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Commensal Streptococci Serve as a Reservoir for β-Lactam Resistance Genes in *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* is a leading cause of pneumonia, meningitis, septicemia, and middle ear infections. The incidence of *S. pneumoniae* isolates that are not susceptible to penicillin has risen worldwide and may be above 20% in some countries. Beta-lactam antibiotic resistance in pneumococci is associated with significant sequence polymorphism in penicillin-binding proteins (PBPs). Commensal streptococci, especially *S. mitis* and *S. oralis*, have been identified as putative donors of mutated gene fragments. However, no studies have compared sequences of the involved *pbp* genes in large collections of commensal streptococci with those of *S. pneumoniae*. We therefore investigated the sequence diversity of the transpeptidase region of the three *pbp* genes, *pbp2x*, *pbp2b*, and *pbp1a* in 107, 96, and 88 susceptible and nonsusceptible strains of commensal streptococci, respectively, at the nucleotide and amino acid levels to determine to what extent homologous recombination between commensal streptococci and *S. pneumoniae* plays a role in the development of beta-lactam resistance in *S. pneumoniae*. In contrast to pneumococci, extensive sequence variation in the transpeptidase region of *pbp2x*, *pbp2b*, and *pbp1a* was observed in both susceptible and nonsusceptible strains of commensal streptococci, conceivably reflecting the genetic diversity of the many evolutionary lineages of commensal streptococci combined with the recombination events occurring with intra- and interspecies homologues. Our data support the notion that resistance to beta-lactam antibiotics in pneumococci is due to sequences acquired from commensal Mitis group streptococci, especially *S. mitis*. However, several amino acid alterations previously linked to beta-lactam resistance in pneumococci appear to represent species signatures of the donor strain rather than being causal of resistance.**

Streptococcus pneumoniae (pneumococcus) is a leading cause of pneumonia, meningitis, septicemia, and middle ear infections (1). According to data from the World Health Organization, *S. pneumoniae* is the fourth-most-frequent cause of fatal infections worldwide (2). For many years, *S. pneumoniae* was considered to always be susceptible to beta-lactam antibiotics, and since the first reports in the late 1970s on beta-lactam antibiotic resistance in clinical pneumococcal isolates, the incidence of nonsusceptible *S. pneumoniae* isolates has risen worldwide and may be above 20% in some countries, especially in southern Europe (3). However, the introduction of the protein conjugate pneumococcal vaccines in treatment of children has reduced the rates of beta-lactam resistance in some countries, e.g., in the United States (4). Generally, the rate of nonsusceptible *S. pneumoniae* isolates correlates with the level of usage of beta-lactam antibiotics (5). In such countries, a high occurrence of nonsusceptible strains of the closely related commensal streptococci, in particular, *S. mitis* and *S. oralis*, has also been observed (6, 7).

Beta-lactam antibiotic resistance in pneumococci and commensal streptococci develops upon the accumulation of mutations in three of the six transpeptidases involved in cell wall biosynthesis, i.e., the so-called penicillin-binding proteins PBP2x, PBP2b, and PBP1a (8). These sequence alterations reduce the affinity of the transpeptidases for the drugs while leaving the enzyme function unaffected and confer an advantage for the mutated bacteria in the presence of antibiotics. While the *pbp* genes of susceptible pneumococci are well conserved, the sequences of the *pbp* genes in nonsusceptible clinical strains of pneumococci show a mosaic structure, with blocks of sequences of different lengths that may differ by up to 20% at the DNA level and by up to 10% at the amino acid level compared to the corresponding regions in susceptible pneumococci (9–11). Although one paper by Dowson et al. (12) concluded that viridans streptococci (*S. oralis*) have obtained altered penicillin-binding protein genes from penicillin-resistant strains of *S. pneumoniae*, commensal streptococci, especially *S. mitis* and *S. oralis*, have been identified as putative donors to the mosaic structure of the *pbp* genes found in resistant pneumococci (13–16). The evolution of resistance in pneumococci and commensal streptococci has been hypothesized to involve at least two steps (13): first, selection of resistant commensal streptococci with point mutations in their *pbp* genes; second, transfer of parts of these resistance genes to competent pneumococci through homologous recombination. This is in agreement with the generally one-directional transfer of genes from *S. mitis* and, to a lesser extent, *S. oralis* to *S. pneumoniae* (17). In addition, mosaic structures have been observed in resistant commensal streptococci (18–20). Comprehensive studies have identified specific mutations in pneumococci that are associated with beta-lactam resistance (21, 22). However, no studies supported the conclusions with reference to *pbp* gene sequences in resistant and susceptible

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TABLE 1 List of primers used in this study and sequence information

Gene(s)	Primer name	Primer sequence (5′ to 3′)	Position(s)[a]	Trimmed amino acid sequence (positions)[b]	No. (%) of variable nucleotide sites/ total no. of nucleotide sits[f]	No. (%) of variable amino acid sites/ total no. of amino acid sites[f]
pbp1a	pbp1a-f	ACIACDGGDATGGAHGTHTA	877–896	318–577	371/780 (48)	98/263(37%)
	pbp1a-r	GTCCADACRGCCATWGMATA	1790–1771			
pbp2b	pbp2b-f	AATGAYCGHGTBGGDACYTC	793–812	440–643	294/612 (48)	79/204 (39)
	pbp2b-r	TTGATRATRTCRCGHGCAAT	2027–2008			
pbp2x	pbp2x-f	AAGTAYATGACIGCDACCTT	862–881	308–554	320/741 (43)	79/247 (32)
	pbp2x-r	TGVAGRTTRAGRGADTCTTT	1865–1846			
murM and *murN*	murMN-f	CAYGARTGGTAYTAYTGGGAA	694–714[c]			
	murMN-r	TGAATRTARCAWCCAGGRCT	62–43[d]			
Ortholog of *murM*	murM_uo5-f	TTGCARAGTAGTGATTGGKCCA	76–97[e]			
	murM_uo5-r	TCCTTTATTYYTTGCAGTTCGGA	556–534[e]			

[a] Nucleotide positions according to the *S. pneumoniae* R6 sequence.
[b] Amino acid positions in *S. pneumoniae* R6. Active sites range from residue 337 to residue 549 (*pbp2x*), 386 to 617 (*pbp2b*), and 370 to 557 (*pbp1a*).
[c] Nucleotide position in the flanking SP_0614 gene in *S. pneumoniae* R6 of the mugsy cluster of *murM* and *murN*.
[d] Nucleotide position in the flanking SP_0618 gene in *S. pneumoniae* R6 of the mugsy cluster of *murM* and *murN*.
[e] Nucleotide position in the ortholog of the *murM* gene in *S. oralis* uo5.
[f] Numbers of all variable sites for all strains included in the sequence analysis.

members of the large populations of potential donor species. Therefore, the purpose of the present study was to investigate the sequence diversity at the nucleotide and amino acid level of the transpeptidase region of the three *pbp* genes, *pbp2x*, *pbp2b*, and *ppb1a*, in 107, 96, and 88 strains, respectively, of susceptible and nonsusceptible species of the Mitis group of streptococci in order to map the natural history of beta-lactam resistance in these streptococci.

