentrations used at the last selection steps are indicated. C, cefotaxime; P, piperacillin; O, oxacillin; B, benzylpenicillin.

[c] 2x, PBP2x; 2a, PBP2a; 1a, PBP1a; 1b, PBP1b; 2b, PBP2b.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. pneumoniae* R6 is a nonencapsulated derivative of the Rockefeller University strain R36A (2). *S. mitis* B6 was isolated from a hospital in Bochum, Germany, and will be described elsewhere (unpublished data). Streptococci were grown in liquid culture in a casein-based semisynthetic medium supplemented with 0.2% yeast extract (26). MICs of β-lactam antibiotics were tested by agar dilution on blood agar plates (3% sheep blood). Since differences between individual transformants can easily escape detection, when twofold dilutions are used, a narrow range of antibiotic concentrations was used especially for high concentrations of antibiotics. *Escherichia coli* INVαF′ was used for cloning (TA Cloning Kit; Invitrogen, Leek, The Netherlands) the PBP-PCR product. SOC medium (42) was used during the cloning procedure; for glycerol stocks and plasmid preparation, cells were grown in Luria broth containing 100 μg of ampicillin/ml.

Transformation experiments. *S. pneumoniae* R6 was transformed essentially according to published procedures by 30 min of incubation in the presence of DNA at 30°C followed by a 2-h phenotypic expression period at 37°C (29, 50). Purification of chromosomal DNA from *S. pneumoniae* (29) and oral streptococci (47) has been previously described. Transformants were selected in blood agar plates at the antibiotic concentrations specified in Results. The transformants were named according to the selective antibiotics (C, cefotaxime; O, oxacillin; B, benzylpenicillin; P, piperacillin). For example, $R6_T$-CCP is a third-step transformant obtained after three successive rounds of transformation and selection with cefotaxime (the first two steps) and piperacillin. Individual transformants are specified by number.

Amplification and sequencing of PBP genes. PBP genes from *S. mitis* B6 and the *S. pneumoniae* R6 transformants were amplified with the indicated oligonucleotide primers as follows: *pbp2x*, Pn2xUP and Pn2xDOWN (to amplify the region between codon 85 and the 3′ end of the gene [31]); *pbp2b*, 5′-TTAAGT TAGAAATGAGAC and 5′-TTTCCTTTCTAGTTCATTGG (13); *pbp1a*, 5′-G AGAGCAAATTAGTCGCAAC and 5′-ACAAACATTTCATCTGGAGCTAC (33); *pbp2a*, 5′-GTGAACTAGAGGACTCTG and 5′-GAAATAGATTGACT ATCG; *pbp1b* (from *S. pneumoniae* R6), 5′-GTGGTATAATAGATAAAGTG AGG and either 5′-CCCTTGAAGAAGAAGGTCG (for *S. pneumoniae* R6) or 5′-CCCTTGACATCAACACCC (for the transformant $R6_{T5}$-CCC). Sequences of the *pbp1b* and *pbp2a* genes were obtained from a collaboration between Hoffmann-La Roche and Human Genome Sciences Inc., Rockville, Md. Amplification of chromosomal DNA with PCR was carried out in a biomed Thermocycler for 30 cycles of denaturation at 96°C for 30 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min, followed by a 3-min delay period at 72°C after the last cycle. The reaction mixture (100 μl) contained 10 pmol of each oligonucleotide primer and 2.5 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.). DNA fragments were purified with the QIAEX II gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. PCR-derived DNA fragments were cloned into the PCR II vector (Invitrogen). DNA sequences were determined for at least two clones. In cases of discrepancies between the two sequences, PCR-amplified chromosomal DNA was directly sequenced by using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer). Automatic cycle sequencing was conducted with an ABI 377 sequencer.

Preparation of peptidoglycan. In contrast to the methodology used previously where the peptide network of *S. pneumoniae* was analyzed after amidase digestion (17), we isolated the murein sacculi with its saccharide backbone and used cellosyl, a muramidase for enzymatic hydrolysis (kindly provided by R. Marquardt, Hoechst AG, Frankfurt, Germany). A 500-ml culture of an exponentially growing culture (80 nephelometry units) was quickly chilled in an ethanol-ice bath. Cells were collected by centrifugation, resuspended in H_2O, and immediately boiled in 4% sodium dodecyl sulfate (SDS). Cell walls were washed in H_2O–1 M NaCl to remove the SDS and then purified by incubation with 200 μg of pronase/ml for 16 h, which was followed by the addition of 200 μg of trypsin/ml for 16 h. The mixture was then washed three times with 1% SDS, 8 M LiCl, and 100 mM EDTA and then was washed at least twice with H_2O. Teichoic acid was removed with HF (7% [vol/vol], final concentration) for 36 h at 4°C prior to digestion with cellosyl (0.3 mg/ml) for at least 24 h at 37°C. Undigested cell wall material was removed by centrifugation (30 min, 40,000 rpm, SW50.1 Ti rotor in a Beckman ultracentrifuge); at least 95% of the material remained soluble.

Separation and analysis of muropeptides. Samples were mixed with equal volumes of borate buffer (0.5 M, pH 9) and reduced with sodium borohydride for 15 min at room temperature. Excess borohydride was destroyed by the addition of H_3PO_4 (20%, wt/vol) up to a pH of 4. Samples were kept at −20°C. Separation of the digested cell wall components was performed according to the procedure of Glauner (24) with some modifications. The high-performance liquid chromatography (HPLC) system consisted of a Merck L6200A pump and a L4250 UV detector, a Waters 717 autosampler, and a Merck D2500 chromato-integrator. Samples were applied to an Interchim (250 by 4.6 mm) reverse-phase column (ODS hypersil C_{18}, 3 μM). The column was eluted at a low rate of 0.5 ml/min for 10 min with 0.05% (vol/vol) trifluoroacetic acid in water and subsequently with a 90-min linear acetonitrile gradient (0 to 20%, vol/vol) in 0.035% trifluoroacetic acid. The column temperature was maintained at 30°C, and the eluted compounds were detected by absorption at 210 nm.

