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Genetics of Oxacillin Resistance in Clinical Isolates of *Streptococcus pneumoniae* That Are Oxacillin Resistant and Penicillin Susceptible

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It has recently been reported that penicillin-sensitive pneumococci may exhibit reduced susceptibility to oxacillin, resulting in their misclassification as being penicillin resistant by oxacillin disk testing. Intermediate oxacillin resistance (MIC, 1.0 μg/ml) in three of these apparently unrelated penicillin-susceptible clinical isolates of *Streptococcus pneumoniae* isolated in the United Kingdom and in four Spanish isolates was shown to be solely due to the acquisition of a gene encoding an altered penicillin-binding protein (PBP), PBP2X. PBP2X genes cloned from typical penicillin-resistant isolates of *S. pneumoniae* that possessed high-level oxacillin resistance were shown to be able to transform susceptible isolates of *S. pneumoniae* to intermediate oxacillin resistance. In all instances, the intermediately oxacillin-resistant PBP2X transformants retained susceptibility to penicillin (MIC, 0.06 μg/ml). Under appropriate selective pressure, the acquisition of a low-affinity PBP2X by penicillin-susceptible pneumococci could result in an increasing number of false positives for penicillin resistance among isolates of *S. pneumoniae* screened with oxacillin. Additionally, these intermediately oxacillin-resistant isolates showed reduced susceptibility to cefotaxime, an agent likely to be prescribed in place of penicillin for the treatment of serious infections due to these apparently penicillin-resistant organisms.

Since its detection in Papua New Guinea and Australia (9), penicillin resistance in *Streptococcus pneumoniae* has now been reported worldwide (1, 11), with high frequencies of isolation in South Africa (12), Spain (7), and Hungary (16).

In clinical isolates of *S. pneumoniae*, penicillin resistance is due to the development of high-molecular-weight penicillin-binding proteins (PBPs) that have a greatly reduced affinity for the antibiotic (8, 21). Highly penicillin-resistant isolates (MIC, >1 μg of benzylpenicillin per ml) may have alterations to at least four of the five high-molecular-weight PBPs. These highly penicillin-resistant isolates are also cross resistant to many other β-lactam antibiotics, including second and third generation cephalosporins (14). Low-affinity forms of PBP1A (15), PBP2B (4, 5) and PBP2X (13) are thought to have arisen by the horizontal transfer and recombination of homologous chromosomally encoded PBP genes from closely related species of streptococci (17). *Streptococcus mitis* has recently been identified as one of the species responsible for the formation of a low-affinity PBP2B in many penicillin-resistant isolates of *S. pneumoniae* (3).

The primary target of a β-lactam antibiotic is the PBP with the highest affinity for that particular antibiotic. If the affinity of this PBP for the antibiotic is reduced, then the PBP with the second highest affinity becomes the primary target. Thus, the primary target influences the MIC of an isolate. If the affinity of this target for the antibiotic is reduced, then there will be an increase in MIC. The term primary target here presupposes not that this is the only killing target but that it is that which influences MIC because of the differential affinities of PBPs for different β-lactam antibiotics (24). For clinical isolates of *S. pneumoniae* challenged by different β-lactams, either as the result of treatment of a pneumococcal infection or during asymptomatic carriage, when a different organism is the desired target there may be selection for the acquisition of different permutations of low-affinity PBPs.

Clinically, it is important to monitor the frequency of penicillin resistance in *S. pneumoniae* so as to determine appropriate chemotherapeutic practice for a given infection. For routine testing of penicillin susceptibility of *S. pneumoniae*, the use of 1-μg oxacillin discs is currently recommended (20, 22). Recently however, six United Kingdom clinical isolates of *S. pneumoniae* that appeared to be penicillin resistant, on the basis that they each exhibited a reduced zone around 1-μg oxacillin discs, have been detected but were found on subsequent testing to be penicillin susceptible (MIC, 0.06 μg of benzylpenicillin per ml) (10). Here, we examine the genetics of oxacillin resistance in several of these isolates in addition to a number of Spanish isolates and show that resistance involves only the alteration of PBP2X. Moreover, PBP2X genes from many typical penicillin-resistant pneumococci are shown to transform susceptible pneumococci to intermediate oxacillin resistance.

