Conjugative Transfer of Multiple Antibiotic Resistance Markers in *Streptococcus pneumoniae*

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Twenty antibiotic-resistant isolates of *Streptococcus pneumoniae* were investigated for conjugative transfer of their drug resistance markers into streptococcal (groups B and D) and pneumococcal (encapsulated and non-encapsulated) recipients. Of these, 7 wild-type donor pneumococci transferred all their resistance markers (except Pc [penicillin], Su [sulfonamide], and Tp [trimethoprim]) into group D *Streptococcus* and non-encapsulated *S. pneumoniae* recipients at a low frequency (10⁻⁸ to 10⁻⁹). The resistance markers transferred were Tc (tetracycline); Tc and Cm (chloramphenicol); Tc and MLS (macrolides, lincosamides, and streptogramin B); Tc, MLS, Km (kanamycin), and Cm. The transconjugants obtained were similar to that of the wild-type group B *Streptococcus* recipient and was found to be similar to those of the wild-type group B *Streptococcus* host (20 × 10⁶).

The emergence of multiple antibiotic-resistant strains of *Streptococcus pneumoniae* is a new phenomenon (2, 19), although resistance to one (6, 10, 13, 36) or two antibiotics (1, 7, 8, 11, 25) has been previously reported in various countries. Multiple resistance has been reported to be plasmid borne in groups A (3, 23), B (15), and D (4) streptococci. In contrast, none of the drug-resistant *S. pneumoniae* clinical isolates so far examined contains detectable plasmid DNA (7, 31, 32, 37). The only plasmid described in *S. pneumoniae* is the 2.0-megadalton cryptic pDP1 harbored by the R36NC and RSF1010 strains (34, 37).

Recently, a genetic exchange of plasmid-borne drug resistance markers between donor and recipient strains by a mechanism similar to conjugation has been reported in streptococci of groups A (24), B (14, 17), D (18, 29), C, and G (L. Bougueleret, G. Bieth, and T. Horodniceanu, submitted for publication). In all these strains, physical evidence of plasmid DNA has been always found. However, more recently, nine group A, B, F, and G streptococcal resistant strains transferred their resistance markers without evidence of detectable extrachromosomal DNA, suggesting that these drug-resistant determinants are chromosome borne (T. Horodniceanu, L. Bougueleret, and G. Bieth, submitted for publication). Similar results have very recently been reported by Shoemaker et al. (33) for two *S. pneumoniae* resistant strains; the genes for chloramphenicol and tetracycline resistance were transferred into non-encapsulated pneumococci by a process that resembles conjugation, but neither the donor strains nor the transconjugants contained detectable plasmids.

The purpose of this study was to provide information on conjugative transfer of multiple drug resistance markers of pneumococci in the absence of detectable extrachromosomal DNA. First, we studied the transfer ability of resistance markers from seven wild-type *S. pneumoniae* strains into streptococcal and pneumococcal recipients and the possible mechanism of transfer. Second, we studied the physical properties of a plasmid DNA (pIP501) transferred into a pneumococcal recipient.

**MATERIALS AND METHODS**

**Bacterial strains.** Bacteria and plasmids used in this study are listed in Tables 1 and 2. Pneumococci isolated from separate clinical specimens were identified by optochin sensitivity, bile solubility, alpha hemolysis, and capsular typing with antipneumococcal serum (Statens Seruminstitut, Copenhagen). Stock cultures were stored at -80°C.

**Media and drugs.** Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with 5% horse serum was used as the nutrient broth, and brain heart infusion agar (Difco) with 5% horse blood or 5% horse serum was used as the solidified culture medium.
serum was used as the nutrient agar. Drugs used for the selection of donor resistance markers were as follows: erythromycin (Abbott Laboratories, North Chicago, Ill.) and tetracycline (Roussel, Paris) at a final concentration of 4 \( \mu g/mL \) each, chloramphenicol (Roussel) at 5 and 25 \( \mu g/mL \) for pneumococcal and streptococcal recipients, respectively, kanamycin (Spécia, Paris) at 1,000 \( \mu g/mL \), trimethoprim (Roche, Paris) at 5 \( \mu g/mL \), and penicillin G (Spécia) at 0.25 \( \mu g/mL \). The following drugs were used to counterselect bacterial donors: rifampin (Lepetit, Suresnes) at a final concentration of 100 \( \mu g/mL \), fusidic acid (Leo, Paris) at 25 \( \mu g/mL \), and streptomycin (Spécia) at 4,000 \( \mu g/mL \). Definitions for drug resistance markers are given in Table 1.

