Novel plasmids in clinical strains of *Streptococcus pneumoniae*

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Received 23 July 1990
Accepted 31 July 1990

Key words: Streptococcus; Cryptic plasmids

1. SUMMARY

Small plasmids were found in two clinical isolates of *Streptococcus pneumoniae* from Spain (strains 671 and 678) and in one strain (SpR) isolated in Germany. All three strains contained one plasmid (2.75 to 3.1 kb) which is related to the only previously described pneumococcal plasmid, pDP1. Strains 678 and SpR carried a second plasmid of 2.6 kb and 2.7 kb, respectively. These two plasmids hybridized neither with each other, nor with pDP1, demonstrating that they represent new types of plasmids not having been found in pneumococci before.

2. INTRODUCTION

Small cryptic plasmids have been isolated only from a few strains of *S. pneumoniae*, and all of them appear to be identical to pDP1, the first pneumococcal plasmid described [1]. pDP1 has been discovered in a series of laboratory strains which are derivatives of a clinical strain isolated in 1916 by Avery [2]. Independent plasmid-bearing clinical isolates have been found in Italy (one strain [3]) and recently in Australasia (eight strains [4]). Plasmids were also described in five South African strains, three of them contained a plasmid larger than 10 kb [5]; the relationship of these plasmids to pDP1 has not been investigated. In none of the cases, could the presence of a plasmid be linked to the antibiotic resistance profiles of the strains.

After screening a collection of clinical isolates of *S. pneumoniae*, three strains were found that contained pDP1 related plasmids of different sizes. Surprisingly, two of the strains carried an additional small plasmid. These plasmids are described in this report.

3. MATERIALS AND METHODS

3.1. Bacterial strains and plasmids

The 77 clinical strains of *S. pneumoniae* which were screened for plasmids were from a collection of strains including isolates from South Africa, Germany, Finland, and Spain held in this laboratory. *S. pneumoniae* cells were grown in C-medium [6] or CAT-medium [7] without aeration. The *E. coli* - *Streptococcus* shuttle vector pDP28 which
contains a 2.5 kb HindIII/SalI pDP1 DNA fragment was obtained from G. Pozzi [8].

3.2. Serotyping and determination of MIC values for *S. pneumoniae*

Serotyping was carried out by J. Hendrikson at the Statens Seruminstitut, Copenhagen (strain SpR) and provided by C. Latorre (strains 671 and 678). MIC values for benzylpenicillin were obtained by incubating cells at low cell density with serial dilutions of the antibiotic as described [9].

3.3. DNA preparation

*S. pneumoniae* chromosomal DNA was prepared as described previously [10]. *S. pneumoniae* plasmid DNA was obtained essentially as described by Birnboim and Doly [11] and further purified by ultracentrifugation in CsCl-ethidium bromide density gradients.

3.4. DNA hybridization

DNA was transferred from agarose gels to nylon membranes (Gene Screen, NEN, Boston) by diffusion [12], using 0.5 M NaOH, 1.5 M NaCl for DNA denaturation during the transfer. For neutralization, 1 M Tris-HCl, pH 7.0, 1.5 M NaCl was used and the DNA crosslinked to the membrane by irradiation with UV light (302 nm for 5 min). Radioactive probes were prepared after electrophoresis (Biotrap, Schleicher and Schüll, Dassel, F.R.G.) of the DNA from agarose gels followed by random oligonucleotide primed synthesis in the presence of [α-³²P]dATP, using a multiprime DNA labeling kit (Amersham, Braunschweig, F.R.G.). Reaction conditions were as recommended by the manufacturer. Hybridization was carried out by standard methods [13]. For restriction endonuclease analysis, enzymes purchased from Boehringer, Mannheim (F.R.G.) were used throughout.

4. RESULTS

4.1. Characterization of the three plasmid-containing *S. pneumoniae* strains

Relevant properties of the three plasmid-bearing strains are listed in Table 1. They all have different serotypes and different MIC values for penicillin. SpR isolated in Germany is a relatively penicillin resistant strain, whereas 671 and 678 isolated both in 1988 in the same hospital can be considered as penicillin sensitive.

*DpnI*, which recognizes A-methylated GATC sites, restricts DNA of strain 671, whereas the DNAs of 678 and SpR are sensitive to *DpnII* endonuclease, i.e. they contain unmethylated DNA like the pDP1 laboratory host strains described earlier [1]. Thus, the three strains are not related to each other, and the presence of plasmids does not correlate with a specific restriction system associated with the host cell.

