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A Novel Resistance Mechanism against β-Lactams in *Streptococcus pneumoniae* Involves CpoA, a Putative Glycosyltransferase

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Piperacillin resistance in *Streptococcus pneumoniae* was mediated by mutations in a novel gene, *cpoA*, that also confer transformation deficiency and a decrease in penicillin-binding protein 1a. *cpoA* is part of an operon located downstream of the primary σ factor of *S. pneumoniae*. The deduced protein, CpoA, and the peptide encoded by the adjacent 3′ open reading frame contained domains homologous to glycosyltransferases of procaryotes and eucaryotes that act on membrane-associated substrates, such as enzymes functioning in lipopolysaccharide core biosynthesis of gram-negative bacteria, RodD of *Bacillus subtilis*, which is involved in teichoic acid biosynthesis, and the human PIG-A protein, which is required for early steps of glycosylphosphatidylinositol anchor biosynthesis. This suggests that the *cpo* operon has a similar function related to cell surface components.

β-Lactam antibiotics are the most widely used antibacterial compounds. Bacteria have evolved various mechanisms of resistance. Two major resistance mechanisms are known: the production of β-lactamases that destroy the antibiotic before it reaches its target (31) and alterations of the targets themselves, the penicillin-binding proteins (PBPs) (47). In gram-negative organisms, changes in outer membrane permeability can also lead to a decrease in susceptibility (39).

Penicillin resistance in *Streptococcus pneumoniae* is a multistep process that involves a series of alterations in the penicillin target enzymes, the PBPs. PBPs function in late steps of biosynthesis of murein, a macromolecule constituting an essential component of the bacterial cell envelope. PBPs, multimodule proteins, become acylated by β-lactams via a serine residue of the catalytic site of the penicillin-binding domain (for a review, see reference 9). In penicillin-resistant strains, altered PBPs have a reduced affinity for the antibiotic (47).

In order to understand the genetic basis of resistance development step by step, a series of spontaneous β-lactam-resistant laboratory mutants of *S. pneumoniae* has been isolated, resulting in several independent mutant lineages consisting of up to six mutants with stepwise increasing resistance (27). Representatives of two classes of β-lactams were chosen for the selection procedure, each triggering a distinct response: piperacillin, a typical lytic penicillin, and cefotaxime, a cephalosporin that does not induce cellular lysis, a phenomenon related to the fact that it does not interact with PBP2b (18).

Different pathways of resistance development became apparent depending on the selective β-lactam used. Not only were different PBPs affected in cefotaxime- versus piperacillin-resistant mutants (17, 28), but distinct "non-PBP" genes also contributed to resistance, demonstrating that gram-positive organisms also have an unanticipated variety of defense mechanisms against such drugs. Whatever these mechanisms are in molecular terms, in every mutant lineage inevitably a deficiency in genetic competence became apparent, indicating that the integrity of the cell wall, which is threatened by β-lactam treatment, is required for competence and transformability (16).

Competence in the pneumococcus occurs during a period of the exponential growth phase. Shortly before the onset of competence in the cell culture, a competence-inducing activity termed competence factor (CF) is produced (49). This activity has been identified as a peptide named CSP (for competence-stimulating peptide) (20). The CSP primary translation product contains an N-terminal double-glycine-type leader peptide (21) which is thought to be secreted and processed by the ABC transporter ComA (23).

Recently, β-lactam resistance and competence deficiency in cefotaxime-resistant mutants have been related to mutations in a gene encoding a histidine protein kinase, CiaH, the first documentation that non-PBP genes are involved in susceptibility to β-lactams (12). *ciaH*, however, was affected only in three of six cefotaxime-resistant mutant lineages and in none of the piperacillin-resistant mutant lineages despite the fact that all of them showed defects in competence development, demonstrating that other, still unidentified genes contribute to this phenomenon.

This report describes the isolation and sequence analysis of a novel gene, *cpoA*, which is involved in the complex phenotype of two independently isolated first-step piperacillin-resistant mutants. Phenotypic characteristics include piperacillin resistance, a defect in competence development, and the presence of small amounts of PBP1a. *cpoA* mutants differ in several properties from *ciaH* mutants described before, and none of the cefotaxime-resistant mutants was affected in *cpoA*, showing again that the two classes of β-lactams act via different primary mechanisms.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* R6 is a nonencapsulated laboratory strain derived from the Rockefeller University strain R36A (2). The piperacillin-resistant mutants are spontaneous mutants isolated on stepwise increasing concentrations of piperacillin on blood agar plates (27). A three-digit number was assigned to each mutant, with the first numeral specifying the selection step and the last numeral specifying the mutant lineage. P104 and P404, therefore, are the first- and the fourth-step mutants, respectively, of lineage 4. MICs for the mutants were determined by using a series of narrow dilutions on blood agar plates. Properties of the mutants are listed in Table 1. *Streptococcus*

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TABLE 1. The piperacillin-resistant mutant families P004 and P006[a]

Strain	Piperacillin MIC (μg/ml)	Mutant gene	Amino acid change	Reference
R6	0.016–0.03	None (parental strain)		
P106	0.02–0.05	*cpoA*		This paper
P206	0.05	*pbp2b*	Gly-617 to Ala	17
P306	0.05–0.1	*pbp2x*	Gly-597 to Asp	25
P406	0.1–0.15	Unknown		
P506	0.2	Unknown		
P104	0.02	*cpoA*	Gly-12 to Val	This paper
P204	0.02–0.05	Unknown		
P304	0.05–0.1	Unknown		
P404	0.1	Unknown		
P504	0.1–0.2	*pbp2b*	Gly-660 to Asp	17

[a] PBP profiles and immunological detection of PBP1a have been published (27).

oralis M3 is an isolate from South Africa (46), *Streptococcus mitis* 661 is a penicillin-resistant isolate from Spain (13), and *S. mitis* NCTC10712 has been described (5). Streptococci were grown in C medium (26), supplemented with 0.2% yeast extract without aeration. For preparation of competent pneumococci, 0.07% albumin was added to the growth medium. *Escherichia coli* INVαF′ was used for propagation of the plasmid PCR II vector (which was used for the cloning of PCR-generated DNA fragments) (Invitrogen, Leek, The Netherlands); other cloning vectors used were pSU2719 (36), pUC18 (37), and pJDC9 (4).