MATERIALS AND METHODS

Strains—collection and identification. A total of 63 commensal streptococcal strains belonging to the Mitis group of streptococci were included the study. Strains were randomly picked from several strain collections, with no prior knowledge of their antibiotic susceptibility (23–25). They were isolated from the upper respiratory tract of healthy subjects or from blood of patients with bacteremia. The strains were identified by multilocus sequence analysis (MLSA) either as described by Bishop et al. (26) or using a combination of at least three of the following genes: *gdh*, *rpoB*, *dnaJ*, *ddl*, *pdgA*, *recP*, and *aroE* (27). Forty-six strains were identified as *S. mitis*, 10 as *S. oralis*, 6 as *S. infantis*, and 1 as *S. pseudopneumoniae*. Twenty-eight isolates were from the United States, 13 from Denmark, 11 from Greece, 5 from Sweden, 4 from Switzerland, and 2 from the United Kingdom. A complete list of the strains is shown in Table S1 in the supplemental material. In addition to the sequences of the 63 strains generated during this study, sequences available in GenBank of *pbp1a*, *pbp2b*, and *pbp2x* from strains of *S. mitis* and *S. oralis* with at least one known MIC value of beta-lactam antibiotics were included in the analysis (see Table S1). Ten sequences from the work of Chi et al. (13) that have not been deposited in GenBank were kindly supplied by Regine Hakenbeck. Furthermore, a total of 20 sequences from susceptible and nonsusceptible strains of *S. pneumoniae* were included for comparison (see Table S1).

Antibiotic susceptibility testing. MICs were determined by the agar dilution method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Strains were grown in Todd-Hewitt broth supplemented with 0.1% pyruvate and incubated at 37°C in a 5% CO_2-enriched atmosphere to a turbidity of a 0.5 McFarland standard (~10^8 CFU/ml). The suspension for each strain was then transferred to a single well on a sterile 96-well microtiter plate. By using an in-house multi-inoculator and applying 1 μl of the suspension, duplicate sets of Mueller-Hinton agar plates supplemented with 5% defibrinated horse blood and appropriate concentrations of antibiotics in 2-fold dilutions were inoculated with up to 96 different strains. MICs were determined after incubation at 37°C in a 5% CO_2-enriched atmosphere for 48 h. *S. pneumoniae* R6 and *S. pneumoniae* ATCC 49619 were used for quality control. The MIC was read as the lowest concentration of antibiotic inhibiting growth of the bacteria. MIC interpretation was done according to the guidelines from EUCAST (28) using the MIC values for "viridans streptococci" or for *S. pneumoniae* when no value for the relevant antibiotic was available for viridans streptococci. The following beta-lactam antibiotics were used for MIC determination: benzylpenicillin, oxacillin, piperacillin, cefotaxime, cefuroxime, and cephalothin.

DNA extraction, PCR amplification, and DNA sequencing. DNA was extracted as previously described (29). Novel primer pairs for the *pbp1a*, *pbp2b*, and *pbp2x* genes were developed by using sequences extracted from publicly available genomes of Mitis group streptococci as the templates (Table 1). All PCRs were carried out in a 25-μl volume containing 10 μl of 5′-Prime Hotmastermix (5 Prime, Hamburg, Germany), 3 μl of each primer (10 μM), 4 μl of PCR-grade H_2O, and 5 μl of DNA. Amplification was achieved with an initial cycle of 2 min of denaturation at 94°C and 30 cycles of 30 s at 94°C for denaturation, 60 s at 52°C (*pbp1a*) or 55°C (*pbp2b* and *pbp2x*) for annealing, and 60 s at 72°C for extension with a final extension step at 72°C for 5 min. Purified PCR products were sequenced on both strands at GATC Biotech, Constance, Germany.

A novel primer pair designed according to an alignment of the flanking regions of the *murM* and *murN* genes extracted from available genomes of *S. mitis* and *S. oralis* in the GenBank database was used to amplify the *murM* and *murN* genes (Table 1). The amplicon resulted in a 3.3-kb band on an agarose gel when both genes were present in a strain. In some strains, however, an ortholog of the *murM* gene, but not the *murN* gene, was located at a different position in the genome and was detected using a novel primer pair that amplifies a 500-bp internal fragment of the *murM* gene (Table 1). Reactions were carried out in a 25-μl volume containing 10 μl of 5′-Prime Hotmastermix (5 Prime, Hamburg, Germany), 3 μl of each primer (10 μM), 7 μl of PCR-grade H_2O, and 2 μl of DNA. Amplification was achieved with an initial cycle of 2 min of denaturation at 94°C and 34 cycles of 30 s at 94°C for denaturation, 40 s at 60°C for

annealing, and 90 s (*murM*) or 3 min (*murM* and *murN*) at 72°C for extension with a final extension step at 72°C for 7 min. PCR products were visualized on a 1% agarose gel by ethidium bromide staining.

Sequence analysis. Sequences of the *pbp* genes were edited, aligned, and subjected to nucleotide and protein analysis using MEGA version 6.0 (30). Sequences were trimmed to cover the active-site motifs of the transpeptidase region, except for the *pbp2b* gene, in which the first active motif, SVVK, is outside the sequence alignment (Table 1). The trimmed sequences were subjected to phylogenetic analysis using the minimum evolution algorithm. Bootstrap tests were performed with 500 replicates. The mosaic structures of the partial sequences of the *pbp1a*, *pbp2b*, and *pbp2x* genes were detected using a modified Linux version of BRATNextGen software (31). Using a window size of 40 bp and an alpha value of 1, the *pbp1a*, *pbp2b*, and *pbp2x* genes were divided into 9, 5, and 8 subclusters which were consistent with the phylogenetic clusters identified by the minimum evolution algorithm (Fig. 1). Segments of sequences potentially originating from recombination events were identified using a *P* value of 0.05 and calculated through 100 replicates of 10 iterations. The possible origin of the resulting recombinational segments was then potentially identified by phylogenetic analyses of the segments and manual sequence comparison. Segments with the possible same origin are unicolored in Fig. 1.

Nucleotide sequence accession numbers. Sequences of the three genes were deposited at GenBank with the following accession numbers: for *pbp2x*, KP732106 to KP732168 and KP984507 to KP984516; for *pbp2b*, KP732232 to KP732294; and for *pbp1a*, KP732169 to KP732231.

