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Penicillin-resistant viridans streptococci have obtained altered penicillin-binding protein genes from penicillin-resistant strains of *Streptococcus pneumoniae*

(horizontal gene transfer/mosaic genes/genetic transformation/*Streptococcus sanguis*/*Streptococcus oralis*)

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ABSTRACT **Penicillin-resistant strains of *Streptococcus pneumoniae* possess altered forms of penicillin-binding proteins (PBPs) with decreased affinity for penicillin. The PBP2B genes of these strains have a mosaic structure, consisting of regions that are very similar to those in penicillin-sensitive strains, alternating with regions that are highly diverged. Penicillin-resistant strains of viridans group streptococci (e.g., *S. sanguis* and *S. oralis*) that produce altered PBPs have also been reported. The PBP2B genes of two penicillin-resistant clinical isolates of *S. sanguis* were identical in sequence to the mosaic class B PBP2B genes found in penicillin-resistant serotype 23 strains of *S. pneumoniae*. Emergence of penicillin resistance appears to have occurred by the horizontal transfer of an altered PBP2B gene from penicillin-resistant *S. pneumoniae* into *S. sanguis*. The PBP2B genes of three penicillin-resistant *S. oralis* strains were similar to the mosaic class B PBP2B gene of penicillin-resistant strains of *S. pneumoniae* but possessed an additional block of diverged sequence. Penicillin resistance in *S. oralis* has also probably arisen by horizontal transfer of this variant form of the class B mosaic PBP2B gene from a penicillin-resistant strain of *S. pneumoniae*.**

The emergence of resistance to penicillin in a number of bacterial species has occurred by the development of altered high molecular weight penicillin-binding proteins (PBPs) that have reduced affinity for the antibiotic (1). These PBPs are encoded by chromosomal genes and catalyze the final stages of bacterial peptidoglycan synthesis (1). One of the best documented examples of this type of resistance is provided by *Streptococcus pneumoniae* (the pneumococcus). Penicillin resistance in *S. pneumoniae* was first reported in Australia and Papua New Guinea in the late 1960s (2, 3). Subsequently, pneumococci with higher levels of penicillin resistance, and multiply antibiotic-resistant strains, were reported from South Africa (4). Penicillin-resistant pneumococci are now found worldwide and have minimal inhibitory concentrations (MICs) of benzylpenicillin up to 1000-fold greater than those of typical penicillin-sensitive strains (5).

Resistance is due to the production of altered forms of the high molecular weight PBPs 1A, 1B, 2A, 2B, and 2X (6, 7). The origins of penicillin-resistant pneumococci have been investigated by comparing the sequences of PBP genes from penicillin-sensitive and penicillin-resistant strains (8, 9). Sequencing of the gene encoding PBP2B from penicillin-sensitive strains of *S. pneumoniae*, and of the amylomaltase gene from both sensitive and resistant strains, revealed that this species is genetically very uniform [sequence divergence between strains of ≤1% (9)]. However, the PBP2B genes from penicillin-resistant strains have a mosaic structure, consisting of blocks of nucleotides that are very similar to those in the PBP2B genes of penicillin-sensitive strains ("sensitive blocks"), and blocks that diverge by 14–23% ("resistant blocks") (9). The emergence of penicillin resistance in pneumococci appears to have involved the replacement, on at least two occasions, presumably by transformation, of parts of the original pneumococcal PBP2B gene with the homologous regions from closely related species (9).

Penicillin resistance has also developed in other species of streptococci. Viridans streptococci are members of the normal mouth flora and are a major cause of bacterial endocarditis. These streptococci have long been considered to be uniformly susceptible to penicillin. However, as early as 1949, penicillin-resistant isolates of α-hemolytic streptococci, which may have been members of the viridans group, were shown to arise in the gingival flora of patients receiving oral penicillin prophylaxis (10). Only in the late 1970s, in South Africa and the U.S., were penicillin-resistant viridans streptococci positively identified to the species level (11). In South Africa in the late 1970s penicillin-resistant isolates of *S. mitis* and *S. sanguis* were found in the nasopharynx of children coincident with the emergence of multiply antibiotic-resistant strains of *S. pneumoniae* (11). Serious infections (e.g., endocarditis) caused by penicillin-resistant viridans streptococci remain rare (12). Resistance to penicillin in these viridans streptococci has been shown to be due to the production of altered PBPs with lowered affinity for penicillin (11, 12).