Transformation. Transformation of *S. pneumoniae* followed published procedures (48). Piperacillin-resistant transformants of *S. pneumoniae* were screened in blood agar plates containing different concentrations of the antibiotic as stated. Erythromycin was used at 1 μg/ml for insertional inactivation with pJDC9. *E. coli* was transformed according to the protocol provided by Invitrogen, from whom competent cells of *E. coli* NVαF′ were obtained.

Assay of PBPs. PBPs were visualized on fluorograms after labeling of cell lysates with [^{3}H]propionylampicillin and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (29).

Cell fractionation. Cells from a 900-ml culture were harvested by centrifugation, washed once with 50 mM sodium phosphate buffer (pH 7.1), and disrupted in a French pressure cell (Amicon). Unbroken cells and cell walls were sedimented at 30,000 × *g* for 30 min in a Sorvall centrifuge (HB4 rotor), and the supernatant was centrifuged in a Beckman Ti60 rotor at 100,000 × *g* for 3 h. The supernatant was used as the cytoplasmic fraction. The pelleted membranes were washed twice in the same buffer. Fractions were stored at −80°C.

Preparation of antibodies against CpoA. In order to obtain sufficient protein for immunization, a DNA segment encoding amino acids 2 to 257 of CpoA was cloned into pMALc2 (33) and transformed into *E. coli* TB1 (24). The only clone obtained that expressed a cytoplasmic protein contained a mutation in the CpoA sequence (CC2525A to CTA changing Pro-123 to Leu), and the attempt to express the product in the periplasm resulted only in unstable constructs. Soluble protein was obtained after induction at 27°C overnight and purified by amylose-affinity chromatography as described by the manufacturer (New England Biolabs). The fusion protein was cleaved with factor Xa, the peptides were separated by SDS-PAGE, and the CpoA fragment was purified by electroelution. One hundred micrograms was used for immunization and injected with complete Freund adjuvant into rabbits, followed by booster injections after 3 to 6 weeks. Western blotting was performed as described previously (15) and CpoA was visualized by using a 1:10,000 dilution of the antiserum in phosphate-buffered saline/Tween and alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) by the method of Blake et al. (3).

RNA isolation and Northern analysis. Cellular lysis and extraction of total RNA were performed essentially as described previously (34), using late-exponential-phase cells of a 10-ml culture grown at 30°C. Between 10 and 30 μg of total RNA was applied per lane. RNA was electrophoresed in a 1% formaldehyde–agarose gel (7). RNAs were transferred onto a positively charged nylon membrane (Hybond; Amersham, Buckinghamshire, England) by capillary blotting, and after prehybridization with yeast RNA, the radioactive probe was hybridized in the presence of yeast RNA (50 mg/ml) overnight at 65°C. The nylon membrane was washed as described previously (7). Quantifications of the transcripts were done from direct scans with the PhosphorImager (Molecular Dynamics) by using ImageQuant software 3.3. Labeling of antisense RNA sequences with [α-^{32}P]UTP (3,000 Ci/mmol; Amersham, Braunschweig, Federal Republic of Germany) was performed in transcriptional reactions with *pbp1a* DNA template covering nucleotides 10 to 870 of the gene (35) by using the in vitro MAXIscript T7 transcription kit from Ambion (Austin, Tex.) under conditions described by the manufacturer. The template DNA in control reactions was a *pbp3* fragment between nucleotides 4 and 734 of the structural gene (24a).

PCR. PCRs were carried out in a Biomed thermocycler for 30 cycles with denaturation for 30 s at 96°C, annealing for 1 min at 52°C, and extension for 1 min at 72°C, followed by a 3-min extension period at 72°C. A 100-μl reaction mixture contained 10 pmol of each oligonucleotide primer, 125 μM deoxynucleoside triphosphates, 6 mM $MgCl_2$, 2.5 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.), and buffer, as described by the manufacturer. For amplification of *cpoA*-specific DNA from other species, primers T127 5′-A2159TGTTGAGT TCAAGTGAG and T128 5′-G928CAGCTTCTAAATAGTCA were used.

DNA sequencing. DNA sequencing of cloned DNA fragments was performed by the dideoxynucleotide chain-termination method (44) with the T7 sequencing kit (Pharmacia, Freiburg, Germany).

Computer-based sequence analysis. All sequence analysis programs are part of the GCG sequence analysis software package, version 8.0, of the University of Wisconsin Department of Genetics (6). Sequence similarity searches were based on the BLAST program (1).

Nucleotide sequence accession number. The nucleotide sequence has been deposited with the EMBL nucleotide sequence database under accession number Y11463.

RESULTS

The mutant lineage P004. The mutant lineage P004 appeared most suitable for identification of a PBP-independent resistance determinant. It consists of five mutants, P104 (the first-step mutant) to P504 (the fifth-step mutant), with increasing piperacillin resistance. The only point mutation detected in a PBP gene, *pbp2b*, occurred at the fifth selection step in P504 (Table 1). Unlike in two other piperacillin mutant lineages, *pbp2x* did not function as a resistance determinant, and although the first-step mutant P104 produced less PBP1a as determined by anti-PBP1a-specific antibodies (27), no mutation in the structural gene or in a region approximately 1 kb upstream of *pbp1a* could be assigned to this phenotype (17). In other words, not one mutation in a PBP gene was detectable during four successive selection steps. Transformation deficiency was apparent already in the first-step mutant P104: it could be transformed only for a short period during the growth cycle with a transformation efficiency that was more than 100-fold lower than that of the parental R6 strain.