**Determination of resistance to antibiotics.** Antimicrobial resistance was tested by the disk diffusion method on Mueller-Hinton agar (Institut Pasteur, Paris) with 5% horse blood. The determination of high-level resistance to aminoglycoside antibiotics was tested by the method of Moellering et al. (28) on brain heart infusion agar with 5% horse blood and at least 1,000 \( \mu g/mL \). The determination of the minimum inhibitory concentration was done as previously described (15). Penicillinase activity was tested by the usual methods (12, 20, 27).

**Antibiotic-resistant mutants.** Mutant pneumococcal recipients (R6 and BM6006) resistant to streptomycin and rifampin were obtained as previously described (17).

**Mating conditions.** Donor and recipient strains were grown in nutrient broth with gentle aeration for 3 and 6 h, respectively, as previously described (17). Matings were done by mixing 0.1 mL of each strain and spreading directly on a sterile membrane filter (type HA, 0.45 \( \mu m \), 47 mm; Millipore Corp., Bedford, Mass.) placed on a horse blood nutrient agar plate. After 18 h of incubation at 37°C, the membrane filter was washed with 0.5 mL of brain heart infusion broth, and dilutions were plated by spreading on appropriate selective horse blood media. Transconjugants were scored after 48 h of incubation at 37°C. Cell-free and chloroform-sterilized preparations, treated by the method of Jacob and Hobbs (18), were mixed with appropriate recipients as described above. The frequencies of transfer were expressed as the number of resistant recipients per donor organism.

**DNase assays.** The sensitivity of transfer to 10

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**Table 1. Bacterial donor strains and plasmids**

<table>
<thead>
<tr>
<th>Strain designation (serotype or serogroup)</th>
<th>Relevant resistance markers</th>
<th>Plasmid designation (host)</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM6001 (19)</td>
<td>Cm Tc</td>
<td>pIP501 (JH2-2)</td>
<td>Strasbourg, 1978</td>
</tr>
<tr>
<td>BM6014 (6)</td>
<td>MLS Tc</td>
<td>pIP501 (R36NC)</td>
<td>This study</td>
</tr>
<tr>
<td>BM6017 (6)</td>
<td>Km MLS Tc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM6020 (6)</td>
<td>Km MLS Tc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM4200 (23)</td>
<td>Cm Km MLS Pc Su Tc Tp*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM6102 (B)</td>
<td>Cm MLS</td>
<td>pDP1 (R36NC)</td>
<td></td>
</tr>
<tr>
<td>BM5201 (D)</td>
<td>Cm MLS Fus' Rif'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM5227</td>
<td>Cm MLS Str'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R36NC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Drug resistance markers: Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Pc, penicillin G; and Su, sulfonamide. MLS, macrolides (erythromycin, oleandomycin, spiramycin), lincosamides (lincomycin, clindamycin), and streptogramin B; Tp, trimethoprim; Fus', fusidic acid resistance; Rif', rifampin resistance; Str', streptomycin resistance.

**Table 2. Recipient bacterial strains**

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Species (serogroup or serotype)</th>
<th>Relevant characters</th>
<th>Origin or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH2-2</td>
<td>S. faecalis (D)</td>
<td>Fus' Rif'</td>
<td>(18)</td>
</tr>
<tr>
<td>BM133</td>
<td>S. faecalis (D)</td>
<td>Str'</td>
<td>(17)</td>
</tr>
<tr>
<td>BM132</td>
<td>Streptococcus agalactiae (B)</td>
<td>Fus' Rif'</td>
<td>(17)</td>
</tr>
<tr>
<td>R6</td>
<td>S. pneumoniae b</td>
<td>Non-encapsulated, endo'</td>
<td>G. Tiraby</td>
</tr>
<tr>
<td>BM124</td>
<td>S. pneumoniae</td>
<td>Mutant of R6 resistant to streptomycin</td>
<td>This study</td>
</tr>
<tr>
<td>BM126</td>
<td>S. pneumoniae</td>
<td>Mutant of R6 resistant to rifampin</td>
<td>This study</td>
</tr>
<tr>
<td>BM6006</td>
<td>S. pneumoniae (1)*</td>
<td>Encapsulated</td>
<td>Institut Pasteur Collection</td>
</tr>
<tr>
<td>BM121</td>
<td>S. pneumoniae (1)</td>
<td>Mutant of BM6006 resistant to streptomycin</td>
<td>This study</td>
</tr>
</tbody>
</table>

* For definitions, see Table 1. Endo', Deficiency in main pneumococcal endonuclease.

