Penicillinase-Producing *Neisseria gonorrhoeae*: a Molecular Comparison of 5.3-kb and 7.4-kb β-Lactamase Plasmids

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Restriction endonuclease analysis and heteroduplex studies indicate that the only difference between the 5.3-kilobase (kb) and 7.4-kb plasmids from β-lactamase-producing *Neisseria gonorrhoeae* is that the latter is the 5.3-kb plasmid with a 2.1-kb insertion. The insertion is bounded by inverted repeats of approximately 300 base pairs. Several plasmids from β-lactamase-producing *N. gonorrhoeae* isolated at different times and in different countries were compared. Nine 5.3-kb plasmids were examined and found to be indistinguishable, as were sixteen 7.4-kb plasmids.

Penicillin-resistant, β-lactamase-producing gonococci were first reported in England in 1976 (16) and in the United States in 1977 (17). Since then they have spread to other countries, and the number of infections with β-lactamase-producing strains of *N. gonorrhoeae* is increasing (24, 25). Penicillin resistance in these organisms is mediated by an ampicillin resistance determinant carried on either a 5.3-kilobase (kb) (3.3 Mdal) or a 7.4-kb (4.6-Mdal) plasmid. Both plasmids carry about 40% of TnA (15, 18, 19), including the bla gene that encodes the TEM-1 β-lactamase (16, 19, 25). The 5.3-kb plasmids originated in β-lactamase-producing *N. gonorrhoeae* strains isolated in Africa (17) and in Britain (8, 21), whereas the 7.4-kb plasmids first appeared in isolates from the Philippines and other Far Eastern countries (21).

DNA-DNA hybridization experiments with these initial isolates indicated that these two plasmids, apart from carrying TnA sequences, are closely related (18). A later study (24) examined 80 β-lactamase-producing *N. gonorrhoeae* strains isolated in the Netherlands and found them to contain an ampicillin resistance determinant, specifying a TEM-1 β-lactamase carried on either a 5.3- or a 7.4-kb plasmid. DNA-DNA hybridization studies showed that these plasmids were also closely related. These results indicate world-wide dissemination of two closely related plasmids carrying the bla gene of TnA.

In this paper we compare more closely the 5.3-kb plasmid and the 7.4-kb plasmid by restriction enzyme and heteroduplex analysis. In addition, several 5.3- and 7.4-kb plasmids isolated from β-lactamase-producing *N. gonorrhoeae* strains from several countries, including the United Kingdom (8), were compared.

**MATERIALS AND METHODS**

**Media.** *N. gonorrhoeae* strains were grown on GC medium base (Difco Laboratories) supplemented with 1% hemoglobin (Difco) and 1% of GC solutions 1 and 2 as described by Kellogg et al. (9). Ampicillin (1 μg/ml) (Beecham Research Laboratories) was added when β-lactamase-producing *N. gonorrhoeae* strains were being cultivated.

**Bacterial strains.** The strains used are listed in Table 1. The plasmid content of the *N. gonorrhoeae* strains was determined by the methods of Guerry et al. (7) and Meyers et al. (14).

**Transfer of β-lactamase plasmids into *Escherichia coli* KH802.** Plasmids were transferred from *N. gonorrhoeae* to *E. coli* KH802 as described by Flett et al. (6).