Cloning of a novel resistance determinant. Chromosomal DNA of P404 (*pbp2b*$^+$ *pbp1a*$^+$ *pbp2x*$^+$) was used to transform the R6 strain to increased piperacillin resistance, and transformants were plated on a range of piperacillin concentrations in order to optimize the selective conditions. The plates were incubated at 30°C, the temperature at which the mutants were selected originally (27). Piperacillin-resistant transformants were obtained only at concentrations as low as 0.04 μg/ml, i.e., up to the MIC for the first-step mutant P104 (0.045 to 0.06 μg/ml compared to 0.03 to 0.04 μg/ml for the R6 strain when determined on agar, which results in values higher than MICs obtained when cells were plated on the agar). The selective concentration was not high enough to completely suppress growth of the recipient R6 cells when plated without added DNAs, but with DNA, the number of colonies was 10-fold higher. Other β-lactams, oxacillin and cefotaxime, were not suitable for selection, probably since the difference in MICs between the recipient strain and transformants was not sufficient.

Chromosomal P404 DNA was digested with various restriction enzymes. After separation on an agarose gel, fractions corresponding to different-size DNA fragments were tested in transformation assays with *S. pneumoniae* R6 as the recipient for the presence of a resistance determinant. Piperacillin-resistant transformants were obtained with the 4- to 5-kb DNA fraction of a *Pst*I/*Hin*dIII digest. By using a larger quantity of DNA and a long preparative agarose gel, this region was further divided into eight fractions. Aliquots were used as donor DNA in transformation assays as above. The DNA fraction

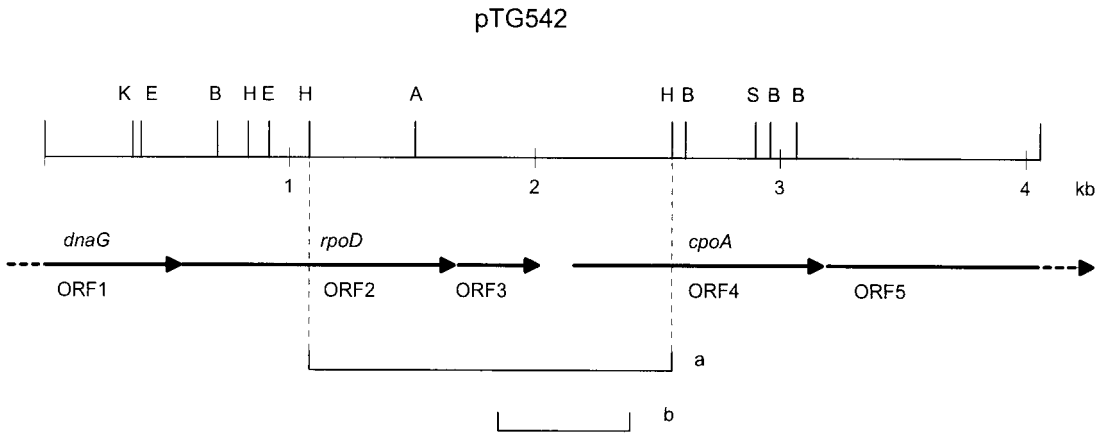

FIG. 1. Map of pTG542. Restriction endonuclease cleavage sites are represented as follows: A, *Acc*I; B, *Bgl*II; E, *Eco*RV; H, *Hin*dII; K, *Kpn*I; S, *Sac*I. Open reading frames are represented by arrows indicating the predicted orientation. The 5′ end of ORF1 and the 3′ end of ORF5 are located outside the cloned fragment and are indicated as dashed arrows. Lines a and b represent the *Hin*dII fragment and the 535-bp PCR fragment, respectively, conferring piperacillin resistance in P104 and P106.

with which the highest number of transformants was obtained (>100) was ligated into the vector pSU2719 and used to transform *E. coli* INVαF′. Of 18 transformants, 1 contained a recombinant plasmid with a 4-kb insert that transferred piperacillin resistance with high efficiency ($>10^4$ transformants) into *S. pneumoniae* R6. The 3′ and 5′ ends of the inserts were identical in both plasmids, and one plasmid, pTG542, was used for further analysis.

Sequence analysis of pTG542. The map of the 4,060-bp DNA fragment of pTG542 and open reading frames (ORFs) are outlined in Fig. 1. The DNA sequence and the deduced amino acid sequences are shown in Fig. 2. All ORFs started with an ATG codon. Between ORF3 and ORF4, a short palindrome was followed by a −35 and a −10 region as well as a Shine-Dalgarno sequence at appropriate distances upstream of ORF4. ORF4 had two potential start sites, A2132TG and A2159TG, but only the latter was preceded by a Shine-Dalgarno sequence and was thus assigned as the start codon. All other ORFs were preceded by a putative ribosome binding site but not by −10 and −35 sequences. This suggests that the cloned DNA fragment contains two transcriptional units: one including ORF2 and ORF3 and the other including at least ORF4 and ORF5. The AT content of all ORFs was between 56 and 60%, and that of the sequence between ORF3 and ORF4 was around 68%, in agreement with findings of a higher AT content in intergenic regions. In the opposite orientation, no ORF encoding more than 90 amino acids was found.