Identification of the peaks. Liquid chromatography-mass spectrometry was performed using a Waters 600 MSHPLC pump system and a Waters model PD1991 with a diode array detector system liquid chromatograph coupled to a Finnigam (San Jose, Calif.) TSQ7000 triple quadrupole mass spectrometer. Data acquisition was performed between 300 and 2,500 Da with scan times on the order of 2 to 3 s. The fact that molecules of higher mass were multiply charged allowed identification of these structures. The muropeptide structures were deduced from mass spectrometry data taking in account the previous described structures and their elution time (17, 46).

Detection of PBPs. Cells of exponentially growing cultures were collected by centrifugation and resuspended in 10 mM Na phosphate buffer (pH 7.2)–0.1% (wt/vol) Triton X-100; in the case of *S. mitis*, 0.5 mg of cellosyl/ml and 0.8 mg of lysozyme/ml were added (40). PBPs were labeled with 10 μl of a cell suspension corresponding to 10^7 cells with ^{3}[H]propionylampicillin for 30 min at 37°C (22); routinely, approximately 1 μCi of radioactive β-lactam was used per sample. The specific radioactivity of ^{3}[H]propionylampicillin was not determined directly and was estimated to be almost that of the ^{3}[H]succinimidylpropionate (90 to 100 Ci/mmol; Amersham, Buckinghamshire, England) used for its synthesis. Proteins were separated by polyacrylamide gel electrophoresis. The final acrylamide concentration of the separation gel was 7.5% with a ratio of acrylamide:bisacrylam-

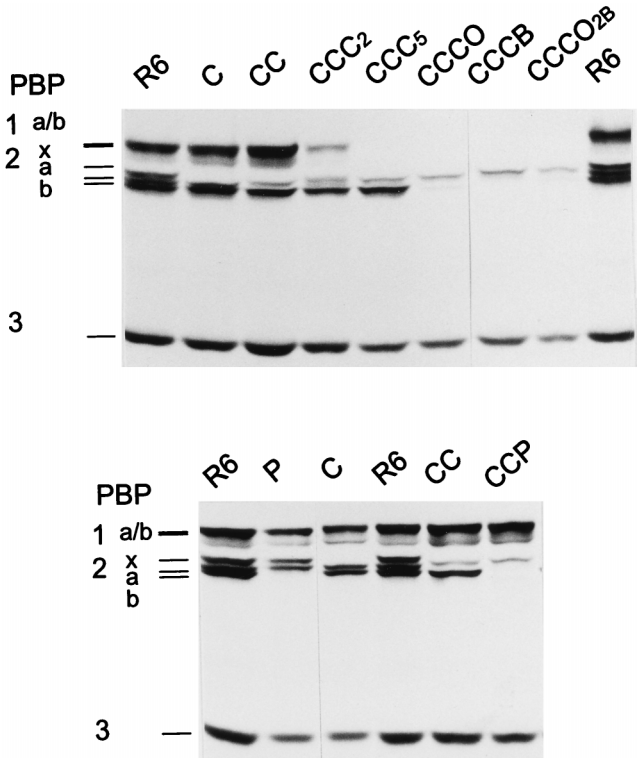

FIG. 1. PBP profiles of β-lactam-resistant *S. pneumoniae* transformants. PBPs in whole-cell lysates were labeled with [^{3}H]propionylampicillin and separated on sodium dodecyl sulfate-polyacrylamide gels followed by fluorography. PBPs of the parental *S. pneumoniae* R6 are indicated on the left. C, CC, and CCC, respective transformants obtained after one, two, and three successive transformations and selections with cefotaxime; other transformants were obtained after selection with P (piperacillin), O (oxacillin) or B (benzylpenicillin). All transformants were obtained with chromosomal DNA except for the transformants $CCCO_{2B}$, where cloned PBP2b of B6 was used as donor DNA. CCC2 ($R6_{T2}$-CCC) and CCC5 ($R6_{T5}$-CCC) were isolated from the same transformation experiment.

ide of 30:1.1, as described for optimal separation of PBP2x, -2a, and -2b (22). PBPs were visualized after fluorography. Alternatively, proteins were blotted onto nitrocellulose filter, and PBPs were detected after immunostaining with rabbit antisera and mouse monoclonal antibodies directed against *S. pneumoniae* PBP1a, -2b, or -2x as described previously (21, 30).

Nucleotide sequence accession numbers. The PBP sequences have been deposited in the EMBL and GenBank databases under the following accession numbers: *S. pneumoniae* R6 *pbp2a*, AJ002292; *S. pneumoniae* R6 *pbp1b*, AJ002291; *S. mitis pbp2b*, AJ002289; *S. mitis pbp2x*, AJ002288; *S. mitis pbp1a* (fragment), AJ002290; *S. mitis pbp1b* (fragment), AJ002293; and *S. mitis pbp2a* (fragment), AJ002294.

RESULTS

Cefotaxime-resistant transformants of *S. pneumoniae* R6. With chromosomal DNA from *S. mitis* B6 and the penicillin-susceptible laboratory *S. pneumoniae* strain R6, three successive transformations were required to obtain transformants which required cefotaxime MICs of 16 μg/ml (Table 1). PBP profiles of the transformants obtained after the first, second, and third selection steps ($R6_T$-C, $R6_T$-CC, and $R6_T$-CCC, respectively) were investigated, and examples are shown in Fig. 1.

So far, only the *pbp2x* and *pbp1a* genes, which could even be transferred simultaneously, have been documented to be sufficient for cefotaxime resistance in *S. pneumoniae* (9, 36). As expected, all first-level transformants contained a low-affinity PBP2x (Fig. 1 and 2). All eight second-level transformants, however, also contained a low-affinity PBP2a, whereas PBP1a could still be perfectly labeled (Fig. 1 and 2). Even after a third transformation with 4 μg of cefotaxime/ml for selection, four of the eight transformants analyzed still contained only low-affinity PBP2x and PBP2a, suggesting that either not all changes relevant for resistance of these PBP genes had been transferred before or that another non-PBP gene may contribute to resistance; the other four transformants contained low-affinity PBP1a as well (not shown). Only with 6 μg of cefotaxime/ml as the selective concentration at the third step did all eight $R6_T$-CCC transformants analyzed have a low-affinity PBP1a; surprisingly, one of them ($R6_{T5}$-CCC) contained a low-affinity PBP1b as well (Fig. 1 and Table 1). None of the cefotaxime-resistant transformants contained an apparent low-affinity PBP2b, since it is not a target for this class of cephalosporins (23).