MATERIALS AND METHODS

Bacterial strains and conditions of growth. Strains and their characteristics are listed in Table 1. Isolates were grown either on brain heart infusion agar supplemented with 4% defibrinated sheep blood and incubated at 37°C in a 5%

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TABLE 1. MICs for *S. pneumoniae* strains and transformants

Strain or transformant[a]	Origin[b]	Serotype[c]	MIC (μg/ml) of:				
			Benzylpenicillin	Oxacillin	Cloxacillin	Methicillin	Cefotaxime
R6	USA	NC	0.016	0.12	0.25	0.25	0.016
7751/89	Spain	6	0.25	2	4	2	4
64147	S. Africa	6	6	≥16	≥16	≥16	NT[d]
SP21	Spain	6	1	≥16	≥16	≥16	0.5
53139/72	Papua	6	2	≥16	≥16	≥16	0.25
29044	CZ	14	8	≥16	≥16	≥16	2
DN87/577	UK	23F	1	≥16	≥16	≥16	0.5
8249	S. Africa	19	4	≥16	≥16	≥16	2
DN87/669	UK	19	1	4	8	8	1
7902.92	UK	6	0.06	1	1	1	0.5
9072.92	UK	6	0.06	1	1	1	0.25
10472.92	UK	NC	0.06	1	1	1	0.25
380/89	Spain	19A	0.06	0.5	1	1	0.06
1413/89	Spain	19A	0.06	1	1	1	0.06
40268/89	Spain	19A	0.06	1	1	1	0.12
54654/89	Spain	19A	0.06	0.75	1	1	0.12
$R6^{7902.92/2X}$		NC	0.06	1	1	1	0.5
$R6^{9072.92/2X}$		NC	0.06	1	1	1	0.25
$R6^{10472.92/2X}$		NC	0.06	1	1	1	0.25
$R6^{380/89/2X}$		NC	0.06	1	1	1	0.06
$R6^{1413/89/2X}$		NC	0.06	1	1	1	0.06
$R6^{40268/89/2X}$		NC	0.06	1	1	1	0.12
$R6^{54654/89/2X}$		NC	0.06	1	1	1	0.12
$R6^{7751/89/2X}$		NC	0.06	1	1	1	0.5
$R6^{SP21/2X}$		NC	0.06	1	1	1	0.25
$R6^{53139/72/2X}$		NC	0.06	1	1	1	0.25
$R6^{29044/2X}$		NC	0.06	1	1	1	0.25
$R6^{DN87/577/2X}$		NC	0.06	1	1	1	0.25
$R6^{8249/2X}$		NC	0.06	1	1	1	0.25
$R6^{DN87/669/2X}$		NC	0.06	1	1	1	0.12
$R6^{7751/89/2X/2B}$		NC	0.25	2	4	2	0.25
$R6^{DN87/577/2X/2B}$		NC	0.25	8	≥16	≥16	0.25
$R6^{29044/2X/2B}$		NC	0.25	8	≥16	≥16	0.25
$R6^{29044/2X/2B/64147/1A}$		NC	2	8	≥16	≥16	2

[a] $R6^{/2X}$ is the nomenclature for R6 transformed with the PBP2X gene from the named strain. $R6^{/2X/2B}$ represents R6 transformed with both PBP2X and PBP2B, and $R6^{/2X/2B/1A}$ represents R6 transformed with PBP2X, PBP2B, and PBP1A.
[b] Abbreviations: USA, United States; CZ, Czechoslovakia; UK, United Kingdom.
[c] NC, noncapsulated.
[d] NT, not tested.

CO_2–95% air atmosphere or in C medium plus yeast extract (23). MICs of β-lactam antibiotics were defined as the lowest concentrations that prevented growth of isolated colonies of *S. pneumoniae* on brain heart infusion agar blood plates.

Cloning and fingerprinting of PBP genes. The PBP1A, PBP2X, and PBP2B genes from strains of *S. pneumoniae* were amplified from chromosomal DNA by PCR using oligonucleotide primers described previously (2, 3). After electrophoresis, the amplified gene fragments were eluted from agarose gels by using Gene Clean (Bio 101). These fragments were then either ligated directly into pCR II (Invitrogen Corp.) or end trimmed with phage T4 DNA polymerase and ligated into the *Sma*I site of M13 mp18. The presence of full-sized inserts in these vectors was checked by comparing the electrophoretic mobility of recombinant supercoiled plasmid against that of pCR II and also by the ability to amplify the full-sized fragment from recombinants by PCR. Various PBP gene fragments were also cloned from the penicillin-resistant pneumococci listed in Table 1.