RESULTS

Antibiotic susceptibility. The data corresponding to susceptibility to six different beta-lactam antibiotics determined with reference to the MIC cutoff values of EUCAST are shown in Table 2 (see also Table S1 in the supplemental material). Among the commensal streptococcal strains, resistance to the three penicillins tested ranged from 35% for penicillin to 52% for oxacillin, with the median MIC value of oxacillin being 4-fold higher (4 μg/ml) than those for penicillin (1 μg/ml) and piperacillin (1 μg/ml). The same was observed for the three cephalosporins, with the median MIC value for cephalothin (8 μg/ml) being higher than those for cefuroxime (4 μg/ml) and cefotaxime (2 μg/ml) (Table 2). Only a few strains showed marked variation in susceptibility to the beta-lactam antibiotics tested, most strains showing either resistance or intermediate or full susceptibility to all six beta-lactam antibiotics. Few strains were susceptible to penicillins but resistant to cephalosporins (e.g., MC8909 and MC8938), while some strains also showed considerable variation in susceptibility to each of the three penicillins (e.g., *S. mitis* SK564 and *S. oralis* SK610) or to each of the three cephalosporins (e.g., *S. mitis* 19B-2 and *S. oralis* SK23/ATCC 35037^T).

Although direct comparison of the different countries is not possible due to the nonsystematic collection of strains, isolates from the United States and Greece were clearly more resistant to most of the antibiotics compared to isolates from Denmark, presumably reflecting different policies for antibiotic usage.

Sequence analyses. The molecular mechanisms of beta-lactam resistance were studied by partial sequencing of the transpeptidase region of the *pbp1a*, *pbp2b*, and *pbp2x* genes for all the included strains (see Table S1 in the supplemental material). Combined with GenBank-derived sequences from relevant *S. pneumoniae* strains and strains of *S. oralis* and *S. mitis* with known beta-lactam susceptibility, a total of 107, 96, and 88 sequences were included in the analyses of *pbp2x*, *pbp2b*, and *pbp1a*, respectively.

All three genes showed remarkable nucleotide variation within the sequenced part of the transpeptidase region with 43% (*pbp2x*), 48% (*pbp2b*), and 48% (*pbp1a*) polymorphic sites, which resulted in 32% (*pbp2x*), 39% (*pbp2b*), and 37% (*pbp1a*) variation at the amino acid level (Table 1).

Phylogenetic analyses. The data for the deep-rooted branches in the phylogenetic trees based on the sequenced part of the transpeptidase regions of the *pbp2x*, *pbp2b*, and *pbp1a* genes (Fig. 1) support the notion of extensive sequence polymorphism. With some exceptions, the sequences clustered according to the antibiotic susceptibility of the respective strains. Susceptible strains of *S. mitis* and *S. oralis* showed remarkable sequence variation and formed several separate clusters in all three genes, in contrast to susceptible strains of *S. pneumoniae*, where all three genes were highly conserved. This is in agreement with the general differences in sequence polymorphisms among *S. pneumoniae*, *S. mitis*, and *S. oralis* genomes (17). Below, the three *pbp* genes are discussed in more detail.

pbp2x. The sequenced part of the transpeptidase region of the *pbp2x* gene showed remarkable sequence variation, with several distinct clusters (Fig. 1a). Generally, strains clustered according to antibiotic susceptibility but not according to species affiliation. A large cluster, designated CX1, consisted mostly of resistant strains of *S. oralis*, *S. mitis*, and *S. pneumoniae*, including the multidrug-resistant *S. pneumoniae* ATCC 700669 strain (Spain 23F-1; PMEN1). Interestingly, the same cluster also contained a subcluster of susceptible *S. mitis* strains, but our mosaic structure analysis performed using BRATNextGen software revealed a small sequence segment located just upstream of the K_{547}SG active motif specific to all resistant strains of the large subcluster of CX1. Amino acid alterations within this small fragment may contribute to the reduced susceptibility among the Mitis group streptococci, including *S. pneumoniae*, in the CX1 cluster. While S. *pneumoniae* strains with reduced susceptibility were found in several clusters, susceptible strains of *S. pneumoniae* were found only in cluster CX7.

pbp2b. Minimum evolution analysis of the *pbp2b* gene revealed several distinct clusters that included strains of *S. mitis*, *S. oralis*, *S. infantis*, and *S. pneumoniae* (Fig. 1b). In contrast to the *pbp2x* gene, susceptible strains of *S. pneumoniae* were found in the same cluster (CB3) as nonsusceptible strains of *S. pneumoniae* and susceptible as well as nonsusceptible strains of *S. mitis*. Although this might indicate that the *pbp2b* gene is less involved in beta-lactam resistance, our mosaic structure analysis revealed a sequence segment at the end of the alignment that was shared by most of the nonsusceptible strains of CB3 and all strains located in clusters CB1 and CB2. These two clusters contained most nonsusceptible strains of *S. mitis* and *S. oralis*. Although no *S. pneumoniae* strains included in this study were found in CB1 and CB2, a BLAST search in GenBank revealed that the *pbp2* genes of several strains of *S. pneumoniae* in the NCBI database do belong to these clusters. Interestingly, all such *S. pneumoniae* strains for which susceptibility data were available in the GenBank database had elevated MIC values against beta-lactam antibiotics (data not shown).

pbp1a. Several distinct clusters, many of which included strains of *S. mitis*, *S. oralis*, *S. infantis*, and *S. pneumoniae*, were identified in the minimum evolution tree of the *pbp1a* gene (Fig. 1c). Most strains of *S. mitis*, *S. oralis*, and *S. infantis* with reduced susceptibility were found in CA1, CA8, and CA9, whereas CA8 and CA9 consisted almost exclusively of nonsusceptible strains. The clustering of many susceptible strains of, in particular, *S. mitis*

