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RESEARCH ARTICLE

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Diversity among clinical isolates of penicillin-resistant *Streptococcus mitis*: indication for a PBP1-dependent way to reach high levels of penicillin resistance

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Abstract A total of 12 non-epidemiologically related clinical isolates of *Streptococcus mitis* that showed different levels of resistance to penicillin were studied. Membrane-protein profiles and penicillin-binding protein (PBP) patterns showed a great polymorphism; and patterns of 4–7 PBPs, with sizes that ranged from ~101 kDa to ~40 kDa, were detected in each strain. No association could be found between PBP pattern and resistance level to penicillin among these isolates. Arbitrarily primed PCR confirmed the genetic diversity among this group of streptococci. One of the isolates of intermediate level of resistance to penicillin, which showed a PBP pattern similar to that of the high-resistance strains, was used as a laboratory model to analyse the mechanism underlying high-resistance acquisition by these strains. A 14-fold increase in penicillin resistance was obtained after a single selection step, which resulted in a decrease in penicillin affinity for PBP1. The size of this PBP (92 kDa) and the differences in PBP profiles of the penicillin-resistant clinical isolates suggest the existence in *S. mitis* of PBP-mediated mechanisms to acquire high-level resistance to penicillin, among which alterations in PBP1 seem to play a main role, in contrast to the PBP2X mediated mechanism described for other streptococci.

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Introduction

Although *viridans* streptococci were generally considered uniformly susceptible to penicillin, strains showing penicillin-resistance were reported in 1962 from patients with gingival infection [20]. Today, the increase in resistance of *viridans* streptococci to penicillin [1] is a cause of concern in the treatment of infectious endocarditis, infections in patients with haematological malignancies receiving cytotoxic chemotherapy and in neonatal infections [3, 4, 5, 6, 14, 15, 25].

Among the *viridans* streptococci, *Streptococcus mitis* has been considered the species with the highest level of resistance to penicillin [1]. Penicillin resistance in clinical isolates of *S. mitis* seems to be due to the production of altered high-molecular-mass penicillin-binding proteins (PBPs) with a low binding affinity for penicillin [2, 11, 22]. These PBPs, encoded by chromosomal genes, are located in the cytoplasmic membrane and catalyse reactions in the final steps of bacterial peptidoglycan synthesis. The implication of *S. mitis* in homologous recombination events that lead to mosaic genes in pneumococci has been extensively analysed [8, 9]. There are few reports, however, on PBP profiles and genes of clinical isolates of *S. mitis* or on their relationships with the levels of resistance to penicillin [2].

In this report, we analyse PBPs and whole-protein profiles of 12 non-epidemiologically related penicillin-resistant clinical isolates of *S. mitis* to study the degree of diversity among PBP patterns.

Materials and methods

Bacterial strains

A total of 12 non-epidemiologically related penicillin-resistant clinical isolates of *S. mitis* strains isolated from blood were provided by the Department of Microbiology, Hospital Ramón y Cajal (Madrid, Spain). Alpha-haemolytic streptococci were identified to species level, using the API 20 Strep system [10]. The strains used in PBP studies displayed similar biochemical profiles. All of the strains fermented raffinose and melibiose. None of the strains fermented mannitol and inulin; and hydrolysis of esculin, arginine and hippurate were negative. None of the strains produced polysaccharides from sucrose. *S. mitis* ATCC 9811 was used as a penicillin-susceptible reference strain and *Escherichia coli* BM-21 was used as control in the PBP profile studies.

Antimicrobial agents

Penicillin, ampicillin, cephalothin, cefotaxime and imipenem were provided by their respective manufacturers. [^{3}H]-benzylpenicillin (77.8 mCi/mg, 8.9 mCi/ml) was provided by Merck & Co. (Rahway, USA).