The cefotaxime resistance increased from 0.02 μg/ml for the sensitive recipient roughly 10-fold in the first-step transformants, and the cefotaxime MIC for the second-step transformants with low-affinity PBP2x and -2a was already up to 2 μg/ml, i.e., values which are generally associated with high-level penicillin-resistant clinical isolates (Table 1). MICs for the third-step transformants, 12 to 16 μg/ml, are very high values for *S. pneumoniae*, but they are still below those of the donor *S. mitis* strain. The differences between the transformants and the donor strain were still enormous for oxacillin (MIC of 0.5 to 1 versus 120 μg/ml) and benzylpenicillin (MIC of <0.06 versus 50 μg/ml).

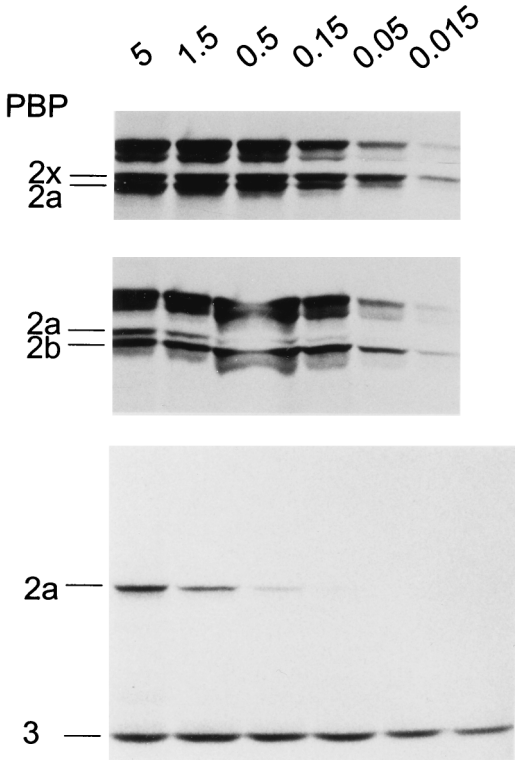

FIG. 2. Affinities of PBPs in β-lactam-resistant transformants of *S. pneumoniae* R6. Cell lysates of the transformants were incubated with the relative [^{3}H]propionylampicillin concentrations (in microcuries) indicated at the top. PBPs of the transformants are indicated to the left. (Top) $R6_{T1}$-P with a low-affinity PBP2b; (middle) $R6_{T2}$-CC with a low-affinity PBP2x; (bottom) $R6_{T4}$-CCCB (all high-M_r PBPs are low-affinity variants and only the low-M_r PBP3 is labeled at all concentrations).

Penicillin-resistant transformants. Since PBP2b plays a major role in resistance to penicillin antibiotics (4, 15, 41), we tested whether selection with penicillins can be used to transfer the *S. mitis pbp2b* into R6. Low-affinity PBP2b variants can be selected in the *S. pneumoniae* R6 background with low concentrations of piperacillin but not with benzylpenicillin or oxacillin (19). Selection of *S. pneumoniae* R6 transformants with chromosomal *S. mitis* B6 DNA was possible between 0.05 and 0.1 μg of piperacillin/ml. The three transformants analyzed contained a low-affinity PBP2b (Fig. 1 and 2). Two of them contained a low-affinity PBP2x as well (not shown). The MIC of piperacillin increased from 0.04 μg/ml for *S. pneumoniae* R6 to 0.15 μg/ml for the transformants, whereas the oxacillin MIC increased only marginally and those of cefotaxime and benzylpenicillin did not change at all (Table 1).

After demonstrating that the gene encoding the *S. mitis* PBP2b could be transferred into *S. pneumoniae* R6, transformants were isolated containing the low-affinity PBP2b in different combinations with other low-affinity PBPs by using different cefotaxime-resistant transformants as recipients as follows: $R6_{T2}$-CC, which contained low-affinity PBP2x and -2a, and $R6_{T5}$-CCC, with low-affinity PBP2x, -2a, -1a, and -1b (Table 1).

With $R6_{T2}$-CC as recipient, transformation with B6 DNA and selection with piperacillin resulted in $R6_T$-CCP transformants, which contained low-affinity PBP2x, -2a, and -2b (Fig. 1). A marked increase in the piperacillin MIC became evident for all four transformants analyzed. The piperacillin MIC varied between 0.5 and 4 μg/ml, and the oxacillin MIC increased up to eightfold to 16 μg/ml (Table 1). The cefotaxime resistance remained basically unchanged. The cefotaxime, oxacillin, and piperacillin MICs for the $R6_T$-CCP transformants were in the concentration range considered to be high resistance, and it should be noted that despite these high values, no changes in the affinity of PBP1a were apparent. In contrast, benzylpenicillin MICs were still rather low and did not exceed 0.15 μg/ml.

In order to construct transformants with affinity changes in all high-M_r PBPs, the $R6_{T5}$-CCC transformant affected in PBP2x, -2a, -1a, and -1b was used as recipient. First, oxacillin was used as the selective antibiotic. Very high concentrations between 200 and 500 μg/ml were required for selection, probably due to the relative instability of the drug. The oxacillin MIC for the five transformants analyzed increased from ≤1 to 60 to 80 μg/ml. The same MIC range was obtained when the cloned *pbp2b* gene was used as donor DNA, demonstrating that it is indeed PBP2b alone that was responsible for the resistance increase ($R6_T$-CCCO2b in Fig. 1 and Table 1). However, in all cases resistance to benzylpenicillin was still low compared to that of the donor strain. Therefore, this compound was used to select another set of transformants, $R6_T$-CCCB, for which MICs of benzylpenicillin were 40 μg/ml and MICs of cefotaxime and oxacillin were also higher compared to those of the oxacillin-selected transformants.

The affinity for the radioactive β-lactam was dramatically reduced in all high-M_r PBPs of the $R6_T$-CCCB transformants, and only PBP2a could be labeled at high antibiotic concentrations (Fig. 1 and 2). In summary, introduction of *S. mitis*-derived low-affinity PBP2x, -2a, -2b, -1a, and -1b resulted in a 1,000- to 4,000-fold increase in β-lactam resistance in *S. pneumoniae*.