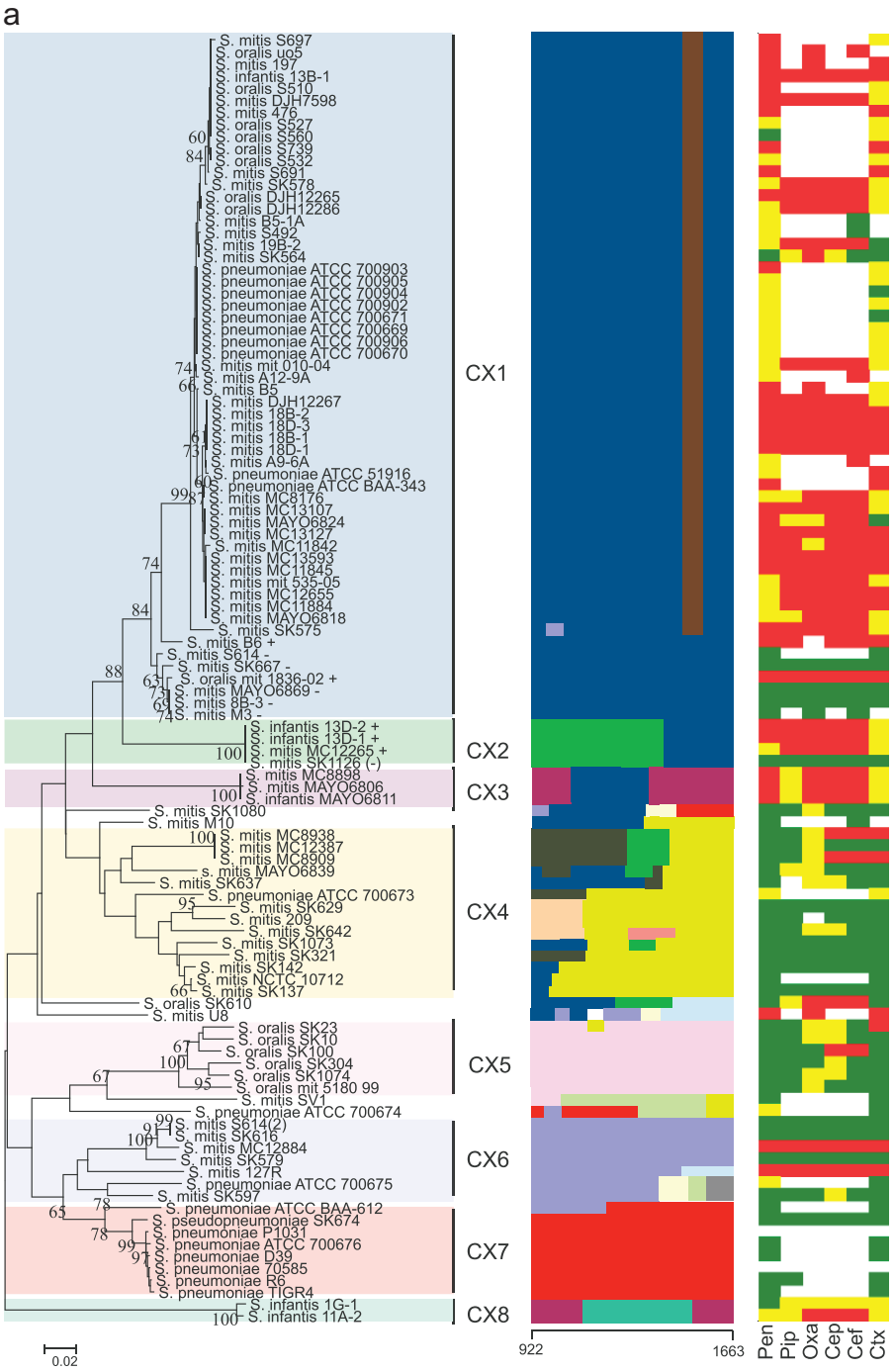

FIG 1 (a to c) Comparative analyses of *pbp* genes in Mitis group streptococci. Left columns show minimum evolution trees generated in MEGA of the transpeptidase region of *pbp2x* (a), *pbp2b* (b), and *pbp1a* (c) genes of susceptible and nonsusceptible strains of commensal streptococci and *S. pneumoniae*. Bootstrap values of 500 replications are shown. Clusters CX1 to CX8 (*pbp2x*), CB1 to CB5 (*pbp2B*), and CA1 to CA9 (*pbp1a*) identified by the BratNextgen software for detecting recombination are highlighted. In the *pbp2x* tree, +, −, or (−) after a strain name denotes the presence of, absence of, or a nonfunctional *murM* gene. The middle columns show recombination segments identified by the BratNextgen software over the length of the sequenced part of the transpeptidase region of the *pbp* genes. Blocks with the same color are hypothesized to be of the same origin. The numbers at the bottom of the middle columns indicate the region of the genes used for the analysis according to the positions in sequences of *S. pneumoniae* R6. Right columns show the susceptibility of six beta-lactam antibiotics (benzylpenicillin [pen], piperacillin [pip], oxacillin [oxa], cephalothin [cep], cefuroxime [cef], and cefotaxime [ctx]) indicated as susceptible (green), intermediate (yellow), and resistant (red) according the thresholds given by EUCAST (28) and shown in Table 2.

in CA1 and *S. pseudopneumoniae* SK674 in CA9 suggests that *pbp1a* has an effect on beta-lactam resistance in Mitis group streptococci only when altered alleles of *pbp2x* or *pbp2b* are present too.

Mosaic structure of the *pbp* genes. Analysis of recombination in the three *pbp* genes using BRATNextGen software revealed extensive inter- and intraspecies recombination resulting in a mosaic structure in all three *pbp* genes (Fig. 1). Interestingly, mosaic structures in the *pbp* genes were found both in susceptible and in