Fingerprinting was carried out as described previously (2). Briefly, the amplified PBP1A, PBP2X, and PBP2B genes were digested with a combination of restriction enzymes (*Hin*fI, *Sty*I, or *Mse*I plus *Dde*I). The fragments were then end labelled with ^{32}P, fractionated on a nondenaturing polyacrylamide gel, and autoradiographed.

Transformation. The penicillin-susceptible pneumococcus R6 was used as recipient in transformation experiments. Pneumococci were made competent as described previously (19) and transformed with either chromosomal DNA or plasmid DNA at final concentrations of about 3 or 0.3 μg/ml, respectively. Transformants were plated on selective media following expression at 37°C for 2 h.

RESULTS

Intermediate oxacillin resistance in *S. pneumoniae* 7902.92 and 9072.92. Both isolates were serotype 6 and in this study required MICs of oxacillin, cloxacillin, and methicillin of 1.0 μg/ml and MICs of penicillin of 0.06 μg/ml, but they differed in their resistance to cefotaxime (7902.92, 0.5 μg/ml; 9072.92, 0.25 μg/ml) (Table 1). (In a previous study, oxacillin MICs were 0.5 μg/ml when tested on Diagnostic Sensitivity Test Agar (Oxoid, Basingstoke, United Kingdom) containing 5% lysed horse blood [10].) Chromosomal DNA from 7902.92 and 9072.92 was able to transform the penicillin-susceptible pneumococcus R6 to intermediate oxacillin re-

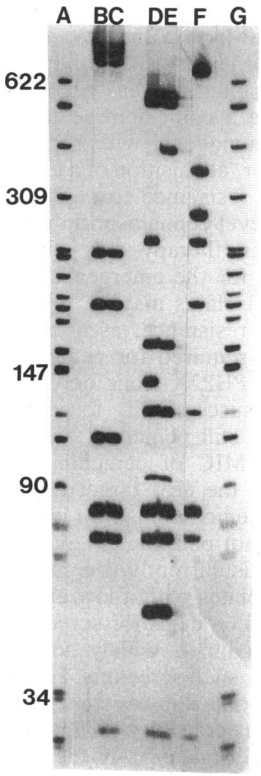

FIG. 1. *Hin*fI fingerprints of the amplified 2,056-bp fragment of PBP2X genes from intermediately oxacillin-resistant pneumococci 7902.92 and 9072.92, and of the penicillin-susceptible pneumococcus R6 transformed with chromosomal DNA from the resistant strains ($R6^{/C}$). Lanes: A and G, molecular size markers (base pairs) (pBR322 digested with *Hpa*II); B, 7902.92; C, $R6^{7902.92/C}$; D, 9072.92; E, $R6^{9072.92/C}$; F, R6.

sistance (MIC, 1.0 μg/ml). These transformants ($R6^{7902.92/C}$ and $R6^{9072.92/C}$) also required MICs of cloxacillin, methicillin, and cefotaxime that were identical to those for the donor strains (Table 1). The frequency of transformation to intermediate oxacillin resistance was about 5×10^{-4}, which was similar to that found previously for the transfer of a single PBP gene (19). Coincident with an increase of resistance to oxacillin, there was also a reduction of susceptibility to penicillin from 0.016 μg/ml to 0.06 μg/ml (Table 1). This was identical to the level of penicillin susceptibility found in the clinical isolates.

PBP1A, PBP2X, and PBP2B genes from two $R6^{7902.92/C}$ and two $R6^{9072.92/C}$ transformants were analyzed by fingerprinting. Fingerprints of the PBP1A and PBP2B genes from all four transformants were found to be identical to those from the penicillin-susceptible strain R6 (data not presented). However, the $R6^{7902.92/C}$ and $R6^{9072.92/C}$ transformants possessed PBP2X gene fingerprints that were different from each other and were very different from those of R6, although they were identical or very similar to the PBP2X fingerprints of 7902.92 and 9072.92, respectively. Representative examples of PBP2X *Hin*fI digests from $R6^{7902.92/C}$ and $R6^{9072.92/C}$ transformants are shown in Fig. 1. The data strongly suggest that both $R6^{7902.92/C}$ and $R6^{9072.92/C}$ transformants possessed altered PBP2X genes and that both 7902.92 and 9072.92 possess different PBP2X genes. In transformation experiments with high-molecular-weight chromosomal DNA, one might expect recombination to introduce the whole of a particular gene, but this is not necessarily always the case, and recombination may occur within the gene being examined. This explains why the PBP2X gene fingerprint of one transformant in Fig. 1 ($R6^{7902.92/C}$) is identical to that of the donor but the fingerprint of the other transformant ($R6^{9072.92/C}$), in which presumably only partial recombination has occurred, is not quite identical to that of the donor.