* This strain is susceptible to antibiotics.
PNEUMOCOCCAL TRANSFERABLE MULTIPLE RESISTANCE

and 100 μg of DNase I per ml was determined by the method of Jacob and Hobbs (18).

Phage techniques. Cell-free supernatants of all wild-type donor strains were tested by the surface spot technique for the presence of prophages (17, 18). Pneumococcal and streptococcal recipients were used as indicator strains.

Curing procedures. Curing by high temperature (42°C) and by chemical agents (acridine orange and ethidium bromide) was done as previously described (15).

Plasmid DNA isolation. For streptococcal transconjugant strains, 2 ml of an 18-h broth culture was inoculated into 100 ml of broth to obtain a turbidity of 15 to 20 Klett units (green filter no. 54) and allowed to grow with gentle aeration at 37°C.

At 100 Klett Units, cultures were treated with 3% (wt./vol.) glycine (Sigma Chemical Co., St. Louis, Mo.), and at 130 Klett Units, cells were harvested and washed. Crude and cleared lysates were obtained as described by Reider and Macrina (30). For pneumococcal transconjugant strains, as well as for strain R36NC, growth, lysis, and treatment of lysates were performed by the method of Smith and Guild (34). In some experiments, after 1 h of growth, cells were labeled for 3 h at 37°C with 1 μCi of [3H]thymidine per ml (specific activity, 25 Ci/mmol; Commissariat à l'Energie Atomique, Yvette, France). Plasmid DNAs were isolated from cleared lysates by centrifugation in cesium chloride-ethidium bromide density gradients.

When no plasmid DNA was visible under UV light, the labeled gradient was fractionated dropwise from the bottom, and 10-μl samples were spotted onto Whatman 3MM filters and counted for radioactivity as previously described (7). Plasmid DNA fractions were dialyzed against 10 mM Tris-hydrochloride-1 mM EDTA (pH 8) and stored at 4°C.

Gel electrophoresis. Electrophoresis was performed on horizontal slab gels with 0.6% agarose (Type II; Sigma) and run at 140 V for 3 h at room temperature in TBE buffer (89 mM Tris base—89 mM boric acid—2.5 mM disodium EDTA [pH 8.3]). Gels were stained with 1 μg of ethidium bromide per ml of water and photographed with Polaroid type 55 film through a red filter under UV transillumination. Molecular weights of plasmid DNAs were calculated from relative mobilities in agarose with DNA from plasmids TP114 (41 megadaltons), RP4 (36 megadaltons), RSA (23 megadaltons), and ColEl::Amp (7.76 megadaltons) as standards.

RESULTS

Drug resistance pattern of wild-type pneumococci. A total of 20 S. pneumoniae antibiotic-resistant strains were tested for drug resistance with susceptibility disks for macrolides (erythromycin, oleandomycin, spiramycin), lincosamides (lincomycin, clindamycin), and streptogramin B, and chloramphenicol, tetracycline, sulfonamide, trimethoprim, and penicillin G. The pattern of high-level aminoglycoside resistance was investigated for all wild-type strains, for streptococcal and pneumococcal transconjugants, and for JH2-2 and BM124. Of the 20 strains investigated, 5 were resistant to tetracycline, 3 were resistant to tetracycline and chloramphenicol, 7 were resistant to tetracycline and macrolides, lincosamides, and streptogramin B, 4 were resistant to tetracycline, macrolides, lincosamides, streptogramin B, and kanamycin, and 1 was resistant to tetracycline, chloramphenicol, macrolides, lincosamides, streptogramin B, kanamycin, penicillin G, trimethoprim, and sulfonamide. The minimum inhibitory concentration of penicillin G for the inhibition of strain BM4200 was 0.5 μg/ml. No penicillinase activity could be detected in strain BM4200. The minimum inhibitory concentrations of kanamycin, neomycin, lividomycin, and ribostamycin for the inhibition of wild-type pneumococci (BM6017, BM6020, BM4200) and their pneumococcal and streptococcal transconjugants were >8,000 μg/ml.