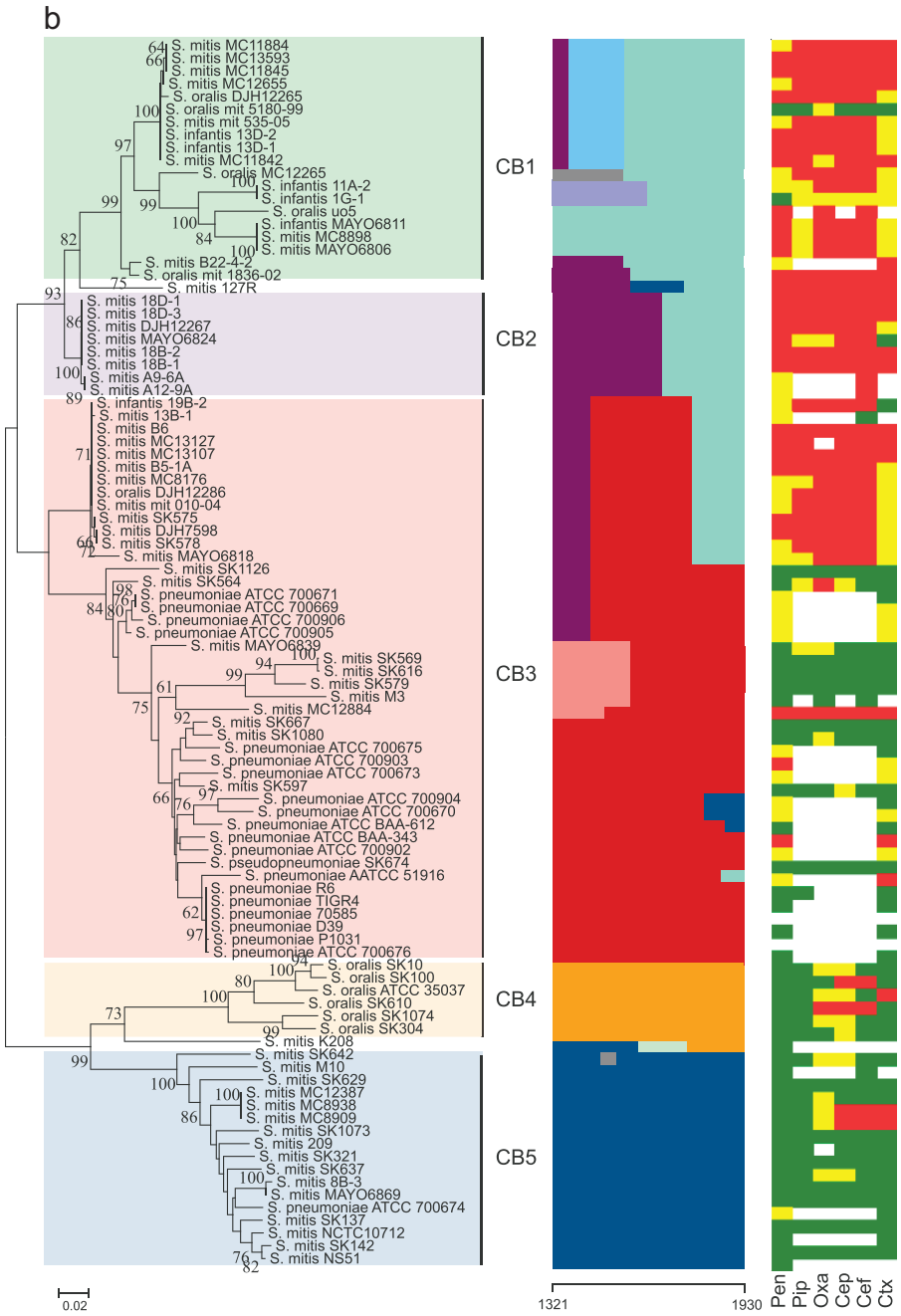

FIG 1 continued

nonsusceptible strains of *S. mitis*, *S. oralis*, and *S. infantis*, indicating that such *pbp* alleles resulting from random recombination, in these three species, were maintained in the absence of selection pressure from antibiotic use. In the *pbp2x* gene, extensive recombination was observed, particularly in cluster CX4, but all of the *pbp2x* genes displayed a mosaic structure. In the CX1 cluster, a small segment of 73 bp near the end of our alignment was observed in all but one strain with reduced susceptibility. Furthermore, phylogenetic and recombination analysis of available nearly full-length *pbp2x* gene sequences revealed that nonsusceptible strains of *S. pneumoniae* in cluster CX1 had similar sequence segments and clustered with susceptible strains of *S. pneumoniae* when the sequence of the *pbp2x* gene outside the transpeptidase region was used for analysis (data not shown). This may indicate that most of the transpeptidase region of *S. pneumoniae* strains found within the CX1 cluster originated in susceptible strains of *S. mitis* and *S. oralis* and had been transferred intact by one or several homologous recombination events (Fig. 1a). A 211-bp sequence fragment in the *pbp*2x gene of *S. mitis* SK1080 that was identical to that in susceptible strains of *S. pneumoniae* in cluster CX7 may represent a recombination event involving transfer of gene fragments from susceptible strains *of S. pneumoniae* to *S. mitis* or remnants of the ancestral allele.

The *pbp2b* gene recombination analysis revealed that strains of

FIG 1 continued

S. mitis, *S. oralis*, and *S. infantis* with reduced susceptibility in the CB3 cluster harbored a 165-bp segment at the end of the alignment that was similar to segments found in strains with reduced susceptibility in CB1 and CB2 (Fig. 1b). Amino acid alterations within this segment may play an important role in beta-lactam resistance in the Mitis group of streptococci, and a BLAST search using this segment as a query revealed that all strains of *S. pneumoniae* harboring this segment for which susceptibility data were available are nonsusceptible (data not shown). In the *pbp1a* gene, we detected several smaller fragments that were a result of recombination (Fig. 1c), as was also the case for the *pbp2x* and *pbp2b* genes. Whether these segments are due to a single recombination event involving very small DNA fragments or are a result of overlapping recombination events involving larger fragments is unknown. Unlike the *pbp2x* and *pbp2b* genes, no segments in the *pbp1a* gene were related to reduced susceptibility to beta-lactam antibiotics.

Analysis of the amino acid sequence of the transpeptidase region of the PBPs. Although extensive recombination resulting in the characteristic mosaic structure of the *pbp* genes is the basis

TABLE 2 MICs of 63 strains of commensal streptococci divided by species designation

Antibiotic and MIC (μg/ml)	No. (%) of strains	No. (%) of strains of species:			
		S. mitis ($n = 46$)	*S. oralis* ($n = 10$)	*S. infantis* ($n = 6$)	*S. pseudopneumoniae* ($n = 1$)
Benzylpenicillin					
≤0.25	30 (48)	21 (46)	7 (70)	1 (17)	1
>0.25–2	11 (17)	9 (19)	1 (10)	1 (17)	
>2	22 (35)	16 (35)	2 (20)	4 (66)	
Median	1.0	1.0	0.1875	4.0	
Piperacillin[a]					
≤0.25	22 (35)	15 (33)	6 (60)		1
>0.25–2	15 (24)	11 (24)	1 (10)	3 (50)	
>2	26 (41)	20 (43)	3 (30)	3 (50)	
Mean	1.0	1.0	0.125	2.5	
Oxacillin[a]					
≤0.25	15 (24)	12 (26)	2 (20)		1
>0.25–2	15 (24)	10 (22)	4 (40)	1 (16)	
>2	33 (52)	24 (52)	4 (40)	5 (84)	
Median	4.0	4.0	1.25	12.0	
Cephalothin[b]					
≤0.5	16 (25)	14 (30)	1 (10)		1
>0.5–2	10 (16)	5 (11)	4 (40)	1 (16)	
>2	37 (59)	27 (59)	5 (50)	5 (84)	
Median	8.0	4.0	3.0	8.0	
Cefuroxime					
≤0.5	24 (39)	18 (40)	5 (50)		1
>0.5–1	1 (2)			1 (16)	
>1	37 (59)	27 (60)	5 (50)	5 (84)	
Median	4.0	4.0	1.25	4.0	
Cefotaxime					
≤0.5	27 (44)	20 (44)	6 (60)		1
>0.5–2	18 (29)	11 (25)	2 (20)	5 (84)	
>2	17 (27)	14 (31)	2 (20)	1 (16)	
Median	2.0	2.0	0.282	2.0	

[a] No resistant breakpoints for the mitis group of streptococci are given by EUCAST. Breakpoints for benzylpenicillin are used for comparison.
[b] No resistant breakpoints for the mitis group of streptococci are given by EUCAST. Breakpoints for cefotaxime are used for comparison.

of reduced susceptibility in pneumococci, it is the amino acid changes that are responsible for the altered susceptibility. Of the many amino acid substitutions detected among our sequences compared to the susceptible *S. pneumoniae* R6 strain, none were universally found in all strains with reduced susceptibility for beta-lactam antibiotics as illustrated for penicillin and cefotaxime in Fig. 2 (see also Table S2 to Table S4 in the supplemental material). Yet several substitutions in all three *pbp* genes were associated with reduced susceptibility (discussed in detail later) (Fig. 2). In addition to substitutions, we also found an insertion of an alanine after I_{366} (according to *S. pneumoniae* R6 amino acid coordinates) in the *pbp2x* gene of the resistant *S. mitis* 127R. Likewise, D_{325} was deleted in the resistant *S. mitis* SK575 strain and G_{545} and R_{546} were deleted in the resistant *S. infantis* 13D-1 and *S. infantis* 13D-2 strains in *pbp1a*. As these indels were found only in nonsusceptible strains, these insertions and deletions may be involved in beta-lactam resistance.