S. pneumoniae and viridans streptococci are naturally transformable. Transformation of chromosomal genes has been shown to occur *in vivo* between genetically distinguishable strains of *S. pneumoniae* and between *S. pneumoniae* and viridans streptococci (13). We have therefore investigated whether penicillin-resistant viridans streptococci isolated recently in the U.K. have emerged by the horizontal transfer of altered PBP genes from penicillin-resistant strains of *S. pneumoniae* into penicillin-sensitive strains of viridans streptococci.‡

MATERIALS AND METHODS

Bacterial Strains. The characteristics of the strains used are given in Table 1. All of the penicillin-sensitive and penicillin-resistant strains of *S. sanguis* and *S. oralis* were from blood cultures, except *S. oralis* 5302, which was from a wound infection. Streptococcal strains were grown at 37°C on brain–

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Abbreviations: PBP, penicillin-binding protein; PCR, polymerase chain reaction.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M32226–M32228).

Table 1. Properties of penicillin-sensitive and penicillin-resistant streptococci

Strain	Species	Sero-type	MIC,* μg/ml	Year of isolation	Origin
R6	*S. pneumoniae*	NC	0.008	≈1930	U.S.
64147	*S. pneumoniae*	6	6	1978	S. Africa
PN87/557	*S. pneumoniae*	23	2	1987	U.K.
1907	*S. sanguis*	—	1	1988	U.K.
2397	*S. sanguis*	—	2	1988	U.K.
7863	*S. sanguis* (T)	—	ND	1935	NCTC
5303	*S. sanguis*	—	<0.06	1989	U.K.
5309	*S. sanguis*	—	<0.06	1989	U.K.
3626	*S. oralis*	—	4	1988	U.K.
5296	*S. oralis*	—	8	1989	U.K.
5302	*S. oralis*	—	2	1989	U.K.
11427	*S. oralis* (T)	—	ND	1965	NCTC
5298	*S. oralis*	—	<0.06	1989	U.K.
5304	*S. oralis*	—	<0.06	1989	U.K.
11436	*S. bovis*	—	ND	1972	NCTC
2674	*S. cecorum* (T)	—	ND	1982	NCFB
2720	*S. cricetus* (T)	—	ND	1983	NCFB
2721	*S. ferrus* (T)	—	ND	1983	NCFB
2722	*S. iniae* (T)	—	ND	1983	NCFB
10708	*S. milleri*	—	ND	1956	NCTC
10449	*S. mutans* (T)	—	ND	1924	NCTC
2723	*S. rattus* (T)	—	ND	1983	NCFB
8618	*S. salivarius* (T)	—	ND	1943	NCTC
2724	*S. sobrinus* (T)	—	ND	1983	NCFB
12166	*S. vestibularis* (T)	—	ND	1987	NCTC
11389	Group K	—	ND	1979	NCTC

NC, noncapsulated strain; (T), type culture of the species; ND, not determined; NCTC, National Collection of Type Cultures, Colindale, U.K.; NCFB, National Collection of Food Bacteria, Shinfield, U.K.

*Minimal inhibitory concentration of benzylpenicillin.

heart infusion agar (Difco) with 4000 units of catalase per ml, or on the same medium plus 4% defibrinated horse blood, in a 5% CO_2/95% air atmosphere. The species of viridans streptococci were determined using the streptococcal API-20 system (API-bioMerieux, U.K.).

Isolation and Sequencing of the PBP2B Gene from Penicillin-Resistant Strains of *S. sanguis* and *S. oralis*. Chromosomal DNA from *S. oralis* was isolated as described (8). For *S. sanguis* this method was modified by adding lysozyme (5 mg/ml of cell suspension) for 10 min at room temperature prior to the addition of sodium deoxycholate. After the addition of deoxycholate, the suspension was frozen in solid CO_2 and thawed at room temperature three times to increase the degree of cell lysis.

A 1.5-kilobase (kb) fragment encoding the transpeptidase domain of PBP2B from *S. sanguis* and *S. oralis* was amplified from chromosomal DNA by the polymerase chain reaction (PCR) and was cloned in each orientation into bacteriophage M13 as described (8). The nucleotide sequence was determined on both strands by dideoxynucleotide sequencing using oligonucleotides that primed at intervals along each strand of the gene. Occasional errors arising from the PCR were resolved by sequencing a third independent M13 clone.