PBP genes of β-lactam-resistant *S. pneumoniae* transformants. The unusually high resistance levels and the fact that all five PBPs, PBP1a, -1b, -2x, -2a, and -2b, appeared as low affinity in the *S. pneumoniae* transformants suggested the transfer of all five PBP genes. In order to clarify this, the DNA sequences of the PBPs of the *S. pneumoniae* transformants containing the PBP variants were examined and compared to those of *S. pneumoniae* R6 and *S. mitis* B6. The *pbp2x*, *pbp2b*, and *pbp2a* genes could be amplified with primers designed according to known sequences of penicillin-sensitive *S. pneumoniae*. The 3′ ends of the *pbp1a* and *pbp1b* genes could not be amplified, probably because the sequences were too divergent to allow priming of the oligonucleotides.

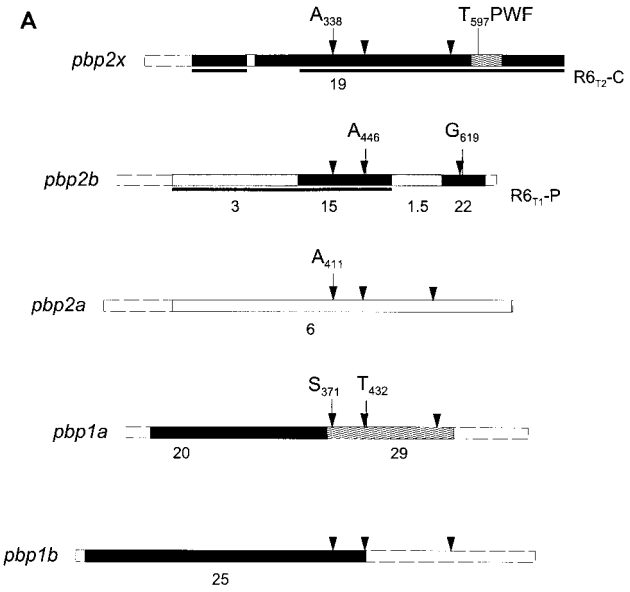

FIG. 3. PBPs of *S. mitis* B6. (A) Schematic representation of the mosaic gene structure. White box indicates sequence that is closely related to the *S. pneumoniae* R6 gene. Black, gray, and hatched boxes mark divergent sequences with the percentages of divergence indicated below. Dashed boxes at the 5′ and 3′ ends indicate regions in the genes of the B6 strain (*pbp2x*, *-2b*, and *-1a*) or the R6 transformant (*pbp2a* and *pbp1b*) that were not sequenced. *pbp2x* and *pbp2b* genes were also examined in the transformant $R6_T$-CCCB; the *S. mitis* sequences present in the *S. pneumoniae* R6 transformants $R6_{T2}$-C (*pbp2x*) and $R6_{T1}$-P (*pbp2b*) are indicated by black lines underneath the *pbp2x* and *pbp2b* sequences. *pbp2a* and *pbp1b* sequences were obtained from the transformant $R6_{T5}$-CCC. Arrowheads indicate the positions of the three active-site motifs in each penicillin-binding domain. Amino acid residues that are discussed in the text are shown. (B) Amino acid sequences. Positions at which the *S. mitis* sequences differ from the R6 sequences are shown (dashes indicate identical residues). The PBP2x of the penicillin-sensitive *S. oralis* M3 is included for comparison. The terminal regions which were not determined in *S. pneumoniae* R6 are in lowercase letters. The three active-site motifs in each penicillin-binding domain are underlined and in boldface. In PBP1a, -2a, and -1b, the conserved regions in the N-terminal transglycosylase domains of the high-M_r PBPs of class A are also underlined and in boldface. T5-ccc, $R6_{T5}$-CCC.

PBP2x. The *pbp2x* gene of the high-level resistance transformant $R6_T$-CCCB was identical to the *pbp2x* gene of the *S. mitis* donor strain. It diverged from the *S. pneumoniae* R6 *pbp2x* by 19.5%. However, it was closely related to the *pbp2x* gene of penicillin-sensitive *S. oralis* M3 (2.5% divergence), which is similar to a major class of *pbp2x* mosaic genes of penicillin-resistant *S. pneumoniae* (47). Only the short region between codons 182 and 197 was almost identical to the R6 *pbp2x* gene; another block (codons 584 to 637) that differed by 31.5% from the M3 gene was not related to any known *pbp2x* gene (Fig. 3). The *pbp2x* gene of the first-step transformant $R6_{T2}$-C contained R6 sequences between codons 182 and 278, indicating that two recombination events had occurred in this particular strain (Fig. 3A). The high-level resistance transformant $R6_{T4}$-CCCB contained the entire *S. mitis pbp2x* gene, documenting that during another transformation step *pbp2x* sequences must have been introduced.