Transfer of resistance markers from pneumococcal wild-type strains. A total of 20 wild-type pneumococcal donor strains were crossed with JH2-2, BM132, BM121, and BM124 recipients on membrane filters. Selection was done separately for all resistance markers carried by the donor strains (Cm, Em, Km, Tc, Pc, and Tp). The transconjugants obtained were crossed with BM133 and BM124 or BM126. Matings in which the transconjugants were donors were designated as retransfers. The time of mating contact was 18 h. When the mating contact was less than 18 h (1, 2, 4, or 6 h), no transconjugants were detected. Similar frequencies of transfer and retransfer were obtained regardless of the antibiotic used for selection (tetracycline, kanamycin, or erythromycin), except that no transconjugants were found when chloramphenicol was the selective agent. The transconjugants obtained in each selection, both after transfers and retransfers, were analyzed for unscored markers, including the chromosomal markers Rif' and Fus', or Str'. Resistance to antibiotics of streptococcal and pneumococcal transconjugants was analyzed by replica-plating and by the disk diffusion method, respectively. An example of a transfer and retransfer experiment after 18 h of mating contact is given in Table 3. The appearance of spontaneous mutations of wild-type S. pneumoniae donor strains to Rif' and Fus' (used together for counterselection) was never observed, and the mutation to Str' (high-level resistance) occurred at a low frequency (4 × 10^-9). Therefore, the number of mutants was negligible in comparison with the number of transconjugants obtained, either in streptococcal or in pneumococcal recipients (see Table 3). The frequency of mutation to Str' of JH2-2 transconjugants was very low (3 × 10^-10). The mutants were very easily differentiated.
from the transconjugants obtained from BM133 as the latter were susceptible to rifampin and fusidic acid and resistant to streptomycin, whereas the mutants were resistant to all these drugs. Moreover, when transfers and retransfers occurred between pneumococci and streptococci and vice versa, confusion of the two species was absolutely excluded; *Streptococcus faecalis* is resistant to optochin and is nonhemolytic, whereas *S. pneumoniae* (including BM124) is very susceptible to optochin and is alpha-hemolytic. Furthermore, colony morphology of the two species is very different. After 18 h of mating on membrane filters, regardless of the antibiotic used for selection (erythromycin, chloramphenicol, tetracycline, or kanamycin), no recipients having received drug resistance markers could be detected in 13 of the 20 wild-type donors studied. In contrast, the transfer of resistance markers occurred in the seven remaining donors, but no transconjugants were obtained when BM132 or BM121 was used as the recipient. The results are shown in Table 4. Relevant findings included the following: (i) wild-type donors transferred their resistance markers at a low frequency (10^{-4} to 10^{-8}) after 18 h of mating contact; (ii) all seven wild-type pneumococci transferred their markers to the JH-2 streptococcal recipient, whereas BM6012 and BM4200 transferred into the BM124 pneumococcal recipient as well; (iii) in all cases (with the exception of BM4200), resistance determinants of donor strains were transferred en bloc. Pc and Tp resistance markers of BM4200 were not detectable either after selection on Pc or Tp or after the analysis of unselected markers; and (iv) retransfer from streptococcal and pneumococcal transconjugants occurred at a very low frequency (10^{-7} to 10^{-8}) into both streptococcal (BM133) and pneumococcal (BM124 or BM126) recipients, with the exception of BM5307, which did not retransfer into BM124. In all cases, with the exception of BM5304 and BM5307, resistance markers of donor transconjugants were retransferred en bloc. The Cm marker of BM5304 and BM5307 was not found after retransfer into BM133.

When chloroform-treated cultures and cell-free supernatants of donor pneumococci (BM6012 and BM4200) were mixed with JH-2, no transferable drug resistance was detected.