The peptide encoded by the partial ORF1 showed homology to DNA primase (DnaG) (not shown), and ORF2 encoded a 369-amino-acid protein with a high level of similarity to primary σ factors; the *S. pneumoniae* genes were therefore named *dnaG* and *rpoD*. Sequence comparison documented that the *S. pneumoniae* σ factor (σ^{42}) clusters within the group of major σ factors of similar sizes of gram-positive bacteria (*Lactococcus lactis*, accession no. D10168; *Enterococcus faecalis*, X86176; and *Bacillus subtilis*, X03897) that are also preceded by a *dnaG* gene. The amino acids known to interact specifically with promoter sequences were all present in the deduced *S. pneumoniae* RpoD as indicated in Fig. 2. Hybridization experiments with an *rpoD*-specific probe showed that no related σ factor was present in the pneumococcal chromosome (not shown). The deduced 109-amino-acid peptide of ORF3 showed similarity to hypothetical proteins of *Mycobacterium leprae* (U00013 and U15180), a *Synechocystis* sp. (D64001), and *Methanococcus jannaschii* (U67555).

A computer search revealed no homologs for the encoded proteins of ORF4 or the 5′ end of ORF5. The deduced 338-amino-acid sequence of the ORF4 protein, with a calculated molecular weight of 38,800 and a pI of 7.3, revealed no hydrophobic segments or signal sequences, indicating that it is a cytoplasmic protein. The ORF4 protein contained, however, regions related to segments present in a variety of proteins from procaryotes, including archaebacteria, and eucaryotic organisms. The N-terminal part of the ORF5 protein also appears to be related as suggested by a BLAST search, but the length of the peptide does not yet allow a clear statement. The domains were specified by several conserved amino acids as indicated in Fig. 3. The majority of the proteins were glycosyltransferases of different specificities (mannosyltransferases, *N*-acetylglucosaminetransferases, glucosyltransferases) that act during lipopolysaccharide (LPS) core biosynthesis of gram-negative bacteria. A related protein of a gram-positive bacterium was represented by the *rodD* (*gtaA*) gene product of *B. subtilis*, which was identified as a uridine diphosphate-glucose poly(glycerol phosphate) α-glucosyltransferase, a teichoic acid biosynthetic enzyme (22). Eucaryotic enzymes were the human PIG-A protein (38), required for synthesis of *N*-acetylglucosaminyl-phosphatidylinositol, the very early intermediate of glycosylphosphatidylinositol anchor biosynthesis which is a membrane attachment structure of a variety of excreted proteins, and its yeast homolog SPT14 (8, 45).

Identification of mutations in P104 and P106. Other independently obtained β-lactam-resistant mutants were screened for the presence of a similar resistance determinant. 5′ and 3′ DNA sequences of the 4-kb insert of pTG542 were used to design oligonucleotide primers for PCR amplification of a 3.7-kb DNA fragment from chromosomal DNA. First, only the mutants representing the last selection step of six cefotaxime- and two piperacillin-resistant lineages were assayed. In all cases, a 3.7-kb PCR product was obtained, but only in the case of P506, the fifth mutant of the P006 lineage, did it transfer piperacillin resistance as efficiently as the 4-kb fragment of pTG542. Second, individual mutants of the lineages P006 and P004 were assayed in order to identify the selection step of the resistance determinant. In both lineages it was present already in the first-step mutants, i.e., P104 and P106.

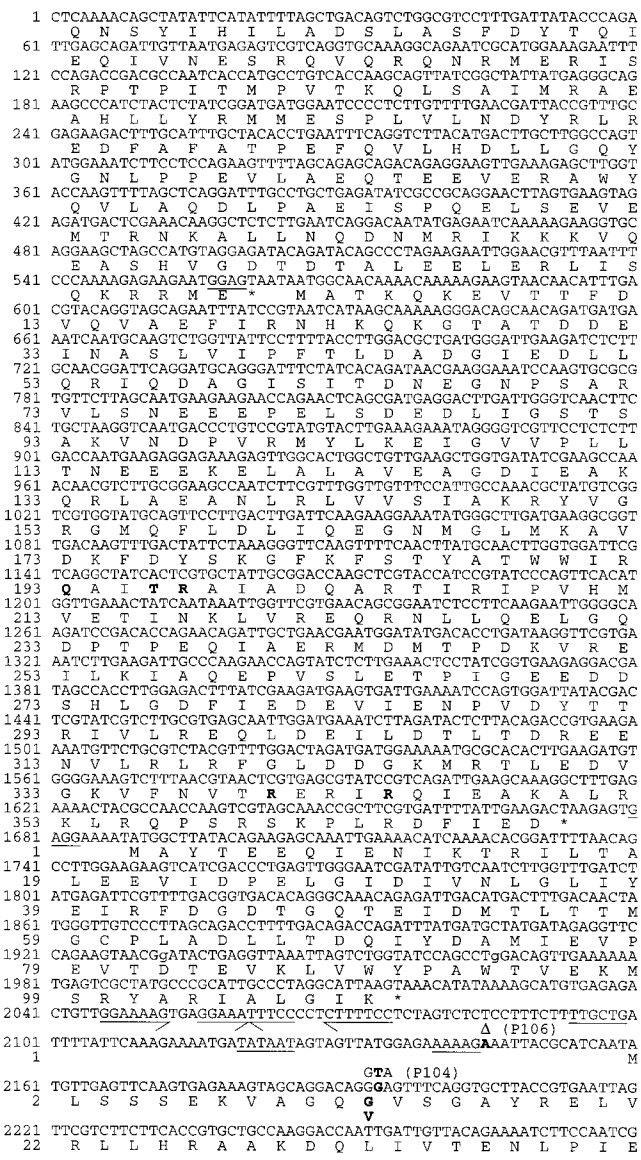

FIG. 2. Nucleotide sequence and deduced protein sequence of pTG542. The numbers on the left side designate the first nucleotide of the respective row; amino acids encoded by the different reading frames are also indicated. Underlined nucleotide sequences represent predicted ribosome binding sites and putative −10 and −35 sequences. Arrows indicate palindromic sequences that may function as transcription terminators. Amino acids marked in ORF2 by boldface letters correspond to region 2.4 (Q193XXTR) and region 4.2 (R340, R344) in main sigma factors according to the description of Lonetto et al. (32). The mutations in P104 and P106 are marked in boldface letters.