Previously, mutations in the *pbp2x* gene corresponding to the S_{337}TMK active-site motif were linked to beta-lactam resistance in pneumococci (22, 32–36). We found that substitutions T_{338}G and M_{339}F were indeed associated with reduced susceptibility in our strain collection and were limited to nonsusceptible strains of *S. oralis*, *S. mitis*, and *S. pneumoniae* from the large CX1 cluster (Fig. 2a; see also Table S2 in the supplemental material). In contrast, T_{338}A and M_{339}L were also found in susceptible strains of *S. mitis*. Furthermore, the M_{400}T mutation, which also has been associated with resistance in clinical isolates of pneumococci (37) and is located in close proximity to the S_{396}SN active-site motif, was in our sequences always found together with the M_{339}F mutations in *S. mitis* strains but not in *S. oralis* strains. Combined, these two mutations may play a crucial role in beta-lactam resistance in *S. mitis* and *S. pneumoniae*. The T_{338}P mutation was found in only two strains, i.e., the *S. mitis* U8 and *S. mitis* 127R singletons located in CX6. The MIC values of all tested beta-lactam antibiotics for these two strains were very high, indicating that this substitution may play an important role in beta-lactam resistance in some strains. The amino acid sequence segment unique to nonsusceptible strains in CX1 included two unique substitutions, L_{510}T and T_{513}N, that had never previously been linked to resistance in *S. pneumoniae*. To

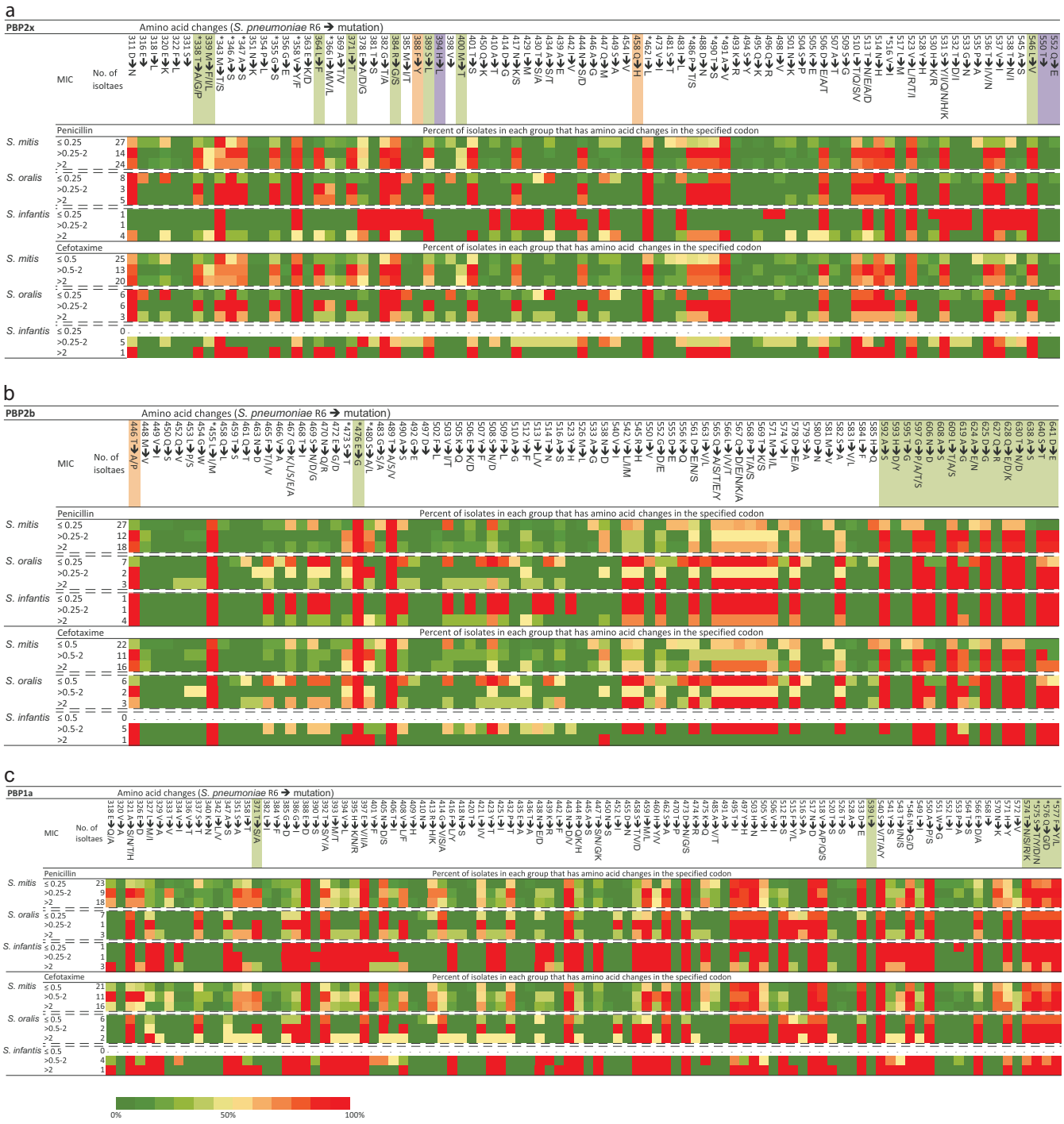

FIG 2 (a to c) Codons with amino acid changes in the transpeptidase region of PBP2x (a), PBP2b (b), and PBP1a (c) compared to the *S. pneumoniae* R6 reference strain. Only amino acids that differ from the reference sequence of the R6 strain are shown. Percentages of isolates in each group that have amino acid changes in the specified position are indicated. Amino acid positions further marked with colors or an asterisk indicate sites previously associated with resistance in *S. pneumoniae* and commensal streptococci (see reference 8). Orange, laboratory mutants; green, clinical isolates; purple, both laboratory mutants and clinical isolates. Amino acid alterations linked to reduced beta-lactam susceptibility in a study by Chewapreecha et al. (21) are marked with an asterisk. The color scale bar represents the percentage of isolates with altered amino acids compared to *S. pneumoniae* R6 in each position.

what extent these substitutions may contribute to resistance has yet to be evaluated.