Isolation and Sequencing of the Amylomaltase Gene. A 929-base-pair (bp) fragment from within the amylomaltase gene was amplified by the PCR and cloned in one orientation into M13mp19, and the nucleotide sequence of 600 bp of the fragment was determined as described (9).

DNA Hybridization. Dense suspensions (≈10^9 bacteria per ml) of each bacterial strain were prepared in brain–heart infusion broth and 3 μl of each suspension was spotted on a sterile nylon membrane (Hybond-N; Amersham) on the surface of a blood agar plate, which then was incubated for 24 hr at 37°C in a 5% CO_2/95% air atmosphere. Membranes containing the bacterial growth were lifted from the agar plates and were processed for DNA hybridization (14). Hybridization with ^{32}P-end-labeled oligonucleotides or [^{32}P]-DNA fragments was carried out by standard techniques using stringent conditions (14).

RESULTS AND DISCUSSION

Detection of Mosaic PBP2B Genes from *S. pneumoniae* in Penicillin-Resistant *S. sanguis* and *S. oralis* by Oligonucleotide Probes. The altered PBP2B (PBP2B^R) genes found in a collection of penicillin-resistant pneumococci have been classified into two groups (9). The class A PBP2B^R gene was found in resistant strains of various serotypes, whereas the class B PBP2B^R gene was found only in serotype 23 strains obtained from Spain and the U.K. The class A genes from different resistant strains varied, both in sequence and in the patterns of alternating "sensitive" and "resistant" blocks. In contrast the class B genes from five resistant strains were identical in sequence (9).

The class A and class B PBP2B^R genes of penicillin-resistant pneumococci differ extensively from each other, and from the PBP2B genes of penicillin-sensitive strains, between nucleotides 1505 and 1567 (Fig. 1 and ref. 9). This region has been found to be altered in all penicillin-resistant pneumococci (9) and the resulting amino acid substitutions are a major contributor to the lower affinity of PBP2B for penicillin (C.G.D., J. A. Brannigan, and B.G.S., unpublished results). Oligonucleotides corresponding to part of this region (nucleotides 1505–1525) were therefore used as probes to search for the pneumococcal class A or class B PBP2B^R genes in penicillin-resistant viridans streptococci. The sequence of the probes for the class A (Pn11) and class B (Pn13) PBP2B^R genes of penicillin-resistant pneumococci and for the PBP2B gene of penicillin-sensitive pneumococci (Pn12) are underlined in Fig. 1, lines B, C, and A, respectively. In addition, the PCR-amplified 1.5-kb fragment from the PBP2B gene of the penicillin-sensitive *S. pneumoniae* strain R6 (nucleotides 812–2316; Fig. 1) was used as a probe.

None of the oligonucleotide probes hybridized to the penicillin-sensitive strains of *S. sanguis* and *S. oralis*. The 1.5-kb probe from the penicillin-sensitive pneumococcus also failed to hybridize to penicillin-sensitive *S. sanguis* strains and bound only very weakly to sensitive strains of *S. oralis*. The sequence of the PBP2B gene of *S. sanguis*, and to a lesser extent of *S. oralis*, appears therefore to be diverged considerably from that of *S. pneumoniae* (see below). However, Pn13 (the probe for the pneumococcal class B PBP2B^R gene) and the 1.5-kb probe from the penicillin-sensitive pneumococcus, hybridized strongly to each of the penicillin-resistant strains of *S. sanguis* and *S. oralis*. None of the other oligonucleotide probes hybridized to the resistant viridans streptococci.

The PBP2B genes of penicillin-resistant strains of *S. oralis* and *S. sanguis*, unlike those of penicillin-sensitive strains, thus contain sequences that are identical, or nearly identical, to sequences found in both the class B PBP2B^R gene of penicillin-resistant pneumococci and the normal PBP2B gene of penicillin-sensitive pneumococci. These results suggest that the PBP2B gene of penicillin-resistant viridans streptococci may be closely related to the mosaic class B PBP2B^R gene found in penicillin-resistant pneumococci. The sequences of the PBP2B genes from resistant *S. oralis* and *S. sanguis* strains were therefore determined.