**Transfer of plasmid pIP501 into pneumococcal recipients.** For obtaining a plasmid-borne *S. pneumoniae* strain to be used as a control for genetic and physical experiments, streptococcal strains BM6102 (wild-type group B donor) and BM5201, both harboring plasmid pIP501 (15), were crossed with BM121 and BM124 pneumococcal recipients. The time of mating contact was 1, 2, 3, 4, 5, 6, and 18 h for the cross BM5201(pIP501) × BM124. Transconjugants were obtained after 4 h, with a maximum of transfer frequency after 18 h of mating contact. Crosses between BM6102 or BM5201 and JH2-2, BM133, and BM132 were used as known mating controls. The significant features of the results obtained after 18 h of mating contact were as follows (Table 5): (i) pIP501 was not transferred into the encapsulated pneumococcal recipient (BM121) either from the wild-type group B (BM6102) donor strain or from BM5201; (ii) the frequency of transfer of pIP501 into BM124 was very low (3 × 10^{-7}), in contrast with the high transfer frequency of this plasmid into streptococcal recipients (10^{-2} to 10^{-4}); and (iii) pIP501 was retransferred from the pneumococcal transconjugant (BM5227) into JH2-2, BM132, and BM126. The frequency of transfer was high into JH2-2 and low into BM132 and BM126. However, the retransfer of pIP501 from

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**Table 3. Mating experiment between wild-type and transconjugant donor strains and streptococcal and pneumococcal recipients (18 h of mating contact)**

<table>
<thead>
<tr>
<th>Donor^a × recipient</th>
<th>Selective donor marker (counter-selection)</th>
<th>No. of donors (CFU/ml)</th>
<th>No. of transconjugants (CFU/ml)</th>
<th>Transfer frequency per donor cell^b</th>
<th>Strain designation of transconjugant</th>
<th>Phenotype of transconjugant^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM4200 × JH2-2</td>
<td>Te (Rif') Fus')</td>
<td>10^6</td>
<td>3</td>
<td>3 × 10^{-6}</td>
<td>BM5320</td>
<td>Cm Km MLS Te Rif' Fus'</td>
</tr>
<tr>
<td>BM4200 × BM124</td>
<td>Te (Str')</td>
<td>10^6</td>
<td>45</td>
<td>4.5 × 10^{-6}</td>
<td>BM5324</td>
<td>Cm Km MLS Te Str'</td>
</tr>
<tr>
<td>BM5320 × BM133</td>
<td>Te (Str')</td>
<td>10^10</td>
<td>3.2 × 10^7</td>
<td>3.2 × 10^{-6}</td>
<td>BM5321</td>
<td>Cm Km MLS Te Str'</td>
</tr>
<tr>
<td>BM5324 × BM126</td>
<td>Te (Rif')</td>
<td>1.2 × 10^9</td>
<td>5 × 10^5</td>
<td>4 × 10^{-7}</td>
<td>BM5325</td>
<td>Cm Km MLS Te Rif'</td>
</tr>
</tbody>
</table>

^a The numbers of donors (colony-forming units [CFU] per milliliter) put on filters before mating were 7 × 10^5, 4 × 10^6, and 8 × 10^6 for BM4200, BM5320, and BM5324, respectively.

^b The numbers of recipients (colony-forming units per milliliter) put on filters before mating were 3 × 10^10 for JH2-2 or BM133 and 2 × 10^9 for BM124 or BM126.

^c For definitions, see Table 1.

^d Similar results were obtained when selection was done on erythromycin, kanamycin, or tetracycline; no transconjugants were obtained when chloramphenicol was the selective agent.

^e Of these transconjugants 10% were chloramphenicol susceptible.
BM5201 into BM133 was 100 times lower than its initial transfer from the wild-type donor strain. No transferable drug resistance was detected when a chloroform-treated broth culture of BM5201 was mixed with BM124. Similar results were recently reported by Smith et al. (35) concerning the transfer of pIP501 into and from non-encapsulated *S. pneumoniae* and *S. faecalis* strains.

**DNase I assays.** The influence of DNase I on transfer frequency was tested by treating the donor and recipient strains, as well as the agar plate which served as the support for the mating membrane filter, with DNase I and MgSO₄. Two crosses, BM4200 × BM124 and BM5201(pIP501) × BM124, were treated. No difference in the frequency of transfer between tests and controls was observed with either 10 or 100 μg of DNase I per ml.

**Production of phages.** Cell-free supernatants of all wild-type donor pneumococcal strains (see Table 1) were tested for the presence of prophages. No plaques were observed with either of the indicator strains used (JH2-2 or BM124).

**Curing experiments.** Clones of BM5301, BM5307, BM5308, BM5313, BM5317, BM5320, and BM5201 were tested for spontaneous loss (100 clones of each strain) of resistance markers at 37°C and by curing (510 clones of each strain) with acridine orange and ethidium bromide at 42°C. No loss (less than 0.2%) of resistance markers was obtained, with the exception of the control strain BM5201(pIP501), in which a curing efficiency of 23% was found after treatment with ethidium bromide at 42°C.