The highly resistant *S. mitis* B6 strain (38) located between the resistant and susceptible subclusters in CX1 may reveal important substitutions involved in beta-lactam resistance. The $N_{417}K$ and $N_{444}S$ substitutions were found in the resistant subcluster and in *S. mitis* B6 but not in the susceptible subcluster of CX1 or in any other of the included susceptible strains. However, none of these

substitutions were previously associated with resistance in *S. pneumoniae*. The multiresistant *S. mitis* MC12884 strain clustered very closely with susceptible strains in cluster CX6. However, this strain harbors two amino acid substitutions, $A_{369}T$ and $I_{454}V$, which are not found in the susceptible strains in cluster CX7. While $A_{369}T$ is found also in the resistant *S. mitis* 127R strain, $I_{454}V$ is unique to *S. mitis* MC12284. Whether or not these mutations are contributing to the observed resistance has to be determined. Interestingly, the $Q_{552}E$ substitution, which was previously associated with reduced susceptibility in *S. pneumoniae* (39), was found only in susceptible strains of *S. mitis* and *S. oralis*.

All nonsusceptible commensal streptococci in CB3 (except for *S. mitis* MC12884) harbored a sequence segment in the *pbp2b* gene similar to that of strains in CB1 and CB2. This was characterized by several amino acid substitutions located close to the active-site motif $K_{615}TG$, including $G_{597}P$ (unique), $N_{606}D$ (unique), $L_{609}T$ (unique), and $A_{619}G$, which was found in most of these strains but also in *S. pneumoniae* ATCC 51916 from CB7 (Fig. 2b; see also Table S3 in the supplemental material). The nonsusceptible *S. pneumoniae* ATCC 51916 strain is particularly informative, as this strain had the same amino acid sequence as that found in the susceptible strains of *S. pneumoniae*, except for six amino acid substitutions from $A_{619}G$ to $D_{641}E$. These six substitutions are identical to the substitutions found in the resistant strains of CB1 and CB2 as well as in some strains in CB3. Although *S. pneumoniae* ATCC 51916 also differs from the susceptible strains of *S. pneumoniae* in the other two *pbp* genes, this may indicate that these substitutions collectively play a role in the resistance against penicillins as also previously suggested (40–42). The $T_{446}A$ and $E_{476}Q$ substitutions, which have been found in most clinical isolates of *S. pneumoniae* with reduced susceptibility (43, 44), were both found in susceptible strains of *S. mitis* and *S. oralis* (Fig. 2b; see also Table S3). Interestingly, the $E_{476}Q$ substitution was found in all strains of *S. mitis* and *S. oralis* regardless of their susceptibility but not in any susceptible strains of *S. pneumoniae*.

The alteration of T_{371} (A/S) in the *pbp1a* gene in the $S_{370}TMK$ active-site motif was found only in strains with reduced susceptibility (Fig. 2c; see also Table S4 in the supplemental material), in agreement with previous observations on clinical isolates of pneumococci (22, 45). Likewise, the $V_{408}L$ substitution was associated with reduced susceptibility although this substitution was found also in a single susceptible strain, *S. mitis* SK616. The four consecutive residues of $T_{574}SQF$ to NTGY have been linked to resistance in *S. pneumoniae* (45) but were found in many susceptible strains of *S. mitis* and *S. oralis*.

Impact of the *murM* gene on beta-lactam susceptibility. In the cluster analysis of the *pbp2x* gene, we observed that some strains clustered very closely independently of their susceptibility to beta-lactams. This was the case for resistant *S. oralis* mit_1836_02, which was very closely related to five susceptible strains of *S. mitis* in a subcluster of CX1 (subcluster a). Another example was observed in CX2, where the susceptible *S. mitis* SK1126 strain in CX2 was genetically identical in the *pbp2x* gene to the resistant *S. mitis* MC12265, *S. infantis* 13D-1, and *S. infantis* 13D-2. Although these strains were dissimilar in the two other genes, this probably does not explain all the observed differences in the beta-lactam susceptibility. On the other hand, it was previously shown that a characteristic mosaic structure in the *murM* gene may contribute to beta-lactam resistance in *S. pneumoniae* strains and that a deletion of this gene in resistant strains of *S. pneumoniae* would change the phenotype of resistant strains to susceptible (46, 47). Whole-genome comparisons of *S. mitis* and *S. oralis* strains revealed that some *S. mitis* and *S. oralis* strains lacked a *murM* gene similar to that found in *S. pneumoniae* (data not shown). Using two specific PCR assays, we found that *S. oralis* mit_1836_02 did harbor the *murM* gene whereas the closely related *S. mitis* M3, 8B-3, SK667, and MAYO 6869 strains in CX2 did not, which perfectly correlates with the susceptibility of these strains (Fig. 1a). Likewise, *S. mitis* MC12265, *S. infantis* 13D-1, and *S. infantis* 13D-2 did harbor a *murM* gene, while sequence analysis of the *murM* gene in *S. mitis* SK1126 derived from genome sequences revealed a stop codon in the *murM* gene of this strain. This pseudogene may be responsible for the susceptible phenotype of SK1126 in spite of the presence of the altered *pbp* genes. Other strains were not tested for the presence of the *murM* gene.

DISCUSSION

Understanding the mechanisms of the evolution of antibiotic resistance in bacteria is crucial. It is generally recognized that resistance to beta-lactam antibiotics in pneumococci is due to alterations in the amino acid sequence of the PBP2x, PBP2b, and PBP1a penicillin-binding proteins (8) driven by selection in the presence of excessive use of antibiotics. PBP alterations are reflected in a mosaic structure in the proteins and the corresponding genes as a result of homologous recombination with other pneumococci and closely related commensal streptococci, most notably, *S. mitis* and *S. oralis*, which share the pharynx as an ecological habitat (9, 10). Although previous studies have shown that *S. mitis* is the main donor of genes acquired by *S. pneumoniae* in homologous recombination events, our data were unable to distinguish between *S. mitis* and *S. oralis* as the main donor, presumably due to prior recombination between the two species (17, 21, 48). To fully understand this interspecies relationship, it is necessary to identify the sequence diversity of the *pbp* genes in commensal streptococci. To our knowledge, this study is the first to identify the sequence diversity of the three major *pbp* genes, *pbp2x*, *pbp2b*, and *pbp1a*, in a large collection of susceptible and nonsusceptible strains of commensal streptococci, in particular, *S. mitis*. We found that the overall number of polymorphic sites generated by spontaneous mutations in all three genes was considerable (up to 39% of all sites) for both susceptible and nonsusceptible strains of *S. mitis*, *S. oralis*, and *S. infantis*. This contrasts with the situation among pneumococci, where only nonsusceptible strains display extensive sequence variations as a consequence of the import of sequences previously diversified in commensal streptococci. This is in agreement with the general population structure of Mitis group streptococci, where pneumococci have a clonal origin, while both *S. mitis* and *S. oralis* consist of multiple evolutionary lineages that serve as a gene reservoir for pneumococci (17, 26, 48). Our phylogenetic analysis of the *pbp* genes revealed that the distances between lineages of *S. mitis* were greater than between those of susceptible pneumococci and *S. mitis*, supporting the hypothesis that *S. pneumoniae* is a successful pathogenic lineage within the *S. mitis* cluster (17, 48).