Nucleotide Sequences of the PBP2B Genes from Penicillin-Resistant Strains of *S. sanguis*. The sequences of part of the PBP2B gene from two penicillin-resistant strains of *S. sanguis* are shown in Fig. 1, together with that of a penicillin-sensitive pneumococcus and those of class A and class B

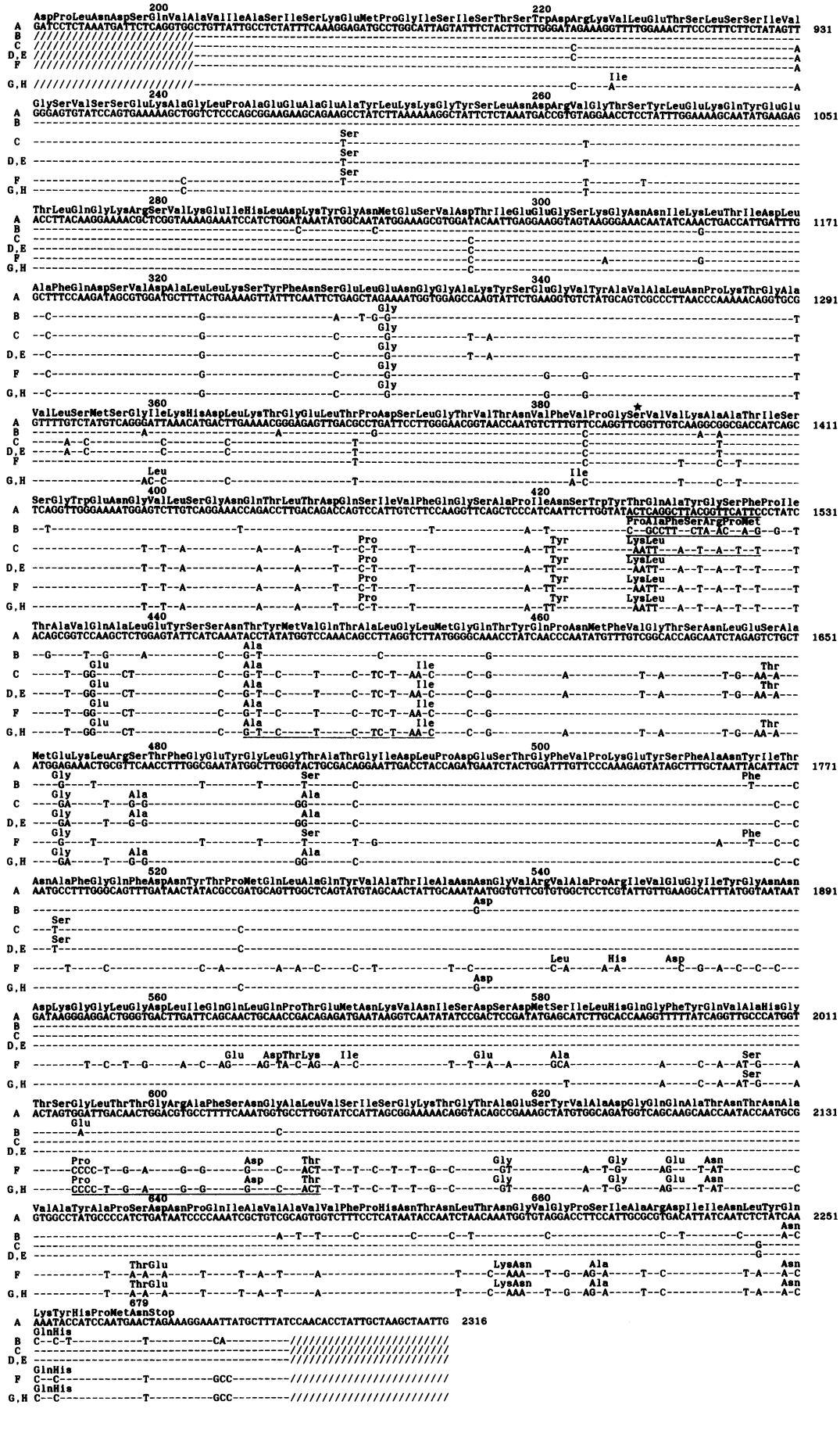

FIG. 1. (*Legend appears at the bottom of the opposite page.*)