B

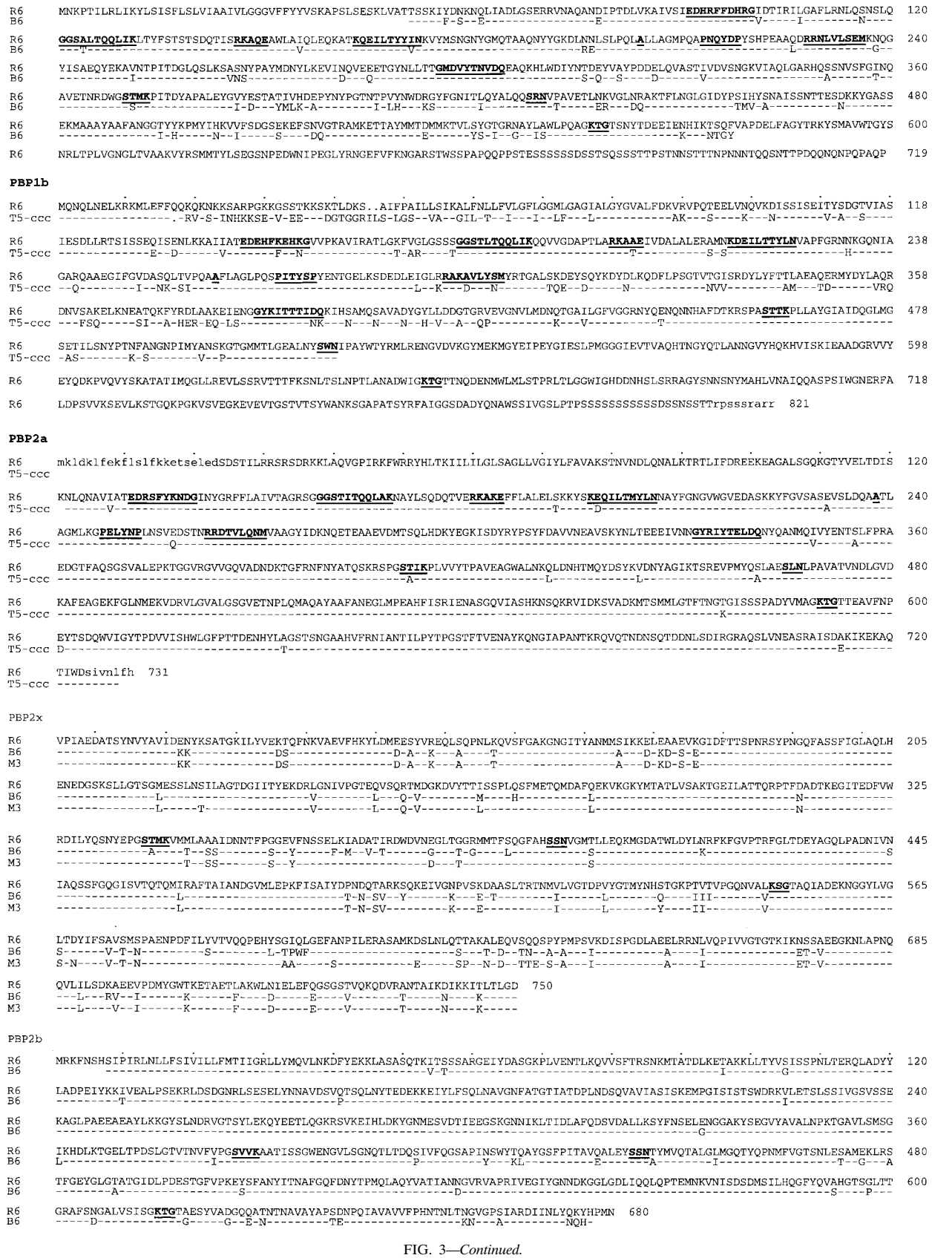

FIG. 3—*Continued.*

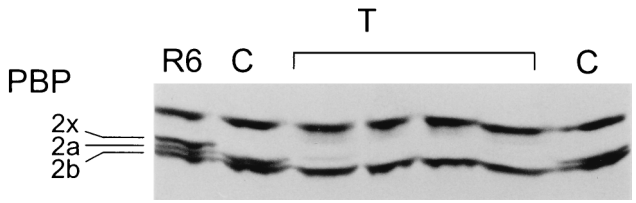

FIG. 4. PBP profiles of cefotaxime-resistant transformants. The *pbp2a* gene of the transformant $R6_{T5}$-CCC was used to transform $R6_T$-C to increased cefotaxime resistance. PBP profiles of three transformants (T) are compared to PBP profiles in the $R6_T$-C recipient (C) and the R6 strain. PBP2x, -2a, and -2b of *S. pneumoniae* R6 are indicated on the left.

PBP2b. The transformant $R6_T$-CCCB also contained the *pbp2b* gene of *S. mitis* B6. It contained two sequence blocks that diverge from *S. pneumoniae* R6 *pbp2b* by 15 and 22%, respectively (Fig. 3). For this transformant it was also the case that the entire *pbp2b* gene was not necessarily transformed in one transformation step: the transformant $R6_{T1}$-P contained only the first divergent sequence block, which included the active-site serine and the SSN (Ser-Ser-Asn) box (Fig. 3A). The gene was distinct from other known *pbp2b* sequences.

PBP1a. The entire *pbp1a* gene could not be amplified, and only partial sequence information was obtained between codons 45 and 385 for the determination of whether it is related to any of the known *pbp1a* genes (Fig. 3). The B6 *pbp1a* gene had a novel mosaic structure: whereas the 5′ end of the gene was closely related to the corresponding region of a variety of *pbp1a* genes, including South African and European penicillin-resistant *S. pneumoniae* (34), the region that includes the penicillin-binding domain after codon 361 was distinct from known *pbp1a* sequences.

PBP2a. PBP2a belongs to the class A high-M_r PBP. In order to see whether the low-affinity *pbp2a* is due to a point mutation or differs from the *S. pneumoniae* R6 gene considerably, the DNA sequence of *pbp2a* of the transformant $R6_{T5}$-CCC was determined. The *pbp2a* gene differed by 6% from the R6 sequence, and the changes were scattered throughout the sequenced region. The *S. mitis* B6 *pbp2a*, which was sequenced between codons 349 and 524, was identical to that of the transformant, demonstrating that the altered *pbp2a* sequences must have been introduced into R6 via transformation.

PBP1b. PBP1b is also a class A high-M_r PBP. The entire *pbp1b* gene of the $R6_{T5}$-CCC transformant could not be amplified with the primers used for the R6 *pbp1b* gene, indicating that the sequence is very different. A DNA fragment covering the 5′ region of the gene was obtained, and the sequence up to codon 520 that included part of the penicillin-binding domain clearly demonstrated a high degree of divergence compared to that of the R6 strain (Fig. 3). In the N-terminal putative transglycosylase domain, one amino acid deletion (Gln17) and one insertion were found (Ile-Leu between Ser44 and Ala45). The divergence from the *S. pneumoniae* gene was 25% on the nucleotide level, corresponding to almost 19% amino acid substitution (Fig. 3B).

PBP2a as resistance determinant. The fact that all transformants from the second selection step with cefotaxime contained a low-affinity PBP2a but no apparent changes in PBP1a indicated that PBP2a plays a role as a resistance determinant. Indeed, when the cloned *pbp2a* gene was used as donor DNA instead of chromosomal DNA from *S. mitis* B6, transformants of the $R6_T$-C recipient strain could be selected with cefotaxime. Twenty transformants were isolated and the cefotaxime MICs for them ranged between 1.2 and 1.8 μg/ml, similar to those for the $R6_T$-CC transformants obtained with chromosomal DNA. PBP profiles were investigated in four transformants, and all of them contained a low-affinity PBP2a (Fig. 4), demonstrating that the *pbp2a* gene was indeed transferred and contributed to cefotaxime resistance. Similar to the situation with *pbp1a*, it could not be selected in the susceptible R6 background.