Susceptibility testing

The minimum inhibitory concentrations (MIC) of penicillin and other β-lactam antibiotics against *S. mitis* strains were determined by microdilution technique, using cation-adjusted Mueller-Hinton broth with 5% lysed horse blood, in accordance with the guidelines of the National Committee for Clinical Laboratory Standards [21]. The inoculum was prepared by suspending several colonies from overnight blood agar culture in sterile 0.9% saline, adjusted to a turbidity equal to 0.5 McFarland standard. The suspension was further diluted to provide a final concentration of bacteria of 5×10^5 colony forming units (cfu) in each well of the microdilution trays. The microtitre plates were incubated in air at 35 °C for 20–24 h. Strains were classified for penicillin susceptibility as follows: susceptible at MIC $\leq$ 0.12 μg/ml, intermediately resistant at MIC = 0.25–2.00 μg/ml and highly resistant at MIC $\geq$ 0.4 μg/ml [21]. *S. mitis* ATCC 9811 and *S. pneumoniae* ATCC 49619 were used as quality control strains.

Isolation of a penicillin-resistant mutant

A spontaneous penicillin-resistant mutant was obtained by spreading 10^9 cfu *S. mitis* 8168 over Todd-Hewitt (Oxoid, Unipath, Basingstoke, UK) agar plates supplemented with 5% sheep blood and a gradient of penicillin concentrations of 0–32 μg/ml. A colony that could grow in the presence of 32 μg penicillin/ml was isolated. The mutant was maintained on Todd-Hewitt agar plates supplemented with 5% sheep blood containing sub-inhibitory penicillin concentrations.

Electrophoresis of soluble membrane proteins

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli [16]. The acrylamide concentration used for the gels was 8% for the general analysis of membrane profiles and PBP patterns of different *S. mitis* isolates (Fig. 1) and 12% for the resolution of similar-sized PBPs (Figs. 3, 4). The gels were stained with Coomassie brilliant blue R0250.

Purification of cell membrane

Plasma membranes from *S. mitis* were obtained using a modification of a described method [13, 29], suitable for β-lactam binding. Two lytic enzymes, mutanolysin (an endo-*N*-acetyl muramidase from *Streptomyces globisporius*) and lysozyme were combined in sequential and controlled steps. All *Streptococcus mitis* strains were grown overnight at 37 °C in Todd-Hewitt broth supplemented with 5% yeast extract (Difco Laboratories, Detroit, Mich.). Growth was monitored by measuring the optical density at 620 nm (OD_{620}), to reach an OD_{620} of 0.4. Cells were centrifuged at 12,500 *g* for 10 min and, after discarding the supernatant, they were resuspended in 10 ml of 50 mM sodium phosphate buffer (pH 6.7) and centrifuged again. The pellet was resuspended in 8.5 ml of an enzymatic solution containing 50 mM Tris-HCl (pH 6.5), 1.2 mM magnesium chloride and 100 μg mutanolysin/ml and incubated for 1 h at 37 °C, with periodic shaking. The tubes were transferred to an ice bath and 8 mg lysozyme/ml was added. After shaking and incubation at 37 °C for 20 min, the suspension was disrupted by ultrasonic treatment using a Branson Sonic B-30 sonicator and then 2 μl of DNase and 4 μl of RNase were added. Unbroken cells were discarded by centrifugation at 9,800 *g* for 5 min. Membranes were sedimented by centrifugation at 245,000 *g* for 1 h, washed in 50 mM sodium phosphate buffer (pH 6.7) and centrifuged three times at 39,200 *g* for 45 min. The last pellet was resuspended in 50 mM sodium phosphate buffer (pH 6.7) at a final protein concentration of 5–6 mg/ml. All centrifugations were run at 4 °C; and the protein concentration was calculated by the Bradford technique [3]. We obtained 0.625 mg membrane protein/l original cell culture.