DNA fragments corresponding to the 5′ end of pTG542 up to position 1960 (Fig. 2) did not confer piperacillin resistance, indicating that *rpoD* is not affected in the piperacillin-resistant mutants. After *Hin*dII digestion of pTG542, only the 1.48-kb fragment that included the 3′ end of *rpoD* and the 5′ end of ORF4 conferred piperacillin resistance upon transformation into the R6 strain (Fig. 1). By using chromosomal DNA of P104 and P106, respectively, a series of PCR-amplified DNA fragments was then tested to narrow down the location of the putative mutation. In both mutants, the resistance-transforming activity was localized on a 535-bp DNA fragment (bp 1755 to 2289) that covered the 5′ end of ORF4 and promoter sequences. The corresponding PCR product from both mutants and the parental R6 strain was cloned into the PCR II vector. All clones containing mutant DNA transferred piperacillin resistance with high efficiency, whereas those containing R6 DNA did not. The DNA sequences were obtained from a pool of between four and seven clones in order to exclude PCR-derived sequence variations. One mutation was found in each of the mutants: in P104, the G2191GA codon was changed to GTA, resulting in the change of Gly-12 to Val; in P106, between the putative Shine-Dalgarno sequence and the ATG start codon one adenine was deleted (position 2144; Fig. 2), suggesting that the transcription of ORF4 was affected.

Phenotypes associated with ORF4 alleles. Since each of the single mutations identified above occurred in the first-step mutants P104 and P106, it should be responsible for the complex phenotype associated with the mutants. In order to verify this, R6 transformants obtained with the cloned 535-bp DNA fragment of either P104 or P106 (three transformants in each case) were tested for susceptibility to various β-lactams, competence development, and PBP profiles. Both alleles conferred

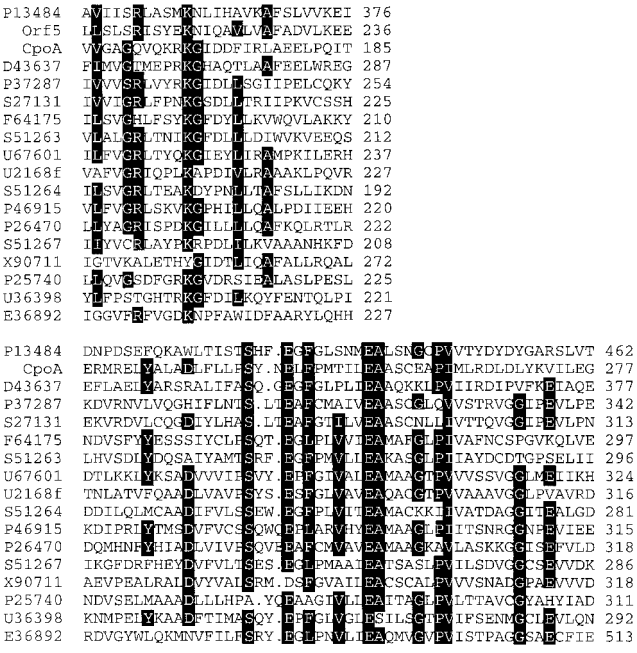

FIG. 3. Common domains of CpoA and ORF5 and a superfamily of glycosyltransferases. Identical and similar amino acids between more than half of the proteins are marked in black. The number of the last amino acid is indicated on the right. D43637, *E. coli* mannosyltransferase A; E36892, *Salmonella typhi* VipC polysaccharide biosynthetic protein; F64175, *Haemophilus influenzae* hypothetical protein; P13484, *B. subtilis* TagE; P25740, *E. coli* LPS core biosynthesis protein; P26470, *Salmonella typhimurium* LPS 1,2-*N*-acetylglucosaminetransferase; P37287, human PIG-A; P46915, *B. subtilis* hypothetical protein; S27131, *Saccharomyces cerevisiae* SP14; S51263, *Yersinia enterocolitica* TrdD; S51264, *Y. enterocolitica* TrsE; S51267, *Y. enterocolitica* TrsH; U2168f, *Mycobacterium leprae* (hypothetical); U36398, *H. influenzae* LPS biosynthesis-related protein; U67601, *Methanococcus jannaschii* LPS biosynthesis-related rfbu-protein; X90711, *Bordetella pertussis* BplH.

MICs of approximately 0.04 to 0.05 μg of piperacillin per ml compared to 0.02 μg/ml for the R6 strain; differences in MIC for cefotaxime and oxacillin between the mutants and R6 were only marginal if expressed at all (0.21 to 0.23 μg/ml versus 0.19 μg/ml for cefotaxime and 0.11 to 0.12 μg/ml versus 0.10 μg/ml for oxacillin). All transformants produced less PBP1a than the mutants (Fig. 4) (27). Competence was observed only during early exponential growth phase, and transformation efficiency was as low as in the mutant P104 (see above) and appeared to be somewhat less in P106 (not shown). It was also noted that with both alleles transformation was even less efficient, at least 10-fold, when cells were grown at 30°C compared to 37°C. The generation time was not significantly longer in mutants and transformants than in the R6 strain. Since the gene locus appeared to be involved in competence and PBP1a production, it was named *cpoA* (for PBP 1 [one] a).

