The aminoglycoside phosphotransferase (APH)(3')(5")-III has been characterized from Streptococcus pneumoniae BM4200, which is resistant to high levels of aminoglycosides. The phosphotransferase was apparently chromosomally-encoded and was responsible for the high-level resistance. The enzyme was not notably pH-dependent, was heterogeneous after isoelectric focusing, with pI values of approximately 4.8 and 5.1, and had an apparent molecular weight of 32500 after SDS-PAGE.

INTRODUCTION

In streptococci, which are naturally resistant to low levels of aminoglycosides, with MICs of approximately 50 to 250 mg l⁻¹, the acquisition of genes coding for aminoglycoside-modifying enzymes confers high-level resistance to a number of these drugs, with MICs well above 2 g l⁻¹. Resistance of this type has spread in recent years among clinical isolates of several streptococcal species where the R genes were frequently, but not always, plasmid-borne (for a recent review see Carlier & Courvalin, 1982). Since 1977, multiple drug resistance has emerged in Streptococcus pneumoniae (Jacobs et al., 1978). The additional acquisition of high-level aminoglycoside resistance in S. pneumoniae (Courvalin et al., 1979) appears unusual because of the particular form of resistance transfer involved, i.e. a conjugation-like process in the apparent absence of extrachromosomal DNA (Buu-Hoï & Horodniceanu, 1980; Guild et al., 1981). It was the purpose of this study to analyse the mechanism of high-level aminoglycoside resistance in pneumococci and to characterize biochemically the R gene product involved.

METHODS

Bacterial strains and growth conditions. Streptococcus pneumoniae strain BM4200 was a clinical isolate, kindly provided by F. W. Goldstein, Hôpital Saint Joseph, Paris, France. Strain MAC was obtained as the standard type F23 strain from the WHO Collaborating Centre for Reference and Research on Pneumococci, Statens Serum Institut, Copenhagen, Denmark. Pneumococci were grown in brain-heart infusion broth (Difco) supplemented with 5% (v/v) human ascites fluid and 5% (v/v) horse serum, and on blood agar plates containing 5% (v/v) defibrinated horse blood. Escherichia coli strain C600(pML21) (Hershfield et al., 1974) and Staphylococcus aureus strain RN450(pSH2) (Courvalin et al., 1978) were used for the production of 3',5"-aminoglycoside phosphotransferases of types I and III, respectively.

Isoelectric focusing in polyacrylamide gels. Isoelectric focusing of 100000 g supernatants (S100) was performed in tube gels as described by Courvalin et al. (1980) and in slab gels containing 6% (w/v) acrylamide, 2% (v/v) ampholines, and 0-4% (v/v) detergent (Nonidet P40). Samples of up to 80 μl were made to 0-5 M with respect to ammonium chloride and to 0-4% with respect to Nonidet P40, and deposited onto squares of filter paper (Whatman 3MM) on the anodic side of the gel. Focusing was carried out for 14 to 16 h at 4 °C and 250 V. A sheet of phosphocellulose paper (Whatman P81) which had been soaked for over 2 h in a solution of neomycin (2 g l⁻¹), rinsed several times in water and twice in TNM buffer (50 mM-Tris, 100 mM-NH₄Cl, 10 mM-MgCl₂, 12 mM-2-
mercaptoethanol, adjusted to pH 7.5 with acetic acid) was squeezed between two sheets of filter paper (Whatman 3MM) to remove excess buffer. It was then laid over the polyacrylamide gel slab. The phosphocellulose sheet was wetted evenly with 1 to 2 ml of buffer containing approximately 20 μCi (740 kBq) [γ-32P]ATP, and incubated for 1 h in a moist chamber at 37 °C. The phosphocellulose sheet was rinsed once in water at 80 °C, four to five times in cold water, dried and autoradiographed.

PAGE. Electrophoresis in polyacrylamide gels containing SDS (Laemmli, 1970), and two-dimensional gel electrophoresis (O'Farrell, 1975) were carried out as described.

Gel filtration. Crude (S100) preparations were applied at 4 °C to a column (1.5 × 190 cm) of Sephadex G-100 superfine, equilibrated with TNM buffer adjusted to 1 M-NH₄Cl.

Determination of MICs. The method of Steers et al. (1959) was used to determine the MICs of the antibiotics.