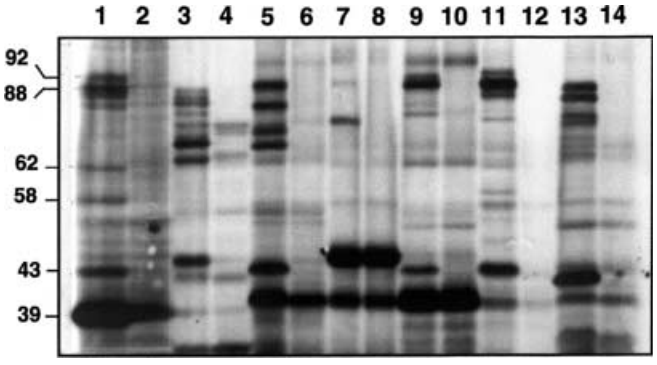

Fig. 1 Penicillin-binding protein (PBP) patterns of *Streptococcus mitis* isolates with different levels of penicillin resistance. *Lanes 1, 2 Escherichia coli* (molecular mass marker), *lanes 3, 4* ATCC 9811 (susceptible), *lanes 5, 6* 1182 (intermediately resistant), *lanes 7, 8* 730 (resistant), *lanes 9, 10*, 8168 (intermediate), *lanes 11, 12* 72244 (resistant), *lanes 13, 14* 38259 (highly resistant). *Lanes 2, 4, 6, 8, 10, 12, 14* are samples that were incubated for 10 min with 10 mg/ml of non-radioactive penicillin before the labelling reaction. The proteins were separated by SDS-PAGE (8% acrylamide) and subjected to fluorography

Labelling of PBPs with radioactive benzylpenicillin and determination of the affinities of PBPs for penicillin

Aliquots (50 μl) of a membrane suspension (0.3–0.4 mg of protein) were incubated for 15 min at 37 °C with 52 μg of [^{3}H]-benzylpenicillin/ml. Binding was stopped by isotopic dilution with benzylpenicillin (final concentration, 10 mg/ml) and samples were further processed as described previously [26].

Isolation and sequencing of DNA

Genomic DNA for use in arbitrarily primed PCR (AP-PCR) [27] and PCR techniques was obtained from isolated bacterial colonies, using the method described by Flamm et al. [12] with modifications. ITS-2 [28] and SM1 [19] primer sequences for AP-PCR, MS1 and MS2 [17, 24] primers for sequencing of genes and PCR parameters

for use in amplification were those previously described [8, 17, 24]. Double-stranded DNA (PCR products) were sequenced by the deoxynucleotide method of DNA sequencing of Sanger et al. [23]. When the PCR products were sequenced, a minimum of two independent PCR products were sequenced to eliminate any errors introduced by PCR. Amplification products were analysed by electrophoresis (0.25 V/cm^2) in 1.2% (w/v) agarose gels stained with ethidium bromide.

Results and Discussion

MIC of the β-lactam antibiotics tested against the *S. mitis* isolates are shown in Table 1. Cefotaxime and imipenem presented the highest activity against all penicillin-resistant strains, whereas ampicillin and cephalothin showed slightly higher MICs than penicillin against those strains. In general, penicillin-resistant *viridans* streptococci also showed a decreased susceptibility to all β-lactam agents tested.

The *S. mitis* strains used in the PBP studies (strains ATCC 9811, 1182, 730, 8168, 72244, 38259) displayed similar biochemical profiles, using the API 20 Strep system. However, great polymorphisms in membrane protein (data not shown) and PBP profiles were found. Fig. 1 shows the PBP profiles of *S. mitis* strains with different resistance levels to penicillin. The PBPs of the organisms exhibited a wide range of molecular weights. The pattern of PBPs obtained by in vitro labelling of resistant stains with [^{3}H]-benzylpenicillin showed important differences with respect to that of the susceptible strains. These include the number of PBPs, the affinity for [^{3}H]-benzylpenicillin and the molecular weight profiles. Even those strains with the same penicillin MIC showed these differences (data not shown). The multiplicity of changes observed in the PBPs of these strains suggests that high-level resistance to penicillin does not involve sequential alterations, as previously described by Zighelboim and Tomasz for *S. pneumoniae* strains [30], but are most probably the consequence of a much more complex process, in which vertical and horizontal gene transfers take place.