In agreement with a previous report (8), homologous recombination affecting *pbp* genes was detected almost exclusively in nonsusceptible strains of pneumococci. In contrast, commensal streptococci showed evidence of homologous inter- and intraspecies recombination in both susceptible and nonsusceptible strains. The finding of interspecies recombination affecting *pbp* genes of *S.*

pneumoniae, *S. mitis*, and *S. oralis* is in agreement with previous reports (9, 13–16). The predominantly unidirectional gene transfer from *S. mitis* to *S. pneumoniae* is in agreement with results of our recent comparative analyses of *S. mitis* and *S. pneumoniae* genomes (17). By this process, pneumococci have exploited the more extensive diversification that already occurs in *S. mitis* lineages as a result of accumulation of mutations and intraspecies recombination. In a few cases, in this study, we observed potential homologous sequence transfer from *S. pneumoniae* to *S. mitis* and *S. oralis*. It is conceivable that these potential exceptions were facilitated by the extensive selective pressure on the *pbp* genes exerted by beta-lactam antibiotic usage (49).

The absence of interspecies recombination affecting *pbp* genes in susceptible strains of pneumococci may be due to reduced fitness of strains displaying mosaic structure in the absence of antibiotic selection pressure. In support of this, Albarracín Orio et al. (50) observed that incorporation of *pbp2b* mutant alleles from commensal streptococci resulted in fitness costs. Incorporation of *pbp1a* and *pbp2x* mutant alleles, however, had a compensatory effect, which may explain why most resistant pneumococci are altered in all three *pbp* genes.

Our recombination analysis suggests that very small (i.e., below 100-bp) sequence fragments were involved in recombination, which would be in agreement with a recent conclusion reported by Mostowy et al. (51) that recombination involving small sequence fragments, termed microrecombination, has an important impact on pneumococcal evolution. Nevertheless, Sauerbier et al. (52) showed that, under *in vitro* conditions, random transformation events occurring between *S. mitis* B6 and *S. pneumoniae* R6 involved from 160 to 23,000 nucleotides. Therefore, a potential explanation is that the small mosaic units observed by Mostowy et al. (51) and us are a result of several independent but partially overlapping recombination events. Either way, these small diverse fragments generated by mutations play a crucial role in shaping the *pbp* genes and, thus, the evolution of beta-lactam resistance in commensal streptococci and *S. pneumoniae*.

Although the collection of strains for this study was not systematic, our data imply that the occurrence of commensal Mitis group streptococci with reduced susceptibility is more common in countries with high-level usage of antibiotics such as the United States and Greece than in countries with a more restricted use of antibiotics such as Denmark. This is in agreement with studies that have shown an increase of the carriage of resistant nonpneumococcal alpha-hemolytic streptococci after antibiotic therapy (53, 54). Apparently, these commensal streptococci may serve as a reservoir long after the occurrence of selective pressure of beta-lactam antibiotic usage because the fitness cost of the resistant phenotype appears to be lower in *S. mitis* and *S. oralis* strains than in *S. pneumoniae* strains.

A recent genome-wide association study by Chewapreecha et al. (21) involving 4,001 pneumococcal isolates aimed at identifying single nucleotide polymorphisms (SNPs) that could confer beta-lactam nonsusceptibility. The study identified 301 SNPs (207 in *pbp* genes), of which 71 (52 in *pbp* genes) were nonsynonymous and associated with beta-lactam nonsusceptibility. Interestingly, of the 20 amino acid alterations shared with our study, all but 1 were detected in susceptible *S. mitis* or *S. oralis* strains (MIC values for penicillin, below 0.032 μg/ml) (Fig. 2). Furthermore, 15 of these substitutions were found in more than 75% of all the *S. mitis* and *S. oralis* strains in our study (Fig. 2). Similar correlations were observed for many of the amino acid alterations associated with beta-lactam resistance in 80 pneumococcal strains found in the study of Hsieh et al. (22). These included several alterations associated with pneumococcal resistance also reported in other studies (8) such as $R_{384}G$ in the *pbp2x* gene, $T_{574}SQF$ to NTGY in the *pbp1a* gene, and $E_{476}G$ in the *pbp2b* gene. These observations clearly suggest that many of the amino acid alterations previously found to be associated with resistance in pneumococci more likely are species signatures of the donor of the sequence rather than the specific alteration causing resistance. Our findings highlight the difficulties in detecting true causal amino acid alterations even in large genome-wide association studies without reference to the large population of closely related Mitis group streptococci.

Our results support the hypothesis that the *murM* gene may contribute to beta-lactam resistance in commensal streptococci, as is the case for *S. pneumoniae* (47, 55). We found that the presence of the *murM* gene was necessary for commensal streptococci to develop a resistant phenotype. In pneumococci, the *murM* gene is always present (46), indicating that the *murM* gene is indispensable in pneumococci but not in commensal streptococci as long as they remain susceptible to beta-lactam antibiotics. To what degree the *murM* gene and, potentially, other genes may contribute to beta-lactam resistance in commensal streptococci needs to be evaluated in more detail.

In conclusion, the transpeptidase regions of *pbp2x*, *pbp2b*, and *pbp1a* in commensal Mitis group streptococci display considerable sequence variation at both the nucleotide and the amino acid levels. In contrast to pneumococci, sequence variation was observed in both susceptible and nonsusceptible strains, reflecting the level of genetic diversification within the species, including consequences of prior intra- and interspecies homologous recombination events. Furthermore, our data support the hypothesis that resistance to beta-lactam antibiotics in pneumococci is due to sequences acquired from commensal Mitis group streptococci, especially *S. mitis*. Several amino acid alterations found to be associated with beta-lactam resistance in pneumococci were also found to be associated with reduced susceptibility in the commensal streptococci. However, other amino acid alterations previously linked to beta-lactam resistance in pneumococci appear to represent species signatures of the donor strain rather than being causal of resistance. This report clearly demonstrates the importance of the commensal Mitis group streptococci as a reservoir of beta-lactam resistance exploited by pneumococci. Further insight into this relationship obtained by whole-genome sequencing of a comprehensive collection of susceptible and resistant strains of Mitis group streptococci will facilitate the exact identification of structural changes in penicillin-binding proteins responsible for resistance to beta-lactam antibiotics. Such information may eventually enable the design of modified antibiotics that circumvent these resistance mechanisms.

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