Assay of aminoglycoside-modifying enzymes. The enzymes were assayed in S100 preparations after sonication, or after column chromatography, using radioactive cofactors (see below) and binding of the modified antibiotics to phosphocellulose (Whatman P81) as described by Haas & Dowding (1975).

Affinity chromatography. A column (1.5 × 15 cm) of acrylamide-agarose beads to which neomycin was covalently linked (E. Collatz, G. Gerbaud and P. Courvalin, unpublished) was used for specific enrichment of aminoglycoside-modifying enzymes. Bacterial lysate in TNM buffer (5 to 10 ml) adjusted to 30 mM-NH₄Cl was applied to the column, previously equilibrated with the same buffer, at 22 °C to 25 °C. The column was washed with four to five column volumes of the equilibration buffer followed by 10 ml buffer adjusted to 0.5 M-NH₄Cl and containing 5 mg neomycin. The column was washed with approximately 10 column volumes of TNM buffer adjusted to 1 M-NH₄Cl and equilibrated with starting buffer before re-use.

Assay for extrachromosomal DNA. Bacteria were assayed for the presence of extrachromosomal DNA as described previously (Courvalin & Davies, 1977; Courvalin & Fianit, 1980; Labigne-Roussel et al., 1981). Centrifugation in caesium chloride–ethidium bromide was carried out for 20 h at 50,000 r.p.m. and 18 °C in a Beckman VTi 50 rotor. The supercoiled DNA was collected and centrifuged a second time for 8 h at 65,000 r.p.m. in a VTi 65 rotor under otherwise identical conditions. In parallel experiments, DNA was sedimented through a glycerol cushion (Labigne-Roussel et al., 1981). Two screening techniques for extrachromosomal DNA employing agarose gel electrophoresis of crude bacterial lysates were used (Barnes, 1976; Eckhardt, 1978).

Chemicals. [1-14C]Acetyl-CoA, [γ-32P]ATP triethylammonium salt, and [U-14C]ATP ammonium salt, were obtained from Amersham. The antibiotics were provided by the following laboratories: gentamicin (Gen) complex (i.e. 26.3% Clay 40.8% C1, 32.9% C2), Schering; kanamycins A, B, C (KanA, By C), and amikacin (Ami), Bristol; neamine (NeoA) and neomycin B (NeoB), Upjohn; paromomycin (Par) and butirosin (But), Parke-Davis; tobramycin (Tob), Lilly; lividomycin A (LivA), Kowa; ribostamycin (Rib), Meiji; streptomycin (Str), Pfizer; nitrocefin, Glaxo.

RESULTS AND DISCUSSION

Antibiotic resistance characters of S. pneumoniae BM4200 and BM4200-1

Strain BM4200 was resistant to chloramphenicol, macrolide–lincosamide–streptogramin B-type (MLS) antibiotics (constitutively), penicillin, sulphonamide, tetracycline and its lipophilic analogues minocycline and chelocardin, and trimethoprim. The strain showed high-level resistance, with MICs of 4 g l⁻¹ or higher (Table 1), to a number of aminoglycosides, including NeoA and NeoB, Par, LivA, Rib, and KanA and B. Strain BM4200 was resistant to lower levels of But and susceptible to Tob and Ami. Strain BM4200-1, a derivative of BM4200, had spontaneously lost its high-level aminoglycoside resistance and its resistance to But, while none of the remaining resistance characters was affected. Its sensitivity to aminoglycosides was indistinguishable from that of the reference strain MAC (Table 1). In 10¹¹ bacteria no reversion

<table>
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<th>MIC of antibiotics (mg l⁻¹)</th>
<th>NeoB</th>
<th>NeoA</th>
<th>Par</th>
<th>LivA</th>
<th>But</th>
<th>Rib</th>
<th>KanA</th>
<th>KanB</th>
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<td>&gt;4096</td>
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<td>8</td>
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<tr>
<td>BM4200-1</td>
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<td>256</td>
<td>512</td>
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<td>128</td>
<td>256</td>
<td>256</td>
<td>128</td>
<td>8</td>
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<tr>
<td>MAC†</td>
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<td>512</td>
<td>128</td>
<td>128</td>
<td>256</td>
<td>256</td>
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* All strains were of serotype 23F.
† Isolated before 1940, standard type 23F strain.
Pneumococcal aminoglycoside phosphotransferase