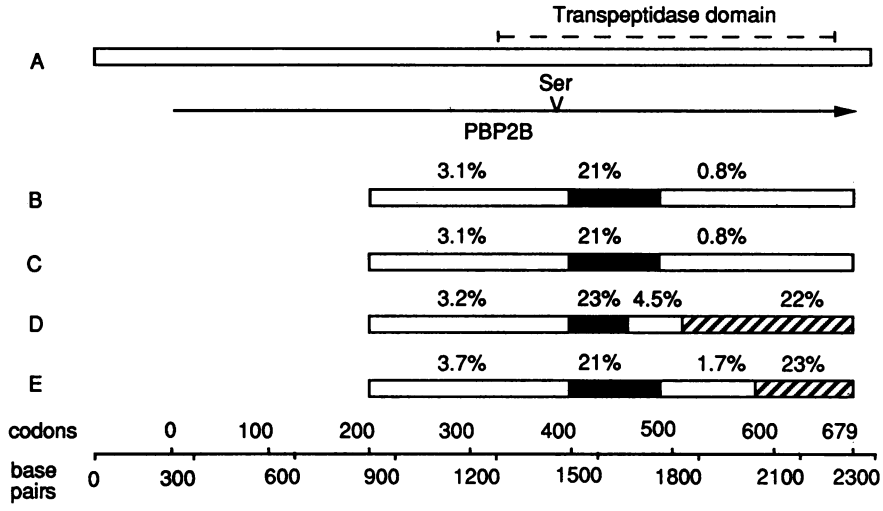

FIG. 2. PBP2B genes of penicillin-resistant streptococci. The PBP2B gene of the penicillin-sensitive *S. pneumoniae* strain R6 is represented in *A*. The thin line terminating in an arrow shows the extent of the coding region. The location of the penicillin-sensitive transpeptidase domain is shown by the dashed line. The sequenced regions of the PBP2B genes of penicillin-resistant strains are shown in *B–E*. The open rectangles show regions where the sequences are very similar to those in penicillin-sensitive pneumococci ("sensitive blocks"). The solid (block 1) and hatched (block 2) rectangles are regions that are highly diverged ("resistant blocks"). (*B*) *S. pneumoniae* strain DN87/577 (class B PBP2B^R gene). (*C*) Penicillin-resistant *S. sanguis* strains 1907 and 2397. (*D*) Penicillin-resistant *S. oralis* strain 3626. (*E*) Penicillin-resistant *S. oralis* strains 5296 and 5302. The figures above the blocks show the percentage sequence divergence from the corresponding regions in penicillin-sensitive pneumococci. The position of the active-site serine residue is marked.

PBP2B^R genes from penicillin-resistant pneumococci. The sequences from the *S. sanguis* strains 1907 and 2397 (Table 1), which were isolated in the U.K., were identical both to each other and to the class B PBP2B^R gene of penicillin-resistant strains of *S. pneumoniae*.

The class B PBP2B^R gene has been found in penicillin-resistant serotype 23 strains of *S. pneumoniae* from the U.K. and Spain (9). Since serotype 23 strains are by far the most common type of penicillin-resistant pneumococci in the U.K. (16), it is likely that the mosaic class B PBP2B^R gene from these pneumococci has been introduced by transformation into *S. sanguis*. However, the possibility that the mosaic class B PBP2B^R gene arose in *S. sanguis*, and has been introduced into pneumococci, must be considered.

The latter possibility is very unlikely, as the two "sensitive blocks" in the mosaic class B PBP2B^R gene (Fig. 2) are almost certainly pneumococcal DNA since their sequences diverge from those of the penicillin-sensitive pneumococcus by only 3.1% and 0.8%, respectively. The failure of the 1.5-kb probe from the PBP2B gene of a penicillin-sensitive pneumococcus to hybridize to penicillin-sensitive strains of *S. sanguis* suggests that these two species are considerably diverged in sequence. Comparison of the sequence of a 600-bp region from the amylomaltase gene of the penicillin-sensitive *S. sanguis* strain 5309 with that of the corresponding region of six *S. pneumoniae* strains showed a divergence of ≈19% between the two species. The "sensitive blocks" in the class B PBP2B^R gene are therefore far too similar to those of *S. pneumoniae* to have originated in *S. sanguis*.

Nucleotide Sequences of the PBP2B Genes from Penicillin-Resistant Strains of *S. oralis*. The sequences of the PBP2B genes of three penicillin-resistant strains of *S. oralis* were determined (Fig. 1). The sequences from isolates 5296 and 5302 (Table 1) were identical and had a mosaic structure, consisting of two blocks that were very similar to those in penicillin-sensitive pneumococci (≤3.7% diverged), and two blocks that were 21% and 23% diverged from those in sensitive pneumococci (Fig. 2). One of the diverged blocks (block 1; nucleotides 1429–1702) was identical to the "resistant block" in the class B PBP2B^R gene of penicillin-resistant pneumococci. The downstream diverged block (block 2; nucleotides 1990–2281) was not found in the class B PBP2B^R gene or in any of the class A PBP2B^R genes (9).