Amino acid changes in low-affinity PBPs. The deduced amino acid sequence of *S. mitis* PBP2x between residues 198 and 583 is only 2.8% divergent from that of the *S. oralis* M3 gene, which does not confer relevant cefotaxime resistance in *S. pneumoniae*. In contrast, the amino acid sequence differed by 4.7% from that of the M3 PBP, i.e., 56% of the nucleotide changes resulted in an amino acid substitution. The region between codons 338 (immediately after the active-site Ser337) and 417 is particularly remarkable in that each of the nine nucleotide changes results in an amino acid alteration, strongly suggesting that this is the result of mutation and selection (Fig. 3b). There are three sites which are potentially relevant for resistance: (i) the Thr338 to Ala change directly after the active-site serine, which change is involved in β-lactam resistance (25a), (ii) the Ile366 to Met change, which is also present in PBP2x of high-level cefotaxime-resistant *S. mitis* and *S. oralis* but not in penicillin-resistant *S. pneumoniae* (40), and (iii) the region between Ser596 and Gly601, which is highly altered in the *S. mitis* B6 PBP2x and has been implicated in cefotaxime resistance in laboratory mutants (28).

The *S. mitis* PBP2b contained the Thr446 to Ala change, which is present in PBP2b of all resistant streptococci analyzed

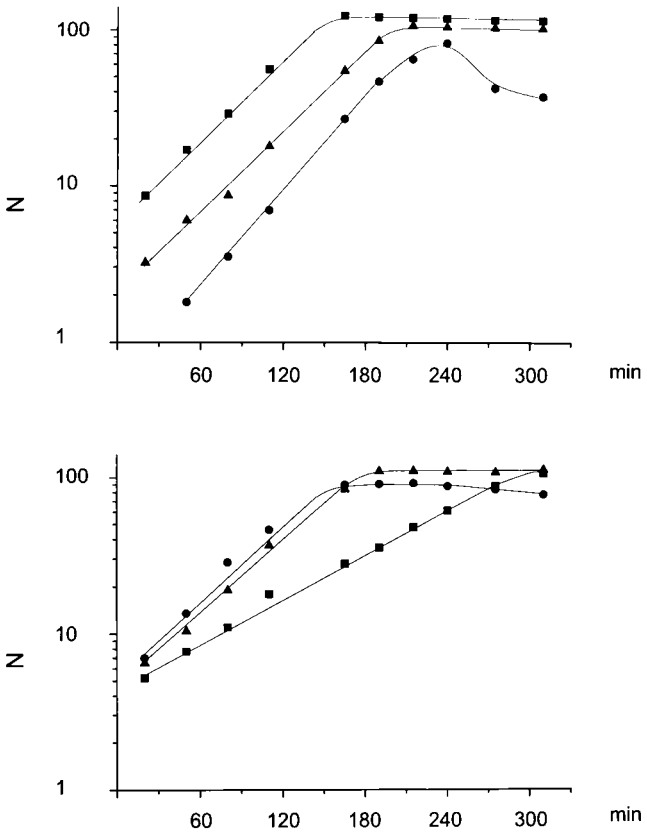

FIG. 5. Growth of *S. pneumoniae* R6 transformants in liquid medium. Cells of an exponentially growing culture were diluted 1:50 in prewarmed C medium, and growth was monitored by nephelometry (N, nephelometry units). (Top) ■, *S. pneumoniae* R6; ●, $R6_T$-P; ▲, $R6_T$-CCCO. (Bottom) ■, *S. mitis* B6; ●, $R6_T$-CCP; ▲, $R6_{T5}$-CCC.