**Plasmid DNA isolation and gel electrophoresis analysis.** All streptococcal and pneumococcal transconjugants were examined for the presence of extrachromosomal DNA. In each experiment, a plasmid-carrying strain was included; BM5201 was used as a control for streptococcal transconjugants, and BM5201 and R36NC were used as controls for pneumococcal transconjugants. Except for the control strains, none of the transconjugants (streptococcal or pneumococcal) were cured.
pneumococcal) contained bands of satellite DNA detectable by dye-buoyant centrifugation analysis. The gradients obtained from labeled lysates of pneumococcal transconjugants (including BM5227) were fractionated and counted for radioactivity. Again, all attempts to isolate plasmid DNA were unsuccessful, except in the case of control strain BM5227. Moreover, the lysates of pneumococcal transconjugants and of the R36NC strain were directly (34) analyzed by agarose gel electrophoresis. Pneumococcal transconjugants (see Table 4) did not contain plasmid DNA detectable by the methods used. However, plasmid pDP1 (molecular weight, \(2.0 \times 10^6\)) was consistently detected in R36NC, as was plasmid pIP501 in BM124. The molecular weight of pIP501 after transfer into BM124 remained unmodified (\(20 \times 10^6\)) (data not shown).

**DISCUSSION**

Conjugative transfer of plasmid-borne resistance markers has been previously reported in different streptococcal groups (14, 17, 18, 24). Shoemaker et al. (33) recently reported a DNase-resistant transfer of chromosomal tetracycline and chloramphenicol markers from *S. pneumoniae* into a non-encapsulated pneumococcal recipient in the absence of plasmid DNA. In the present study, we describe the ability of multiply resistant *S. pneumoniae* strains to transfer their resistance markers by a mechanism similar to conjugation without a demonstration of detectable plasmid DNA. Based on the data presented here, several features appear significant: (i) wild-type donor antibiotic-resistant pneumococci transferred their resistance markers at a low frequency (\(10^{-3}\) to \(10^{-4}\)) into streptococcal or pneumococcal recipients or both; (ii) the same strains transferred neither into a group B *Streptococcus* nor into an encapsulated *S. pneumoniae* recipient; (iii) Pc and Tp markers were neither transferred independently nor cotransferred with other resistance markers; (iv) pIP501 was transferred at a low frequency into the non-encapsulated BM124, but was not transferred into the encapsulated BM121 pneumococcal recipient. pIP501 was retransferred from the transconjugant BM5227 at a high frequency into a group D (*S. faecalis*) recipient and at a low frequency into group B and non-encapsulated pneumococcal recipients; and (v) plasmid DNA of pIP501 could always be isolated from BM5227, whereas no detectable extrachromosomal DNA could be found either in streptococcal or in pneumococcal transconjugants obtained after the transfer of resistance markers from wild-type *S. pneumoniae* strains. Furthermore, two wild-type strains, BM6001 (7, 34) and BM4200 (data not shown), were examined for the presence of plasmid DNA, but no detectable extrachromosomal DNA was found.

As close contact between a very high number of viable cells is necessary to realize an efficient transfer, the genetic exchange of resistance markers between pneumococcal strains is probably a mechanism similar to that of conjugation. The same hypothesis has previously been suggested for the conjugative transfer in streptococci of groups D (18, 29), B (14, 17), A (24), C, and G (Bougueleret et al., submitted for publication). Although the transfers presented here are DNase resistant (for the two crosses studied), a genetic exchange by transformation cannot totally be excluded as the number of viable cells of encapsulated pneumococcal wild-type donors was decreased 100-fold between 4 and 18 h of incubation on the membrane mating filter (data not shown). Shoemaker et al. (33) reported similar results in matings between non-encapsulated pneumococci; the donor titers were 100-fold less after 24 h on filters, in comparison with the number of donors after 5 h. However, the number of recombinants obtained by them was threefold higher after 24 h than after 5 h. Therefore, a spontaneous release of transforming DNA from the donor strains is likely and might be an explanation for the successful transfer of resistance markers from wild-type donor pneumococci (two strains) into BM124. However, all wild-type donors (seven strains) transferred only after 18 h of mating contact into JH2-2, which is known to be a nontransformable recipient. The fact that only two wild-type donors could transfer directly into the BM124 recipient remains to be explained as the retransfer from streptococcal transconjugants into pneumococcal recipients was obtained in all instances but one (BM5307). On the other hand, the transfer into JH2-2, but not into BM132, might be explained by the closer homology between the chromosomes of *S. pneumoniae* and *S. faecalis* than between those of *S. pneumoniae* and group B *Streptococcus*. Another explanation could be that JH2-2 excretes a sex pheromone which induces donor cells to become adherent, generating the cell-to-cell contact necessary for both plasmid and chromosome transfer (9). From the results presented here, it is evident that all *S. pneumoniae* donor strains transfer their resistance markers into *S. faecalis* recipients, in contrast with the results reported by Shoemaker et al. (33), who obtained transfers only into non-encapsulated *S. pneumoniae* recipients.