**Isolation of plasmids for restriction analysis and heteroduplex studies.** The KH802 strains with either the 5.3- or the 7.4-kb *N. gonorrhoeae* plasmid were grown overnight in 500 ml of nutrient broth with 100 μg of ampicillin per ml. Cells were harvested and suspended in 10 ml of sucrose-Tris (25%, 50 mM), and 0.5 ml of lysozyme (10 mg/ml) was added, followed by 2.5 ml of 0.2 M EDTA (pH 7.0), 1.5 ml of 20% sodium laurylsulfate, and 6 ml of 5 M NaCl. This mixture was stored overnight at 4°C and then centrifuged at 17,000 × g for 60 min at 1°C. Sodium acetate (2 ml, 4 M) was added to the supernatant, which was then resuspended for 5 min at 12,000 × g. Isopropanol (15 ml) was added to the supernatant fluid, and the precipitate was recovered by centrifugation at 14,000 × g for 15 min. The pellet was suspended in Tris-EDTA (10 mM Tris, 1 mM EDTA), treated with RNase, and then chloroform extracted. Plasmid DNA was isolated from this solution on a cesium chloride-ethidium bromide density gradient. After banding, plasmid DNA was recov-
TABLE 1. Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of strains or genotype</th>
<th>Yr isolated (country)</th>
<th>Plasmid content (kb) or designation</th>
<th>β-Lactamase plasmid(s)</th>
<th>Source or reference</th>
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<tr>
<td><strong>β-Lactamase-producing N. gonorrhoeae</strong></td>
<td></td>
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<tr>
<td>2</td>
<td>1976 (England)</td>
<td>4.2, 5.3</td>
<td>pLV11, pNG2</td>
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<tr>
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<td>4.2, 7.4</td>
<td>pLV10</td>
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<tr>
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<td>pLV12</td>
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<td>pNG16</td>
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<td>pNG21</td>
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<td>pNG10, pNG12</td>
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</tr>
<tr>
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<td>pNG13, pNG14</td>
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<td>pNG7, pNG9, pNG11</td>
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<td></td>
<td>pLV11::Tn723</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Sources: A, J. R. Saunders, Department of Microbiology, University of Liverpool, England; B, A. E. Jephcott, Regional Public Health Laboratory, Bristol, England; C, R. Schmitt, Lehrstuhl fur Genetik, Universitaet Regensburg, Federal Republic of Germany.

<sup>b</sup> Tn723 is contained in the chromosome. Tn723 is derived from the minor transposon in Tn721 (1), now designated Tn722, by the in vitro insertion of a determinant for kanamycin resistance into the single PstI site (R. Schmitt, personal communication).

ered in approximately 1 ml of cesium chloride solution. To this was added 9 ml of water, 1 ml of 4 M sodium acetate and 20 ml of ethanol. After 18 h at -20°C, the precipitated DNA was recovered by centrifugation and dissolved in 1 ml of Tris-EDTA (10 mM Tris, 1 mM EDTA). This solution was phenol and chloroform extracted, and the DNA was precipitated with 4 M sodium acetate and ethanol as described above. Finally, the plasmid DNA was dissolved in 200 µl of Tris-EDTA and stored at -20°C. For isolation of adequate plasmid DNA from strains containing the 5.3-kb plasmid, it was usually necessary to start with 1,500- to 2,000-ml nutrient broth cultures rather than 500-ml cultures.

Restriction endonuclease analysis. Alul and HpaII were obtained from Bethesda Research Laboratories. BamHI, PstI, HindIII and EcoRI were prepared in this laboratory. Alul digestions were performed in 6 mM Tris-hydrochloride-6 mM MgCl<sub>2</sub>-50 mM 2-mercaptoethanol; digestions with other enzymes were performed in 50 mM Tris-hydrochloride-5 mM MgCl<sub>2</sub>. Agarose gels were made with high-gelling-temperature agarose from Miles Laboratories at concentrations of 0.7 or 2%, as required.

Analysis of plasmid DNA by electron microscopy. Plasmid size was determined by the method of Robinson et al. (20). DNA heteroduplex analysis was performed essentially as described by Lai and Nathans (10).

RESULTS

Electron microscope analysis. Plasmids pLV11 and pLV10 were determined to be 5.3 and 7.4 kb, respectively, from contour length measurements obtained by electron microscope analysis. These values agreed very well with measurements made by other investigators (23, 24).

Roberts et al. (18), using DNA-DNA hybridization, reported that the β-lactamase plasmids isolated from β-lactamase-producing strains of *N. gonorrhoeae* contained nucleotide sequences amounting to 40% of the ampicillin transposon Tn4, specifically Tn3. Similar studies are in agreement with these results (11, 12). Heteroduplexes between the 5.3-kb plasmid pNG18 (Table 1) and RSF2124 (22) showed a region of homology between the two plasmids of 1.5 to 1.6 kb (data not shown). The region of homology was localized by repeating the heteroduplex
study with pNG18 cut with PstI (which cuts within the β-lactamase gene of pNG18) and RSF2124 cut with EcoRI. The results (Fig. 1) show that the PstI site of pNG18 lies within the region of homology, indicating that this homology represents TnA sequences. The duplex region in these heteroduplex molecules is reduced to 1.3 kb, consistent with the position of the PstI site in the β-lactamase gene of TnA.