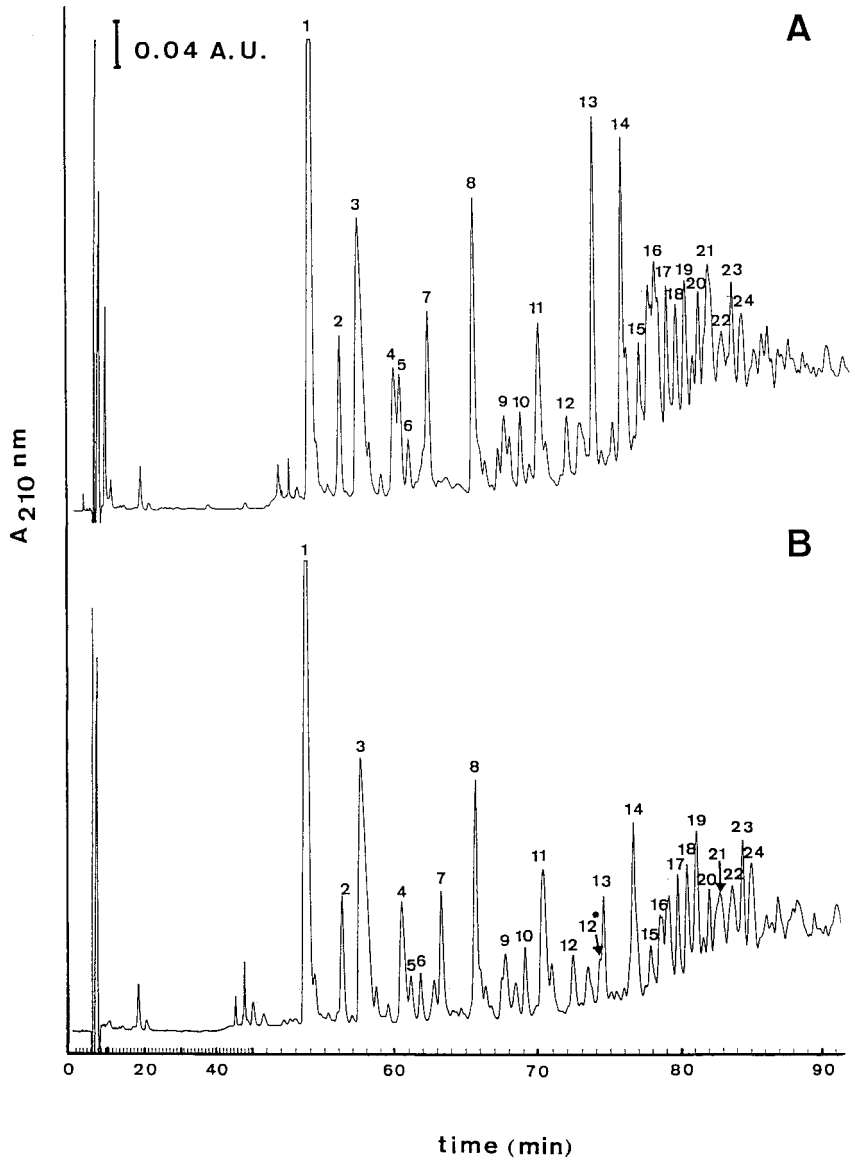

FIG. 6. HPLC analysis of cellosyl-digested cell walls of *S. pneumoniae* R6 and the transformant $R6_T$-CCP. HPLC chromatography of cellosyl-digested cell walls was performed as described in the text. The peaks are numbered according to their position during elution (see Table 2 for analytical data). (Top) *S. pneumoniae* R6; (bottom) transformant $R6_{T3}$-CCP.

(11) and which is responsible for both low-level resistance and a reduced lysis rate (19). The second block of the *S. mitis* PBP2b which has been introduced in the higher-level transformants includes the conserved motif K615TG. There is a change from Ala619 to Gly, and similar substitutions in PBP2x Thr550 to Ala or to Gly were shown to be significant in β-lactam resistance (9, 19).

It is remarkable that in the low-affinity PBP2a, a Thr411 to Ala change after the active-site serine occurred as in the case of PBP2x, and PBP1a also contained a change at the corresponding site (Thr371 to Ser), suggesting that these changes indeed may account for altered enzymatic activities of the PBPs.

Effects of low-affinity PBPs. The replacement of five important enzymes involved in biosynthesis of a crucial cellular component, peptidoglycan, seems to be critical for the cell, especially if the homologs have altered activities at least in terms of their enzymatic interaction with an inhibitor. Two parameters were investigated to see whether the low-affinity PBPs have any effect on the *S. pneumoniae* transformants. Cellular growth was monitored, and the cell wall biochemistry was analyzed in two transformants which contained different sets of low-affinity PBPs: the transformant $R6_{T3}$-CCP, containing low affinity PBP2x, -2a, and -2b; and the transformant $R6_{T5}$-CCC, containing low-affinity PBP2x, -2a, -1a, and -1b.

Cellular growth of the transformants. *S. mitis* B6 had a longer generation time (65 min) compared to *S. pneumoniae* R6 (36 min) (Fig. 5). All transformants, independent of the number and the combination of altered PBPs, grew with generation times of 35 to 38 min, which is identical to that of the parental R6 strain. Only in the case of the $R6_{T1}$-P transformant, which contained a low-affinity PBP2b, was an enhanced lysis rate observed as soon as the transformant reached the end of the exponential growth phase. This was not seen in another

TABLE 2. Molecular mass and composition of the muropeptides of *S. pneumoniae* R6 and transformant $R6_{T3}$-CCP after separation by HPLC

Peak no.[a]	Structure[b]	M_r		Size of peak/total size of all peaks (%)	
		Observed	Calculated	R6	$R6_{T3}$-CCP
1	DS-tri	825.4	825.3	19.01	20.81
2	DS-tri(OH)	826.4	826.3	3.18	3.28
3	DS-tri(−42)	783.4	783.3	12.44	15.11
4	DS-tri(OH)(−42)	784.4	784.3	3.15	4.40
5	DS-tetra	897.2	896.4	2.67	1.18
6	DS-S-tri	912.2	912.4	0.81	1.03
7	DS-penta	967.4	967.3	4.75	3.52
8	DS-A-S-tri	983.6	983.5	7.70	7.12
9	DS-penta(−42)	925.7	925.5	0.92	0.60
10	DS-A-A-tri	967.6	967.5	1.38	1.63
11	DS-A-S-tri(−42)	941.6	941.2	5.16	6.21
12	DS-A-S-tri(OH)(−42)	942.5	942.2	1.62	1.73
12*	DS-A-A-tri(−42)	925.8	925.5	0	1.96
13	BisDS-tetra-tri	1704.4	1703.8	6.65	2.46
14	BisDS-tetra-A-S-tri	1862.7	1862	6.1	4.13
15	BisDS-A-S-tetra-tri	1862.8	1862	1.66	1.15
16	BisDS-tetra-tri(−42)	1662.6	1662.9	3.05	3.16
17	BisDS-A-S-tetra-A-S-tri	2020.4	2020.1	2.58	2.15
	BisDS-A-A-tetra-A-S-tri	2004.3	2004.1		
18	BisDS-tetra-A-S-tri(−42)	1820	1820	2.21	2.72
19	BisDS-A-S-tetra-tri(−42)	1820.8	1820.1	2.71	3.46
20	BisDS-A-A-tetra-A-S-tri(OH)	2005.2	2005.1	1.80	1.26
21	BisDS-tetra-tri(−42x2)	1620.5	1619.7	4.54	2.89
22	BisDS-tetra-A-A-tri(−42)	1804.8	1804.8	1.62	2.47
23	BisDS-A-S-tetra-A-S-tri(−42)	1978.6	1978.1	2.31	2.50
24	BisDS-tetra-A-S-tri(−42x2)	1778.2	1777.9	1.98	3.07

[a] Peaks are shown in Fig. 6.

[b] DS, disaccharide (GlcNAc-MurNAc); bis, dimeric form; tri, tripeptide (L-Ala-D-Gln-L-Lys); tetra, tetrapeptide (L-Ala-D-Gln-L-Lys-D-Ala); penta, pentapeptide (L-Ala-D-Gln-L-Lys-D-Ala-D-Ala). OH indicates the presence of Glu instead of Gln. S, serine; A-A, Ala-Ala; A-S, Ala-Ser; these amino acids are branched to the ε amino group of the Lys residue and are also present in the interpeptide bridge. Peak 17 contained two compounds as described which could not be separated. −42, the mass that was obtained after cleavage of an acetyl radical from the GlcNAc moiety of the DS during preparation of the peptidoglycan; −42x2, two acetyl radicals were missing, one on each DS of the dimer.

transformant, $R6_{T2}$-P or in higher-resistant transformants of the CCP or CCCO class with a low-affinity PBP2b (Fig. 5). This particular transformant, $R6_{T1}$-P, contained only part of the *pbp2b* gene (Fig. 3). Lysis has been correlated with inhibition (i.e., nonfunction) of PBP2b (19), and thus the phenotype in the transformant may indicate that in this case the encoded protein is not functioning sufficiently well.