PBP2X genes cloned from 7902.92 and 9072.92 were found to transform R6 to intermediate oxacillin resistance. Both $R6^{7902.92/2X}$ and $R6^{9072.92/2X}$ transformants required MICs for β-lactams that were identical to those for the donor strains 7902.92 and 9072.92 (Table 1). In contrast, R6 transformed with PBP1A and PBP2B genes from 7902.92 and 9072.92 did not show increased oxacillin resistance. These data strongly suggest that an altered PBP2X alone is responsible for the intermediate oxacillin resistance found in strains 7902.92 and 9072.92, and that the different levels of resistance to cefotaxime between 7902.92 and 9072.92, and between the $R6^{7902.92/2X}$ and $R6^{9072.92/2X}$ transformants, reflect intrinsic differences in the affinities of PBP2X for cefotaxime in these isolates.

Intermediate oxacillin resistance in five other penicillin-susceptible pneumococci, one from the United Kingdom (10472.92) and four from Spain (380/89, 1413/89, 40268/89, and 54654/89) from a sample of 40 isolates (classed in the United Kingdom as penicillin resistant by oxacillin screening), was also shown by gene cloning and transformation (Table 1) to be solely due to an altered PBP2X. Each of these isolates also possessed elevated cefotaxime resistance compared with strain R6 (Table 1). Although the four Spanish isolates were all of serotype 19A, they varied in their initial levels of resistance to oxacillin (although the $R6^{/2X}$ transformants from these isolates all required apparently the same MIC of oxacillin), their levels of resistance to cefotaxime (Table 1), and their levels of resistance to erythromycin (E), chloramphenicol (C), and tetracycline (T) (380/89, E^r C^s T^s; 1413/89, E^r C^r T^r; 40268/89, E^s C^r T^r; 54654/89, E^s C^s T^s). PBP2X gene fingerprints were found to differ for each of the four isolates both in their restriction pattern for *Hin*fI and *Dde*I plus *Mse*I digests (data not presented). These data suggest that the four serotype 19A isolates are not of a single clonal origin and have independently acquired altered PBP2X genes.

Transfer of intermediate oxacillin resistance from typical penicillin-resistant strains of *S. pneumoniae*. PBP2X genes were cloned from seven penicillin-resistant clinical isolates of *S. pneumoniae*, 7751/89, SP21, 53139/72, 29044, DN87/577, 8249, and DN87/669 (Table 1). Strain DN87/577 is a representative of the multiresistant serotype 23F clone found in Spain, the United States, and the United Kingdom (18). Gene fingerprinting and sequence analysis has shown that at least six of the seven PBP2X genes from these isolates are different from one another (PBP2X from 7751/89 has the same gene fingerprint as SP21) (data not presented). PBP2X genes from each of these seven penicillin-resistant isolates were able to transform the sensitive strain R6 to intermediate oxacillin resistance (MIC, 1.0 μg/ml) and intermediate cefotaxime resistance (MIC, 0.12 to 0.5 μg/ml). However, these $R6^{/2X}$ transformants all remained penicillin susceptible (MIC, 0.06 μg/ml) (Table 1).

PBP1A and PBP2B genes cloned from penicillin-resistant strains of *S. pneumoniae* DN87/577, 7551/89, and 64147 were individually unable to transform the susceptible strain R6 to increased oxacillin resistance even though these genes encoded altered, low-affinity forms of PBP1A and PBP2B (18,

19). This indicates that neither PBP1A nor PBP2B is the primary target for oxacillin.

However, the PBP2B gene from penicillin-resistant strain 7551/89 was found to transform $R6^{7551/89/2X}$ transformants to full levels of oxacillin, cloxacillin, and methicillin resistance (MIC, 2 μg/ml) (Table 1). From these results, it would appear that for strain 7551/89 full oxacillin resistance can be attained by alterations to PBPs 2X and 2B alone. For isolates DN87/577 and 29044, which possess much higher levels of resistance to penicillin (MICs, 1 μg/ml and 8 μg/ml, respectively, as opposed to 0.25 μg/ml for strain 7551/89) and oxacillin (MICs, ≥16 mg/ml as opposed to 2 μg/ml), other PBPs in addition to PBP2X and PBP2B may be involved in this very high level of resistance to oxacillin. The evidence for this comes from the observation that genes encoding a low-affinity PBP2B from DN87/577 and 29044 did not appear to transform $R6^{DN87/577/2X}$ and $R6^{29044/2X}$ to the full level of resistance to oxacillin possessed by DN87/577 and 29044 (8 μg/ml as opposed to ≥16 μg/ml). However, PBP2B from these strains did transform $R6^{DN87/577/2X}$ and $R6^{29044/2X}$ transformants to full resistance to cloxacillin and methicillin (≥16 μg/ml). For $R6^{29044/2X/2B}$, transformation with an altered PBP1A gene from strain 64147 did not further increase the level of resistance to oxacillin; it did however, increase the levels of resistance to penicillin and cefotaxime (Table 1).