Supernatant (S100) preparations from strains BM4200 and BM4200-1 were assayed for aminoglycoside-modifying enzymes. In strain BM4200 there was phosphorylation of the neomycins and kanamycins but no acetylation or adenylylation, and there was no phosphorylation of streptomycin or spectinomycin. The substrate profile of the phosphotransferase is shown in Fig. 1. Since KanB was modified but Tob (3'-deoxykanamycin B) was not, it is inferred that the 3'-hydroxyl group is the site of phosphorylation. The fact that both But and LivA were modified indicates that the enzyme is of type III, i.e. APH(3')(5")-III (Courvalin & Davies, 1977). No aminoglycoside-modifying activity was detected in strain BM4200-1.

In an attempt to determine the pH optimum for enzyme activity we found no optimum between 5 and 9 (data not shown).

The phosphotransferase was analysed after isoelectric focusing of an S100 preparation on a slab gel. Its enzymic activity was revealed by autoradiography after in situ phosphorylation of neomycin bound to phosphocellulose paper (Fig. 2). The enzyme activity did not focus into one distinct band but rather into two zones. The corresponding isoelectric points were found to be 4.8 ± 0.1 and 5.3 ± 0.1.

Isolelectric focusing in slab gels, in combination with the phosphocellulose paper binding procedure (Haas & Dowding, 1975) and subsequent autoradiography, appears to be a suitable technique for the characterization of aminoglycoside phosphotransferases. The APH(3')(5")-III of strain BM4200 and that coded for by plasmid pSH2 (Courvalin et al., 1978) which are closely related if not identical (Collatz et al., 1983), were indistinguishable, while the APH(3')(5")-I, coded for by pML21 (Hershfield et al., 1974), was clearly distinct.

We tried to verify the apparent heterogeneity of the APH(3')(5")-III under denaturing conditions (O'Farrell, 1975), after identification of the enzyme on two-dimensional electropherograms. We compared S100 preparations from strains BM4200 and BM4200-1 but were unable to ascribe the phosphotransferase to an additional spot on the electropherogram of BM4200 (not shown), possibly because of a small amount of the enzyme in this strain. However, when we compared the proteins from Staphylococcus aureus RN450, either plasmid-free or containing the multicopy plasmid pSH2 (Stiffler et al., 1974), we observed in the latter an additional protein, appearing as two incompletely separated spots (Fig. 3). Since the protein was acidic and confined to the strain containing pSH2, and since it migrated just above the 30000 molecular weight marker (cf. below), we believe that the two spots correspond to the two zones of enzyme activity visible in Fig. 2.
Fig. 2. Isoelectric focusing of aminoglycoside phosphotransferases in slab gels. Focusing was of S100 preparations from the strains indicated at the top. The phosphorylation of neomycin, bound to a sheet of phosphocellulose paper, was as described in Methods, and was revealed by autoradiography. *Escherichia coli* strain C600(pML21) and *S. aureus* strain RN450(pSH2) are producers of APH(3')(5") type I and type III, respectively. The zones of APH(3')(5")-III activity are indicated (>).

Fig. 3. Two-dimensional PAGE of S100 preparations from *S. aureus* strains RN450(pSH2) and RN450. The protein encoded by pSH2 is indicated by arrows. The molecular weight markers added in the second dimension were carbonic anhydrase (30000), trypsin inhibitor (21500), and myoglobin (17200).
Fig. 4. Purification of the APH(3')(5") from *S. pneumoniae* BM4200. (a) Affinity chromatography. Approximately 10 ml of an S100 preparation in TNM buffer, adjusted to 30 mM-NH₄Cl, was applied at 23 °C to a column (1.5 × 15 cm) of agarose–acylamide–neomycin, and 2 ml fractions were collected. The enzyme was eluted with 10 ml buffer, adjusted to 0.5 M-NH₄Cl and 0.5 g neomycin 1⁻¹. No protein was detected past fraction 50. Neomycin and [γ−³²P]ATP were added to 40 µl samples of column fractions, and phosphotransferase activity (●) was determined by the phosphocellulose binding assay as described by Haas & Dowding (1975).

(b) SDS-PAGE of the fraction containing the peak of enzymic activity after affinity chromatography. The molecular weight markers were BSA (66,200), alcohol dehydrogenase (36,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), myoglobin (17,200) and lysozyme (14,200).