Composition of muropeptides in transformants. We chose the transformant $R6_{T3}$-CCP, with altered PBP2x, PBP2a, and PBP2b and the $R6_{T5}$-CCC transformant with altered PBP1a, -1b, -2a, and -2x for examination of the muropeptide composition. Isolated cell walls were digested with a muramidase, and muropeptides were separated by HPLC analysis. The pattern of muropeptides obtained from the transformants compared to that of the R6 strain revealed no qualitative differences (not shown). Muropeptides from $R6_{T3}$-CCP were analyzed in more detail (Fig. 6). The removal of teichoic acid with HF was necessary for optimal separation of the peaks, which allowed quantitative determination and identification of the muropeptides by mass spectrometry. As before, the transformant contained no muropeptides that did not exist in the R6 strain. The proportion of disaccharide-tripeptide monomers, however, was significantly higher (63 versus 54% of the total muropeptides), but the direct cross-linked dimers were lower (8 versus 14%) (Table 2). We cannot rule out some variation in the multimeric fractions; however, they constituted only a minor portion (<3%) of the recovered material.

DISCUSSION

We demonstrate here that all high-M_r PBPs, PBP1a, -1b, -2a, -2x, and -2b, can be changed into variants with low affinity to penicillin by successive transformation with chromosomal DNA of an *S. mitis* with high resistance levels to β-lactam antibiotics. Previous reports clearly demonstrated that, at least in the particular clinical isolates investigated, the combination of only *pbp2x*, *pbp2b*, and *pbp1a* are required for penicillin and cefotaxime resistance (9, 36). In contrast, the setting here differs in important respects: (i) the *S. mitis* B6 isolate used as donor is extremely resistant to a variety of β-lactams, and (ii) it contains *pbp2x*, *pbp1a*, and *pbp2b* genes that, although clearly homologs to the *S. pneumoniae* genes, have novel mosaic structures and contain sequences not described so far.

The low-affinity PBP2a appeared in all transformants analyzed prior to acquisition of a low-affinity PBP1a, suggesting it may be a prerequisite for transfer of this particular *pbp1a*. The *pbp2a* gene from the transformant encoding a low-affinity PBP2a variant could easily be transformed into a low-level resistant strain with an altered *pbp2x*, confirming its potential role as resistance determinant. Unlike *pbp2x* and *pbp2b*, however, *pbp2a* did not confer resistance in the susceptible R6 background, i.e., it is not a primary resistance determinant (19).

The role of the *pbp1b* gene in resistance was not analyzed since only the 5′ end (which does not cover the entire penicillin-binding domain where relevant mutations are to be expected) could be amplified from the transformant. Thus, it is not clear whether *pbp1b* was incidentally transferred into this particular transformant or whether it represents indeed another resistance determinant. PBP1b variants with reduced affinity have been described before in interspecies transformations to penicillin resistance (7), and a low-affinity PBP2a was noted in several penicillin-resistant clinical isolates of *S. pneumoniae* (31) as well as in cefotaxime-resistant transformants obtained with chromosomal DNA from *S. mitis* and *S. oralis* (40, 41), suggesting that alterations in the respective genes may be more common although not necessarily present in all penicillin-resistant isolates.

The fact that high-level oxacillin resistance in the $R6_T$-CCCO transformants does not correlate with high-level resistance to benzylpenicillin is curious. Low-level oxacillin-resistant strains that are not penicillin resistant have been described (15), but it is not clear whether the same mechanism, changes in *pbp2x*, applies for the high resistance levels as well.

The generation time of the *S. pneumoniae* transformants was identical to that of the parental *S. pneumoniae* R6, independent of the combination of low-affinity PBPs present, indicating that all PBP variants from the *S. mitis* donor strain are functioning sufficiently well in the different genetic background. The only effect noted, an early onset of stationary-phase lysis, concerned one particular transformant, $R6_{T1}$-P, with an altered PBP2b.

No qualitative changes in the biochemistry of murein, the substrate and product of PBP activity, were detected in the pneumococcal transformants containing *S. mitis* low-affinity PBP variants. Substantial muropeptide changes have been reported for penicillin-resistant Hungarian and South African *S. pneumoniae* isolates (18, 46). *S. pneumoniae* R6 derivatives

have been obtained that also produced an altered cell wall after transfer of penicillin resistance from such isolates into the R6 background (16). Our results indicate that such changes do not automatically occur during transfer of resistance determinants from one species to another. It is possible that the different biochemistry of the peptidoglycan is an intrinsic property of the clones analyzed, and the recent report that penicillin resistance and concomitant PBP changes could be separated from the cell wall changes indicates that indeed, other non-PBP genes could be responsible for this effect (44), similar to our findings. An increase in the ratio of monomeric versus cross-linked peptides has also been observed for laboratory mutants with altered high-M_r PBPs (45), but it cannot be deduced from the analyses which one of the PBPs is responsible for this effect.

The ease with which all altered high-M_r PBPs, being after all essential enzymes, could be transferred between two species is remarkable. It is equally remarkable that all five PBPs contain almost a completely identical number of codons, and this property may contribute significantly to their ability to be exchangeable in total, or in parts, between different species. The experiments described here were done under laboratory conditions that are feasible in vivo, giving rise to a frightful perspective of future therapeutic problems in geographic areas where penicillin-resistant pneumococci are still rare.

ACKNOWLEDGMENTS

We thank R. Marquardt from Hoechst AG for providing cellosyl, Werner Fischer for his advice in teichoic acid hydrolysis, and Richard Reinhard for his help in automated DNA sequencing.

This work was supported by the BMBF (grant no. 01KI9402), an EMBO fellowship to R.H., and by INSERM (CRI 95061).

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