Plasmids pNG18 (5.3 kb) and pNG10 (7.4 kb) were compared by forming heteroduplexes from them. The results (Fig. 2) showed that these plasmids are largely homologous. The only apparent difference between the two was that the 7.4-kb plasmid carries an insertion of 2.1 kb. This sequence is bounded by inverted repeats of approximately 300 base pairs. Since BamHI cuts the 5.3-kb plasmid twice to generate fragments of 2.4 and 2.9 kb, whereas the same enzyme cuts the 7.4-kb plasmid twice to give fragments of 2.4 and 5.0 kb, the 7.4-kb plasmid is related to the smaller 5.3-kb plasmid by insertion of a 2.1-kb sequence into the larger of the two BamHI fragments of the 5.3-kb plasmid. The position of the insertion was localized by repeating the heteroduplex analysis with (i) plasmid DNA cut with PstI and (ii) plasmid DNA cut with BamHI. The former study indicated that the insertion was situated 1.75 kb from the single PstI site. The latter experiment placed the insertion unambiguously at coordinate 1.4 on the 5.3-kb plasmid (Fig. 3).

Comparison of several 5.3- and 7.4-kb plasmids. All of the 7.4-kb plasmids (Table 1) were digested with restriction endonucleases HindII, BamHI, HpaII, AluI, and HindIII. BamHI cut each plasmid twice to yield two bands of 2.4 and 2.9 kb; HpaII generated three fragments, 0.3, 0.6, and 4.4 kb; HindII cut the molecules at a single site; and AluI

patterns on 2% agarose gels that were indistinguishable from one plasmid to another.

Likewise, restriction endonuclease digestion of all of the 5.3-kb plasmids (Table 1) gave the same patterns of bands for BamHI, HpaII, AluI, and HindII. BamHI cut each plasmid twice to yield two bands of 2.4 and 2.9 kb; HpaII generated three fragments, 0.6, and 4.4 kb; HindII cut the molecules at a single site; and AluI

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generated about a dozen fragments, yielding band patterns on 2% agarose gels that were indistinguishable from one plasmid to another, but which could be distinguished from the AulI pattern of the 7.4-kb plasmid (data not shown). A restriction map of the 5.3-kb plasmid pNG18 is shown in Fig. 3. At two places, where two restriction sites are very close (i.e., HpaII/PstI and Hpal/HindII), it was difficult to order these sites with respect to one another. The HindII cut was deduced to be on the right-hand side of the HpaII cut because in a BamHI plus HindII double digestion the 0.6-kb HpaII fragment was shortened. The position of the PstI site on the left of the other HpaII site was deduced from the pattern of the BamHI plus PstI double digestion.

DISCUSSION

β-Lactamase-producing *N. gonorrhoeae* strains contain either a 5.3- or a 7.4-kb β-lactamase plasmid (5, 8, 13, 15–18, 21, 23, 25) which are closely related (11, 12, 17). The heteroduplex studies reported in this paper show that the whole of the 5.3-kb plasmid is contained within the 7.4-kb plasmid. The results indicate that the 7.4-kb plasmid is the 5.3-kb plasmid with a 2.1-kb insertion at coordinate 1.4 (Fig. 3). Furthermore, this extra DNA appears to be bounded by small inverted repeats (estimated at 300 base pairs from electron microscope data) reminiscent of those found on insertion elements and transposons (2), suggesting that the 7.4-kb plasmid might well have been derived from the 5.3-kb plasmid by transpositional acquisition of an insertion element.

Our restriction data for the larger β-lactamase plasmids were in complete agreement with the restriction map of the 7.4-kb β-lactamase plasmid pFA3 (15, 23; T. E. Sox, Ph.D. thesis, University of North Carolina at Chapel Hill, 1978) and probably the same as a similar plasmid isolated in France (13). All of the 7.4-kb plasmids examined were identical. Similarly, all of the 5.3-kb plasmids examined gave indistinguishable restriction patterns. Furthermore, the restriction map of the 5.3-kb plasmid was contained within that of the 7.4-kb plasmid, as expected from the heteroduplex analysis.

A derivative of the 5.3-kb plasmid, pLV11, generated by transposing onto it Tn1723, when heteroduplexed with a second 5.3-kb plasmid (pNG18) showed total homology between the two original sequences (data not shown), as expected from the restriction data. We conclude, therefore, that the β-lactamase plasmids of the same size are identical even though they were isolated in different countries at different times (Table 1), and the strains from which they were isolated belong to several different auxotypes, typed as proposed by Catlin (3). We detected no changes in the plasmids as has been reported by van Embden et al. (24).

It seems reasonable to suggest, therefore, that these two β-lactamase plasmids have been spread among *N. gonorrhoeae* strains on a world-wide scale.

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