Based on the data presented here, a transduction mechanism is unlikely as no phages were detected in donor strains, and no recipient cells acquired resistance markers when mixed with
donor cell-free supernatants. However, Porter et al. (28) recently reported a possible phage-associated gene transfer system for S. pneumoniae called pseudotransduction that has many properties of generalized transduction.

Detectable extrachromosomal DNA could not be demonstrated in either streptococcal or pneumococcal transconjugants, in contrast with the consistent isolation of pDP1 and pIP501 plasmid DNAs harbored by R36NC and BM5227, respectively. These results suggest that resistance markers in wild-type pneumococci, as well as in the transconjugants, could be chromosome borne and linked to genes which are able to mediate the conjugative transfer. Resistance markers may be inserted into the host chromosome as transposable elements lacking the sequences for autonomous replication. Recently, we obtained similar results with nine antibiotic-resistant group A, B, F, and G streptococci and discussed several hypotheses for this apparently general phenomenon occurring in streptococci (Horodniceanu et al., submitted for publication). Of 20 drug-resistant pneumococci, 13 were unable to transfer their resistance determinants. These results suggest that in these cases, the transposable elements may be inserted into the host chromosome without transfer genes. Shoemaker et al. (32) reported that two wild-type pneumococci isolated in France (7) and in Japan (25) contain Cm and Tc genes integrated into the chromosome in the form of adjacent insertions of heterologous DNA.

High-level resistance to streptomycin has been reported in S. pneumoniae clinical isolates (19), but this resistance is not mediated by the production of a streptomycin-modifying enzyme (31). In contrast, Collatz et al. (personal communication) have observed that high-level resistance of BM4200 to kanamycin was mediated by a type III aminoglycoside 3'-phosphotransferase which modifies kanamycin, neomycin, amikacin, butirosin, ribostamycin, and lidodomyacin A antibiotics. An increased emergence of high-level resistance to aminoglycoside antibiotics (minimum inhibitory concentration, ≥8,000 µg/ml) in clinical isolates of S. faecalis carried by conjugative plasmids has recently been reported (16, 18, 21). These plasmids code for the synthesis of aminoglycoside-modifying enzymes (5, 22). The results presented in this study suggest that the genes which code for the synthesis of this 3'-phosphotransferase may be located on the S. pneumoniae chromosome.

Plasmid-borne antibiotic-resistance is rather a common phenomenon in clinical pathogen-resistant streptococci (3, 4, 15, 16, 21), with the exception of the S. pneumoniae strains so far examined (7, 31, 32, 37). However, plasmid pIP501 (15) is transferred either by transformation (32) or by conjugation (35, this study) both from its original group B Streptococcus host and from an S. faecalis transconjugant into a non-encapsulated pneumococcal host. In addition, pIP501 is retransferred from this new S. pneumoniae host into both streptococcal (group B and S. faecalis) and non-encapsulated pneumococcal recipients. pIP501 is also conjugally transferred into other streptococcal recipients, such as groups A, C, and G (unpublished data). These results suggest that pIP501 has a wide host-range specificity and is capable of autonomous replication in different streptococcal hosts, including S. pneumoniae. In contrast, both the entry of pIP501 and the transfer of resistance markers from wild-type pneumococci were prevented into an encapsulated pneumococcal recipient (BM121). This would suggest either that transferred DNA (plasmid or chromosomal) succumbs to nuclease digestion after penetrating the capsule of the recipient strain or that the capsule itself represents an impenetrable barrier. This might explain the lack of R plasmids in naturally occurring encapsulated pneumococci.

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LITERATURE CITED