Due to the high heterogeneity found in the protein profiles of the different strains, it was difficult to assess the implication of a particular PBP in penicillin resistance, as was proposed for the role of PBP2X in *S. pneumoniae* [7, 18] and other species [9, 24]. Moreover, our data support that a PBP similar in size to the PBP2X in pneumococci is not always detected among the profiles of the strains tested (i.e. lane 7 of Fig. 1). If alterations in PBP2X-like protein were not the main mechanism to reach penicillin resistance, it should be possible to check this hypothesis through the analysis of highly penicillin-resistant spontaneous mutants of one

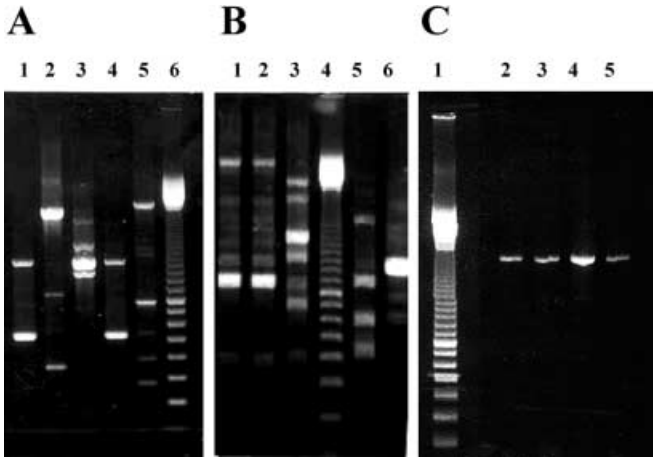

Fig. 2A–C Arbitrarily primed (AP)-PCR. **A** AP-PCR profiles from *S. mitis* isolates with ITS-2 primer. *Lane 1* ME-32, *lane 2* 38259 (highly resistant), *lane 3* 68989 (highly resistant), *lane 4* 8168 (parental strain), *lane 5* ATCC 9811 (susceptible), *lane 6* size marker (100 bp). **B** AP-PCR profiles from *S. mitis* isolates with SM1 primer. *Lane 1* ME-32 (resistant mutant), *lane 2* 8168 (parental strain), *lane3* ATCC 9811 (susceptible), *lane 4* size marker (100 bp), *lane 5* 38259 (highly resistant), *lane 6* 68989 (highly resistant). **C** PCR amplification of chromosomal DNA samples from *S. mitis* 8168 (*lanes 2, 3*) and the ME-32 mutant (*lanes 4, 5*) with MS1 and MS2 primers. The lanes contained 10 µl of a reaction mixture with sample diluted 1:10 (*lanes 2, 4*) or 1:100 (*lanes 3, 5*). *Lane 1* contained 1 µg/ml of a molecular mass marker (100 bp ladder)

Table 1 Minimum inhibitory concentration (*MIC*) of β-lactam antibiotics against *Streptococcus mitis* strains

S. mitis strain	MIC (µg/ml)				
	Penicillin	Ampicillin	Cephalothin	Cefotaxime	Imipenem
8168	2.0	8.0	8.0	2.0	1.0
1182	4.0	4.0	4.0	2.0	0.1
16785	4.0	8.0	16.0	0.5	0.1
16453	8.0	16.0	32.0	1.0	0.5
730	8.0	16.0	8.0	2.0	1.0
48427	16.0	16.0	16.0	4.0	1.0
34986	16.0	32.0	16.0	2.0	2.0
72244	16.0	32.0	32.0	4.0	2.0
60829	16.0	32.0	32.0	8.0	0.5
5693	16.0	32.0	> 32.0	4.0	≤ 0.01
68989	32.0	> 32.0	> 32.0	32.0	4.0
38259	32.0	16.0	16.0	2.0	0.03
ATCC 9811	≤ 0.03	≤ 0.06	≤ 0.03	≤ 0.06	≤ 0.12