An attempt was made to enrich specifically the APH(3')(5")-III of BM4200 by affinity chromatography using immobilized neomycin (Fig. 4a). Virtually all the enzymic activity bound to the column, and was eluted with neomycin and salt. The fraction containing the peak of enzyme activity was analysed by PAGE in the presence of SDS (Fig. 4b). It had a prominent band and a few minor contaminants. After gel filtration through Sephadex G-100 (not shown), the enzyme activity was associated with the prominent protein. Its molecular weight was estimated to be approximately 32,500. Recently, Trieu-Cuot & Courvalin (1983) have established the nucleotide sequence of the APH(3')(5")-III gene carried by the plasmid pJH1, originally isolated from *Streptococcus faecalis* (Jacob & Hobbs, 1974). The derived protein, molecular weight 29,200, would contain 32% of glutamic acid, an amount which might prevent saturation of the protein with SDS (Nelson, 1971) and account for the relatively high apparent molecular weight after electrophoresis in the presence of this detergent. The determination of the molecular weight by filtration through Sephadex G-200 superfine (not shown) yielded an underestimation, 22,500, probably due to some binding of the enzyme to the gel matrix.

**Search for a plasmid-borne aminoglycoside phosphotransferase (aph) gene**

Because the *aph* gene is frequently extrachromosomal (see Carlier & Courvalin, 1982), we made various attempts to identify a plasmid-borne *aph* gene in strain BM4200. Two methods were used to look for extrachromosomal DNA. Both yielded negative results, whereas plasmid DNA could be observed under identical experimental conditions in pneumococcal strains with an established plasmid content. The ultracentrifugation analysis of total DNA in CsCl–ethidium bromide is shown in Fig. 5. While the presence of plasmids pIP501 and pDP1 is evident in *S. pneumoniae* strains DP3200 and D39S, respectively (Shoemaker *et al.*, 1979; Smith & Guild, 1979), there was no supercoiled DNA in strain BM4200. Using techniques for plasmid
Fig. 5. Density gradient ultracentrifugation in caesium chloride-ethidium bromide. Fractions containing (or suspected to contain) covalently closed circular DNA (see Methods) were collected after centrifugation in a Beckman VTi 50 rotor and re-centrifuged in a VTi 65 rotor. The strains from which the DNA was prepared are indicated at the top. Streptococcus pneumoniae strain D39S contained plasmid pDPl (Smith & Guild, 1979) and S. pneumoniae strain DP3200 (Shoemaker et al., 1979) contained plasmid pIP501.

analysis by agarose gel electrophoresis of crude bacterial lysates, plasmids pIP501 and pDPl were again revealed, but there was no evidence for the presence of extrachromosomal DNA in strain BM4200 (not shown). This observation is in agreement with conclusions reached previously by Buu-Hoï & Horodniceanu (1980) and Guild et al. (1981). The aph gene was cloned into a ColE1 factor derivative and found to reside, together with the tetracycline and MLS R determinants, on a transposable element designated Tn1545 (Carlier & Courvalin, 1982). Recent analysis of Tn1545 has revealed that this transposon is conjugal and that it can insert into the chromosome, or into a subsequently introduced plasmid, in a Rec- strain of S. faecalis (P. Courvalin & C. Carlier, unpublished work).

In addition to the conjugal transfer in pneumococci (Buu-Hoï & Horodniceanu, 1980; Guild et al., 1981) a similar resistance transfer in the apparent absence of extrachromosomal DNA has been observed in β-haemolytic streptococci (Horodniceanu et al., 1981), in Bacteroides fragilis (Macrina et al., 1981; Malamy & Tally, 1981) and in Clostridium difficile (Smith et al., 1981). Conjugal transfer of resistance transposons has been described in S. faecalis (Franke & Clewell, 1981) and in Streptococcus agalactiae (Smith & Guild, 1982).

From protein analysis and nucleic acid annealing studies we concluded that the aph gene of S. pneumoniae BM4200 is widespread among Gram-positive cocci where it may exist apart from, or adjacent to, other R determinants (Collatz et al., 1983). The characterization of APH(3′)(5″)-III should facilitate further studies of the distribution of the corresponding gene.

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REFERENCES