Insertional mutagenesis of *cpoA*. A gene fragment covering the first 229 codons from *cpoA* was amplified by PCR and cloned into the PCR II vector prior to subcloning into the *Eco*RI-digested pJDC9. The pJDC9 vector can replicate in *E. coli* but not in *S. pneumoniae* and contains an erythromycin resistance marker that can be expressed in both organisms. Erythromycin-resistant transformants of *S. pneumoniae* can be obtained only by homologous recombination of the cloned DNA fragment with the chromosome, resulting in insertional duplication mutagenesis (40). No erythromycin-resistant transformants were obtained from this *cpoA* DNA fragment, and attempts to interrupt the gene further downstream were also unsuccessful. In contrast, with a control plasmid containing an internal DNA fragment from a nonessential gene (<500 bp), more than 200 transformants were obtained. This suggests that *cpoA*, or other genes of the operon, are essential.

The CpoA protein in parental and mutant strains. In order to obtain antibodies against CpoA, a DNA segment encoding the first 257 amino acid residues was fused to the maltose binding protein gene and overexpressed in *E. coli* (see Materials and Methods). After purification of the fusion protein and cleavage with factor Xa, the CpoA derivative was used as an antigen. The antiserum reacted with a protein of approximately 38 kDa in cell lysates of *S. pneumoniae* R6, in agreement with the predicted size of CpoA (38.8 kDa). Despite the fact that a purified CpoA derivative was used for immunization, the antiserum strongly reacted also with the *E. coli* maltose binding protein (Fig. 5). The *S. pneumoniae* homolog MalX shows a high degree (30%) of amino acid identity (43). We therefore assume that the pneumococcal protein with an apparent molecular mass of 42 kDa that is also recognized by the antiserum is MalX (Fig. 5).

P104 contained the same amounts of CpoA as R6. Remarkable was the fact that CpoA could not be detected in P106 cells as could P104 or R6, suggesting that the 1-bp deletion in the assumed promoter region of *cpoA* indeed affects the quantity of CpoA (Fig. 5a). The cross-reacting maltose binding protein served as an internal control, confirming that equal amounts of total protein were loaded per sample.

Since CpoA was apparently involved in expression of competence, which occurs only during early exponential growth phase and not at all during stationary growth, the amount in the R6 strain was assayed in samples taken at various times from a growing culture. Equivalent amounts of proteins of whole-cell lysates were separated by SDS-polyacrylamide gels and, after Western blotting, probed with CpoA-specific antibodies (not shown). Thus, the amount of CpoA per cell equivalent appeared constant during the growth cycle. The same result was obtained for P104. After separation of cytoplasmic proteins from the membrane fraction by ultracentrifugation of a cell lysate, the immunostain revealed that the main portion of CpoA was associated with the membrane fraction (Fig. 5b).

Transcription of *pbp1a*. The reduced amount of PBP1a conferred by the *cpoA* alleles could be the result of an effect at either the transcriptional or the translation level. In order to distinguish between these possibilities, *pbp1a*-specific mRNA

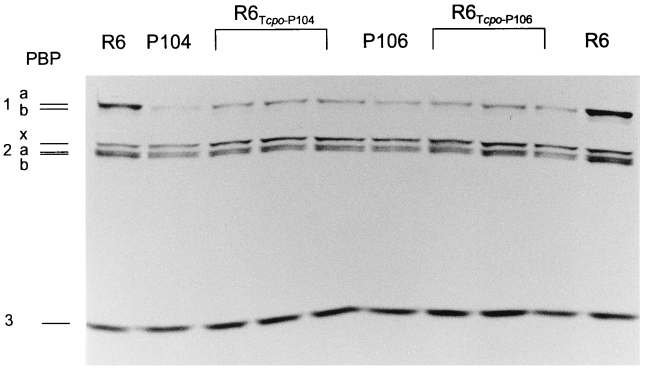

FIG. 4. Effect of *cpoA* mutations on PBP profile of *S. pneumoniae*. Shown is a fluorogram of [^{3}H]propionylampicillin-labeled cell lysates of the parental R6 strain, the mutants P104 and P106, and three R6 transformants obtained with *cpoA* of P104 ($R6_{Tcpo\text{-}P104}$) and P106 ($R6_{Tcpo\text{-}P106}$). PBPs are indicated on the left side. PBP1b can be recognized only as a faint band; the strongly labeled PBP that has the slowed electrophoretic mobility in the R6 strain is PBP1a.