4.2. Relationship of the plasmids to pDP1

In unrestricted plasmid preparations of strain 671 only one supercoiled form was detected (Fig. 1A, lane 5), whereas in strain 678 two well separated DNA bands were apparent (Fig. 1A, lane 3) while SpR contained two plasmids of almost the same size (Fig. 1A, lane 1). Judged from analyses of total DNA preparations, the amount

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Year isolated</th>
<th>Type</th>
<th>MIC a</th>
<th>DpnI/DpnII b</th>
<th>Plasmid (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpR</td>
<td>Berlin</td>
<td>1986</td>
<td>6A</td>
<td>0.25</td>
<td>−/+</td>
<td>pPR1 (2.75)</td>
</tr>
<tr>
<td>678</td>
<td>Barcelona</td>
<td>1988</td>
<td>23</td>
<td>0.015</td>
<td>−/+</td>
<td>pPR2 (3.0)</td>
</tr>
<tr>
<td>671</td>
<td>Barcelona</td>
<td>1988</td>
<td>1</td>
<td>0.06</td>
<td>+/−</td>
<td>pPR3 (3.1)</td>
</tr>
</tbody>
</table>

a for Benzyl-Penicillin (µg/ml)
b DNA-restricted (+) or not restricted (−).
Fig. 1. Presence of pDP1 related plasmids in the three clinical strains SpR, 671, and 678. (A) Ethidium bromide stained agarose gels. Plasmid DNA from strain SpR (lanes 1 and 2), 678 (lanes 3 and 4), and 671 (lanes 5 and 6) was separated on a 1% agarose gel without endonuclease digestion (1, 3, 5) or after digestion with *Hind*II (2) or *Sal*I (4 and 6). The size in kb of marker DNA fragments (M) is indicated on the left. Arrows mark the fragments that gave positive signals after Southern blotting using a 32P-labeled 2.5 kb *Hind*III/*Sal*I pDP1 DNA fragment as a probe. (B) Autoradiogram of the Southern blot.

of each plasmid per cell appeared to be fairly similar, estimated to be five to ten copies per cell.

The existence of two different plasmids in strains 678 and SpR was further supported by restriction analyses and preliminary hybridization experiments. In order to investigate whether all the plasmids are related to the pneumococcal plasmid pDP1, as with other previously identified plasmids, a 2.5 kb pDP1-probe was used in a hybridization experiment. Plasmid preparations were restricted in such a way that the two plasmids in SpR were clearly separated: *Hind*II linearized one plasmid (2.75 kb) and restricted the other into two fragments of 1.1 and 1.6 kb (Fig. 1A, lane 2). The 678 plasmids (2.6 kb and 3.0 kb) and the 671 plasmids (3.1 kb) were linearized with

<table>
<thead>
<tr>
<th>Plasmid (strain)</th>
<th><em>Hind</em>III</th>
<th><em>Cla</em>I</th>
<th><em>Hinc</em>II</th>
<th><em>Hind</em>II</th>
<th><em>Sal</em>I</th>
<th><em>Acc</em>l</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPR1 (SpR)</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pPR2 (678)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pPR3 (671)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>pCZ1 (SpR)</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pCZ2 (678)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>
SalI (Fig. 1A, lanes 4 and 6). The Southern blot shown in Fig. 1B clearly demonstrates that only one plasmid of each strain hybridized with pDP1. The same result was obtained using the 3.1 kb plasmid of strain 671 as hybridization probe. The pDP1 related plasmids were named pPR1 - pPR3 (Table 1).

In order to see whether the non-pDP1-like plasmids (pCZ1 and pCZ2) are related to each other, pCZ2 DNA was used in a second hybridization experiment. Fig. 2 clearly shows that it hybridized only to itself, and not to pCZ1, and this unrelatedness was further confirmed by their totally different restriction pattern (data not shown). As expected, the pCZ2 probe did not hybridize to the DNAs of pPR 1–3 (Fig. 2), nor did the pCZ2 (or pCZ1) restriction pattern show any similarity to that of pPR1 - 3 (see also Table 2), confirming the uniqueness of pCZ2 (and pCZ1).

4.3. Restriction endonuclease analysis of the pDP1 related plasmids

The three plasmids that hybridized with pDP1 DNA varied in sizes between 2.75 and 3.1 kb. The restriction map shows that, correlating with the decreasing size of the plasmids, they contained an AccI fragment of variable length, ranging from approximately 0.9 kb in pPR 3 to 0.55 kb in pPR1. In pPR1, the unique Clal site was missing. All other restriction sites shown in Fig. 3 correspond to the published map of pDP1 [4,8], with the exception of a missing HinfI site in pPR2 (Table 2).

5. DISCUSSION

In the present study we have shown that pDP1-related plasmids occur in pneumococci isolated in Germany and Spain. The frequency of plasmid-containing strains in this screening (4%) involving 77 strains is in the same order of magnitude as reported recently for a collection of strains isolated in Australasia (8 out of 500 strains screened [4]), or for the South African strains investigated (5 out of 50 [5]). The data further confirm that the strains harboring these plasmids do not show any correlation between various properties, including their restriction systems, and that so far no function can be assigned to the plasmids. No hybridization to the chromosomal DNA of different strains, including the R6 strain, was found (unpublished results), indicating that no recombination between the chromosome has taken place which, for example, could explain the variable part in the pDP1-related plasmids.

In two independently isolated strains, the presence of a second, cryptic plasmid, neither related to pDP1 nor to each other, could be demonstrated. The origin(s) of the various plasmids is unclear. It is curious that all these plasmids are fairly small (around 3 kb), and that despite the low frequency of plasmid-containing pneumococci, two of the three strains described contained two plasmids at the same time. It remains to be clarified whether these rare strains possess (or have lost) a function rendering them suitable acceptor and host strains for plasmids.

ACKNOWLEDGEMENTS

We thank J. Hendrikson for serotyping of the strain SpR, G. Pozzi for kindly providing plasmid pDP28, and B. Egan for valuable criticism.

REFERENCES