DISCUSSION

In this study, we have examined the genetics of oxacillin resistance in a number of clinical isolates from the United Kingdom and Spain that appeared to be penicillin resistant when screened by oxacillin but were in fact penicillin susceptible (MIC, 0.06 μg/ml). In each instance, oxacillin resistance was solely due to the acquisition of a low-affinity PBP2X. These isolates were also cross resistant to cloxacillin and methicillin and possessed intermediate-level resistance to cefotaxime. Alterations to other PBPs are not required for this intermediate-level resistance. From a sample of 40 Spanish pneumococci from the Hospital General Gregario Maranon which were thought to be penicillin resistant by oxacillin screening in the United Kingdom, 10% were found to be false positive.

Horizontal gene transfer and the development of resistance. PBP2X genes in the intermediate oxacillin-resistant isolates examined in this study were shown to have a range of gene fingerprints and were found in various serotypes. Moreover, different PBP2X genes encoded alterations which gave different β-lactam resistance profiles. This would suggest that numerous PBP2X genes have been involved in independent recombination events during the development of resistance among these isolates. Further evidence for this hypothesis comes from the observation that six different PBP2X genes from typical penicillin-resistant pneumococci were able to transform penicillin-susceptible pneumococci to intermediate oxacillin resistance (MIC, 1.0 μg/ml). Both inter- and intraspecific recombination have been shown to be important in the formation and subsequent distribution of low-affinity PBPs in *S. pneumoniae* and other naturally transformable streptococci (2, 3, 4, 5, 6). Altered PBP2X genes in the intermediate-oxacillin-resistant clinical isolates of *S. pneumoniae* may have arisen by direct recombination with species of *Streptococcus* that possess a low-affinity PBP2X rather than by indirectly acquiring the material via a mosaic PBP2X gene from a typical penicillin-resistant pneumococcus.

Selection pressures, resistance, and implications for oxacillin screening and β-lactam therapy. It is possible that the widespread clinical use of isoxazolylpenicillins such as flucloxacillin and oxacillin to treat infections due to staphylococci may provide a coincident selection pressure for the emergence of pneumococci with reduced susceptibility to oxacillin. Moreover, acquisition of a low-affinity PBP2X also confers increased resistance to cephalosporins and is the first step to high-level cephalosporin resistance (19). Therefore, cephalosporin therapy may also contribute to the selection pressure for the emergence of these isolates. Alternatively, these isolates may be relics from the development of penicillin resistance, as alterations to PBP2X are also the first step required for penicillin resistance. However, alteration of PBP2X alone only results in a relatively small decrease in susceptibility to penicillin, and such isolates are clinically still penicillin susceptible (MIC, ≤0.06 μg/ml). This low MIC of penicillin for these isolates is presumably due to the next target PBP having a relatively higher affinity for penicillin than for isoxazolylpenicillins. At the moment, it is not possible to say which of these antibiotics has been, or is currently, the most important agent in the selection of isolates with a low-affinity PBP2X.

The use of oxacillin discs for screening pneumococci for resistance to penicillin is widely accepted and remains a suitable method for such screening. However, in view of the finding that some oxacillin-resistant pneumococci may remain susceptible to penicillin it is important to determine the prevalence of such organisms in the clinical setting. If the number of false-positive penicillin-resistant pneumococci is significant, it may needlessly tip the balance from the routine use of penicillin for serious pneumococcal infections to the increased use of cephalosporins. This would be doubly unfortunate, since these apparently penicillin-resistant isolates retain susceptibility to penicillin but are generally less susceptible to cephalosporins. In addition, the increased use of cephalosporins may hasten their acquisition of a low-affinity PBP1A, thereby conferring high-level cephalosporin resistance (19). We reiterate the recommendation of Johnson et al. (10) that isolates showing reduced susceptibility to oxacillin should be tested for resistance to penicillin by determination of penicillin MIC.

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