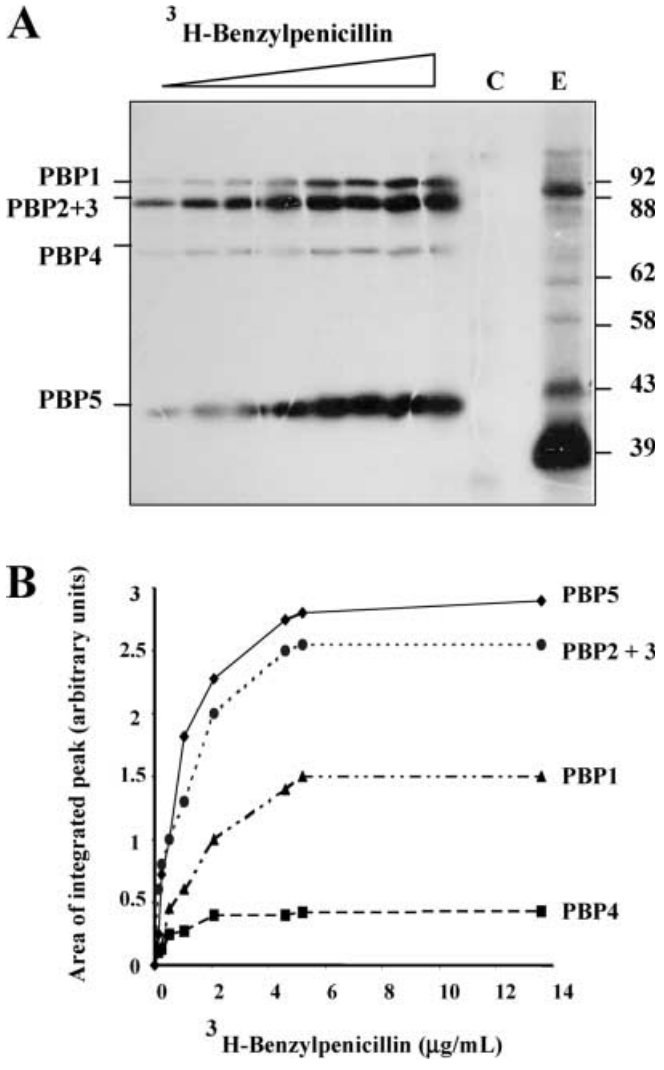

Fig. 3A, B Affinity of *S. mitis* 8168 PBPs for [^{3}H]-benzylpenicillin. Increasing concentrations of [^{3}H]-benzylpenicillin were incubated for 10 min at 30 °C with membrane aliquots isolated from *S. mitis* 8168. After stopping the reaction with an excess of cold penicillin, the proteins were separated by SDS-PAGE (12% acrylamide) and subjected to fluorography. **A** Fluorogram of the experiment. *Lane C* Control for non-specific binding, *lane E E. coli* membranes used as molecular mass marker of the sizes *indicated at the right*. **B** Densitometric analysis of the experiment shown in **A**. The fluorogram from **A** was quantified by microdensitometry; and the intensity of each band was plotted versus the concentration of labelled benzylpenicillin