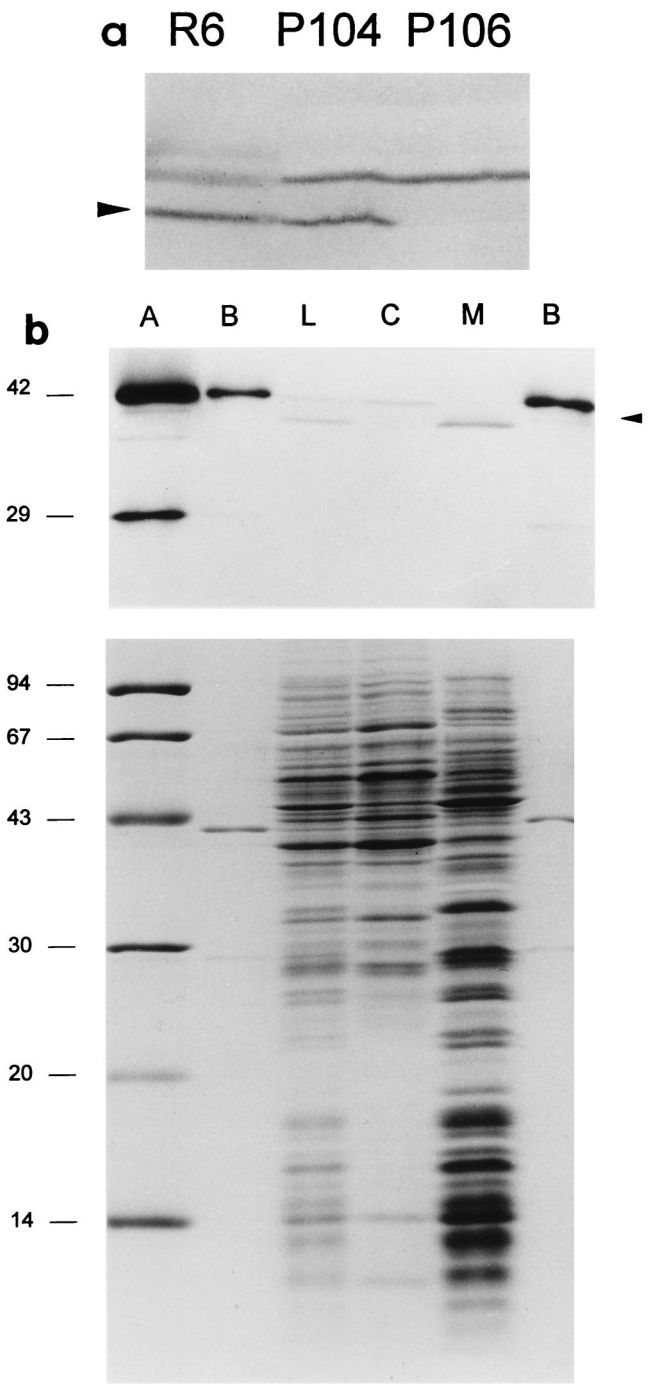

FIG. 5. Detection of CpoA with specific antibodies. CpoA protein was detected after separation of proteins on SDS-PAGE on Western blots by using rabbit anti-CpoA antiserum at a dilution of 1:10,000. (a) CpoA in cell lysates of R6, P104, and P106. The arrow indicates the position of CpoA. The protein band above corresponds presumably to the *S. pneumoniae* MalX protein. (b) CpoA in cell lysates of R6. (Top) Immunoblot of cell fractions. Lanes A and B, factor Xa-treated CpoA-MalE fusion protein. The 42-kDa band represents the maltose binding protein; the CpoA derivative has a molecular mass of 29 kDa. Lanes L, C, and M, whole-cell lysate, the cytoplasmic fraction, and the membrane fraction, respectively. The position of CpoA is marked by an arrow on the right. (Bottom) Coomassie blue-stained SDS gel. Marker proteins are indicated on the left side.

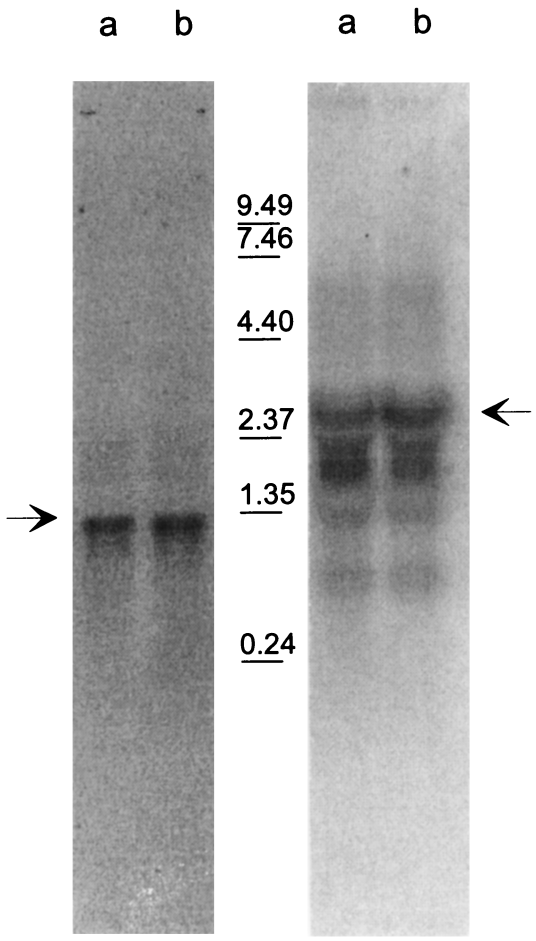

FIG. 6. Northern blot analysis for *pbp1a* and *pbp3* mRNA. RNA from *S. pneumoniae* R6 (lanes a) and P106 (lanes b) was probed with an RNA probe corresponding to an internal *pbp3* DNA fragment (left side; 10 μg of RNA) and an RNA probe corresponding to an internal *pbp1a* DNA fragment (right side; 30 μg of RNA). The sizes, in kilobases, of marker fragments are indicated.

was determined in the mutant P106 versus the R6 strain. Total RNA was isolated from P106 and R6. *pbp1a* transcript was probed with a specific RNA probe. As a control, a *pbp3*-specific probe was used since the amount of PBP3 was not affected by the *cpoA* mutations, and the amount of *pbp3* transcript should be in the same range as that of *pbp1a* in wild-type cells. As shown in Fig. 6, no significant difference in the amount of either *pbp1a* or *pbp3* between the mutant and parental strains could be detected. In contrast to the *pbp3* signal, the *pbp1a* probe revealed multiple bands, with the largest one being 2.37 kb. It is not clear whether the multiple bands indicate specific degradation of *pbp1a* RNA or reveal the presence of genes with sequences related to *pbp1a*. The size of the largest *pbp1a* transcript is larger than the *pbp1a* gene itself, indicating that like the *B. subtilis* PBP1 gene (*ponA*), which is also preceded by a small ORF, *prfA* (42), the *pbp1a* transcript starts from the promoter upstream of the start codon as has been suggested (35) and thus includes the *prfA*-related *S. pneumoniae* gene.