The PBP2B gene of the third resistant *S. oralis* strain (3626) had a similar mosaic structure but possessed a slightly truncated form of block 1 and an extended form of block 2 (Figs. 1 and 2). Apart from the differences in length, blocks 1 and 2 were identical in sequence in all three resistant *S. oralis* strains.

The PBP2B genes of the resistant *S. oralis* strains were therefore similar to the class B PBP2B^R gene of penicillin-resistant serotype 23 strains of *S. pneumoniae* but contained an extra resistant block. The resistant blocks in the PBP2B genes of penicillin-resistant pneumococci are believed to have been introduced by transformation from the homologous genes of related streptococcal species (9). Both blocks 1 and 2 of the resistant *S. oralis* strains show a similar level of divergence from *S. pneumoniae* (≈22%) and may originally have been introduced into pneumococci from the same source to create a variant form of the class B PBP2B^R gene that has not so far been identified in penicillin-resistant pneumococci. Subsequently, this mosaic PBP2B^R gene appears to have been introduced into *S. oralis*.

Comparisons of the sequences of 600 bp of the amylomaltase genes from three penicillin-sensitive strains of *S. oralis* with those from six *S. pneumoniae* strains (7) showed a divergence of 4–6%. However, there was considerably more intraspecies variation among the *S. oralis* amylomaltase

Fig. 1 (*on opposite page*). Nucleotide sequences of part of the PBP2B genes of penicillin-sensitive and penicillin-resistant streptococcal strains. The sequence of nucleotides 812–2316 of the PBP2B gene of the penicillin-sensitive *S. pneumoniae* strain R6 is shown in full in line A. Positions where the sequences of the PBP2B genes of other strains differ from those in line A are shown. Lines: B, penicillin-resistant *S. pneumoniae* strain 64147 (class A PBP2B^R gene); C, penicillin-resistant *S. pneumoniae* strain PN87/577 (class B PBP2B^R gene); D,E, penicillin-resistant *S. sanguis* strains 1907 and 2397; F, penicillin-resistant *S. oralis* strain 3626; G,H, penicillin-resistant *S. oralis* strains 5296 and 5302. The PBP2B gene is numbered according to ref. 15. The sequences of the oligonucleotides that were used as probes are underlined (see text). //////////, Positions of the PCR primers; *, active-site serine residue.

sequences (3–8%) than among the pneumococcal sequences (≤0.5%). As *S. oralis* is so closely related to *S. pneumoniae*, it is not easy to exclude the possibility that the mosaic PBP2B^R gene of resistant *S. oralis* strains was formed by the introduction of the diverged blocks into a penicillin-sensitive strain of *S. oralis*. The best evidence that it originated in *S. pneumoniae* is that the 1.5-kb probe from the penicillin-sensitive strain of *S. pneumoniae* hybridized strongly under stringent conditions to penicillin-resistant strains of *S. oralis* but only very weakly to penicillin-sensitive strains. The PBP2B genes of the resistant *S. oralis* strains therefore contain sequences (the sensitive blocks) that are more similar to those in penicillin-sensitive pneumococci than to those in penicillin-sensitive strains of *S. oralis*. We therefore favor the view that the variant mosaic PBP2B^R gene found in the resistant *S. oralis* strains has been introduced by transformation from penicillin-resistant pneumococci.

Attempts to Identify the Source of the Resistant Blocks. The oligonucleotides Pn26′ (nucleotides 1565–1594) and Pn29′ (nucleotides 2018–2056), which correspond to regions within block 1 and block 2, respectively (underlined in Fig. 1, line G,H), were used to probe chromosomal DNA from each of the streptococcal species listed in Table 1, in an attempt to identify the source(s) of these diverged blocks. The probes failed to hybridize to DNA from any of the penicillin-sensitive strains of *S. oralis* or *S. sanguis*. As expected, Pn26′ hybridized to each of the resistant *S. oralis* and *S. sanguis* strains and to *S. pneumoniae* DN87/557, which possesses the class B PBP2B^R gene, and Pn29′ hybridized to the resistant *S. oralis* strains. However, neither probe hybridized to any of the other streptococcal strains listed in Table 1, and the origin(s) of the diverged blocks remains unknown.

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