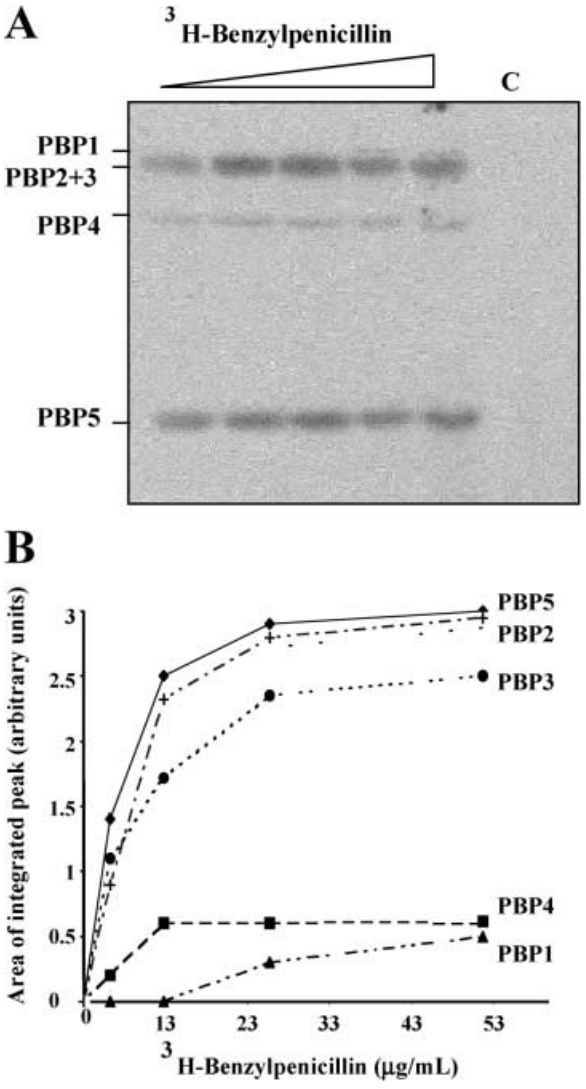

Fig. 4A, B Affinity of *S. mitis* ME-32 (mutant strain) PBPs for [^{3}H]-benzylpenicillin. Membrane preparation aliquots isolated from the *S. mitis* ME-32 were incubated with increasing concentrations of [^{3}H]-benzylpenicillin as above; and the proteins were separated by SDS-PAGE (12% acrylamide). **A** Fluorogram showing the results of the labelling. **B** Densitometric analysis of the results shown in **A**

of the clinical strains with a low level of penicillin resistance. In this sense, strain 8168 (lane 9) was selected as the parent for selection, since it had a PBP pattern similar to that of the highly resistant strain, 38259 (lane 13, MIC = 32 µg/ml), but a lower MIC (2 µg/ml) for penicillin. A resistant mutant named ME-32 was obtained, for which the benzylpenicillin MIC was 64 µg/ml. Increases in the MIC of cefotaxime >8 µg/ml) and imipenem (4 µg/ml) were also observed in the mutant strain.

The ME-32 strain showed biochemical and membrane-protein profiles identical to its parental strain (data not shown). Moreover, AP-PCR of both strains with primers ITS-2 and SM1 also gave identical profiles. However, a comparison of these AP-PCR profiles to those of other resistant clinical isolates revealed great differences among them, supporting the existence of a large genetic diversity among penicillin-resistant *S. mitis* isolates (Fig. 2A, B).

Characterization by saturation studies of both parental and mutant strains showed five PBPs with approximate apparent sizes of 92, 88, 85, 79 and 43 kDa (PBP1–PBP5; Figs. 3A, 4A). The apparently different PBP patterns in Figs. 1, 3A and 4A are a consequence of the different gel concentrations used. Analysis of PBPs in ME-32 and in its parental strain indicated an association between resistance and

decreased affinity of PBP1 for penicillin. No saturation of PBP1 in the mutant strain was observed, even when the antibiotic used was six-fold more labelled than that in its parent (Figs. 3B, 4B). Thus, the increasing level of resistance correlates with the decrease in affinity of the high-molecular-weight PBP1. These data are in agreement with the recent work of Amoroso et al. [2], in which they found a much lower binding of [^{125}I]-penicillin to all the high molecular mass PBPs in a clinical isolate of *S. mitis* with high resistance to β-lactams, and show in a laboratory model that a 16-fold increase in the resistance level to penicillin can be achieved by a change in PBP1 in a single selection step.

Furthermore, due to the high molecular weight of the PBP1 of the ME-32 strain, it probably has no genetic correspondence with the so-called PBP2X of *S. pneumoniae*, previously reported [2] as the main protein responsible for streptococci interspecies distribution of the penicillin-resistant phenotype [8, 9, 24]. However, as in many membrane proteins, the mobility of PBP1 in SDS-PAGE could not reflect their actual molecular size, thus making a putative relationship between them still possible. To check this, we amplified and sequenced the gene encoding the PBP2X-like protein from the genomes of the ME-32 and its parental strain. A ~2.2-kbp product was amplified from each strain, using MS1 and MS2 primers (Fig. 2C), and subsequently sequenced (data not shown). The comparison of both sequences revealed a total identity between them. In addition, the sequence obtained encoded an amino acid sequence which corresponded to the PBP2X from sensitive strains of *S. pneumoniae* [7, 9, 17, 24] (data not shown). Consequently, the penicillin resistance of our *S. mitis* ME-32 strain was not due to a mutation in a PBP2X-like gene.

In conclusion, our data support that clinical isolates of penicillin-resistant *S. mitis* present a high phenotypic and genotypic heterogeneity and, the studies performed on our resistant isolate suggest that at least a mutation of a PBP gene different from that of the well known PBP2X could suffice to produce a high level of resistance. Nevertheless, more penicillin-resistant *S. mitis* strains need to be evaluated in order to support these results.

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