***cpoA* in other strains and species.** PCR-amplified DNA of the expected size (0.77 kb corresponding to the first 257 codons) was obtained with oligonucleotide primers T128 and T127 (see Materials and Methods) and chromosomal DNA isolated from a variety of penicillin-resistant *S. pneumoniae*

clinical isolates, *S. oralis* M3, and two *S. mitis* strains (NCTC10712 and a Spanish resistant isolate, 661), none of which conferred piperacillin resistance when tested in transformation assays using the R6 strain as the recipient.

DISCUSSION

Two classes of β-lactams can be distinguished with respect to the biological activity on *S. pneumoniae*: lysis and rapid killing is induced by the majority of these drugs, including penicillins, whereas growth inhibition only is evoked by broad-spectrum cephalosporins such as cefotaxime or by the monobactam aztreonam (18). The critical difference between these two classes of β-lactams appears to be their interaction with the classical target enzymes, the PBPs: cefotaxime does not bind to PBP2b; in other words, as long as PBP2b can function, lysis is not triggered.

The results described here add further evidence that the two classes of β-lactam antibiotics act by distinct mechanisms. Resistance to these drugs involves not only different PBP alterations but also different non-PBP genes. Whereas changes in PBP2x and the histidine protein kinase CiaH are selectable with cefotaxime, piperacillin selection affects primarily PBP2b and, as shown here, CpoA. Both *ciaH* and *cpoA* alleles mediate resistance in cells that contain no mutations in PBP genes; i.e., PBPs are inhibited at the same concentrations as in the wild type. β-Lactams interact at sub-MICs with PBPs (51), and secondary effects such as lysis and shedding of membrane components also occur at such low antibiotic concentrations (14, 19, 50). Mutations in the non-PBP genes may counteract such effects in the pneumococcus and allow the cell to continue to grow even if PBPs are partly inhibited. As in the case of the *ciaH* alleles, the mutations in *cpoA* conferred increased resistance in the parental background; i.e., no other mutations are required for expression of this phenotype. The increase in resistance may appear only marginal; i.e., the MICs for the mutants increased only by a factor of two compared to the MIC for the parental strain. One should be reminded, however, that a single point mutation in PBP2x confers generally at most a fourfold increase in MIC for cefotaxime, and with PBP2b, the piperacillin MIC increases just twofold or even less (11, 52). The high-level resistance phenotype of laboratory mutants or clinical isolates is due to a combination of mutations in several genes.

It is remarkable that both mutations in *ciaH* of the cefotaxime-resistant mutants and the *cpoA* alleles identified here in piperacillin-resistant mutants interfere with the susceptibility to β-lactam antibiotics and also affect the development of competence. Only in the case of *cpoA* mutants is the amount of a PBP—PBP1a—affected, but how much this change contributes to the MIC change is not known. The competence deficiency appears to be due to distinct mechanisms: the *ciaH* C306 allele conferred complete loss of transformability, and the mutant did not produce CF. Neither the growth medium nor wash fractions of C306 contained detectable amounts of competence-inducing activity (12, 52). In contrast, *cpoA* mutants have residual competence activity, and CF activity was detected in the growth medium of P506 containing the *cpoA*-P106 allele although very late during the growth cycle (not shown [10]), showing that the *cpoA* mutation allows for CSP synthesis but not necessarily at the normal time during the growth cycle. Another main difference with *ciaH* is that the *cpoA* gene or another gene of the *cpoA* operon appeared to be essential to the cell under laboratory conditions whereas insertion duplications within the *cia* genes could readily be obtained (12).

The present findings stress again the fact that cell wall metabolism and genetic competence are linked via still unknown mechanisms. We assume that, since β-lactam antibiotics affect the integrity of the cell wall as outlined above, this reflects that components required for competence induction or transformation depend on the proper biochemistry of the cell envelope.

The function of CpoA is still unknown. The gene could be amplified from other strains of *S. pneumoniae* and also from other *Streptococcus* spp., demonstrating that a homolog is present in related species. Although the protein does not contain pronounced hydrophobic segments, it fractionated with the membrane, suggesting that it associates with membrane components.

CpoA contains domains with homology to glycosyltransferases. This superfamily consists of proteins from procaryotes and eucaryotes, including gram-negative and gram-positive organisms, archaebacteria, yeast, and mammals (Fig. 3). Since a variety of sugars are the substrates of the transferases, the common domain must be related to some function other than the recognition of specific sugar residues. Common to all these enzymes is the transfer of a sugar moiety to a membrane-associated acceptor molecule that is finally localized at the outer surface of the cell membrane. A similar role for ORF4 (and presumably ORF5) can therefore be suggested, e.g., formation of the linkage unit in teichoic acid biosynthesis (41) or polymerization of teichoic acid precursors that may take place prior to translocation through the cytoplasmic membrane, as has been suggested by the recent identification of a possible translocation apparatus of teichoic acids or their precursors in *B. subtilis* (30). The *cpoA* alleles may result in a modified synthesis of the polymer, thereby altering indirectly the property of the cell envelope and counteracting the activity of cell wall antibiotics.

CpoA, CiaH, PBP2b, and PBP2x are not the only compounds that contribute to β-lactam resistance in the pneumococcus. Several mutants of the cefotaxime-resistant lineages, as well as of the piperacillin-resistant lineages, contain still unknown mutations that confer drug resistance or a competence defect as well. Characterization of these genotypes will help to unravel the link between cell wall metabolism and genetic competence in the pneumococcus.

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