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A Novel Type of Resistance Plasmid in *Haemophilus influenzae*

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Resistance plasmids of a novel type were found in two *Haemophilus influenzae* clinical isolates. pPJ301 and pPJ302 are 10.0 kilobases in size, carry a Tn*2*-like transposable element, and are related only by their common beta-lactamase genes to the other two types of resistance plasmids known to occur in *H. influenzae*.

Antibiotic resistance plasmids of the species *Haemophilus influenzae* have been shown to belong to at least two different groups. The first group is characterized by 7.1-kilobase (kb) genetic elements similar to those found in *Neisseria gonorrhoeae*; they code for ampicillin resistance through the synthesis of TEM-1 beta-lactamases specified by a derivative of a Tn*2* transposon which had undergone a 33% deletion (10, 12, 18). These plasmids are infrequent in *H. influenzae*.

The second group is characterized by extrachromosomal elements larger than 45 kb: they specify resistance to different antibiotics, such as ampicillin (through a functional Tn*2* transposon), chloramphenicol, tetracycline, or kanamycin, alone or in combination (11, 17, 25). These plasmids share strong homology with each other owing to multiple insertion events of different resistance transposons, originating likely from the enteric pool, into a unique core plasmid. pW266, isolated and characterized by Laufs et al., is such a recipient core plasmid (15, 16, 19). Genetic elements belonging to this group are the most frequent in *H. influenzae*.

There is also evidence supporting the existence of a third group of plasmids which are integrated in the bacterial chromosome; these genetic elements appear to be larger than 45 kb and code also for different antibiotic resistances (23, 24).

During a recent survey on the *H. influenzae* isolated in Switzerland, we found that of 20 resistant clinical isolates, 13 carried plasmids larger than 45 kb and 5 were devoid of any detectable extrachromosomal elements; furthermore, two ampicillin-resistant strains, *H. influenzae* 301 and 302, harbored plasmids of the unusual size of 10.0 kb, as determined by electron miroscopy and agarose gel electrophoresis: these plasmids were called pPJ301 and pPJ302. In this note, we report their characterization.

H. influenzae 301 was isolated from a Libyan patient transported and admitted to the intensive care unit of a Bern, Switzerland, hospital after a road accident in his country; the patient was suffering from pneumonia following tracheal intubation. *H. influenzae* 302 was isolated in Geneva, Switzerland, from an Ethiopian child suffering from conjunctivitis. Both isolates were shown to be resistant only to ampicillin.

Resistance to ampicillin was due to beta-lactamase production, as indicated by nitrocefin hydrolysis assays (2); resistance was related to the presence of plasmids pPJ301 or pPJ302, as demonstrated by transformation experiments (20) into *Escherichia coli* C600, using plasmid DNA extracted (5, 8) from *H. influenzae* 301 and 302. DNA obtained from more than 20 ampicillin-resistant transformed clones and analyzed by agarose gel electrophoresis showed the constant presence of 10.0-kb plasmids; their identity to the *H. influenzae* genetic elements was confirmed by endonuclease restriction analysis. Plasmids pPJ301 and pPJ302 were stably maintained in *E. coli* C600: 100% of the bacteria tested were still resistant to ampicillin after 50 generations of growth in the absence of antibiotics.

Although pPJ301 and pPJ302 were not self-transmissible plasmids, they could be mobilized by a conjugative genetic element present in the same cell. Construction of an *E. coli* strain harboring one of the *H. influenzae* resistance plasmids together with pUB307 (3), a derivative of RP1 used for mobilization (obtained from J. Frey), resulted in the transfer of pPJ301 or pPJ302 to a susceptible recipient *E. coli* strain at a frequency of about 10^{-2} per donor cell.

Homoduplex structures prepared (7, 9) from pPJ301 and pPJ302 linearized with *Eco*RI and examined in the electron microscope revealed the presence of a circular single-stranded loop of about 5.0 kb at 1.9 and 3.0 kb from the

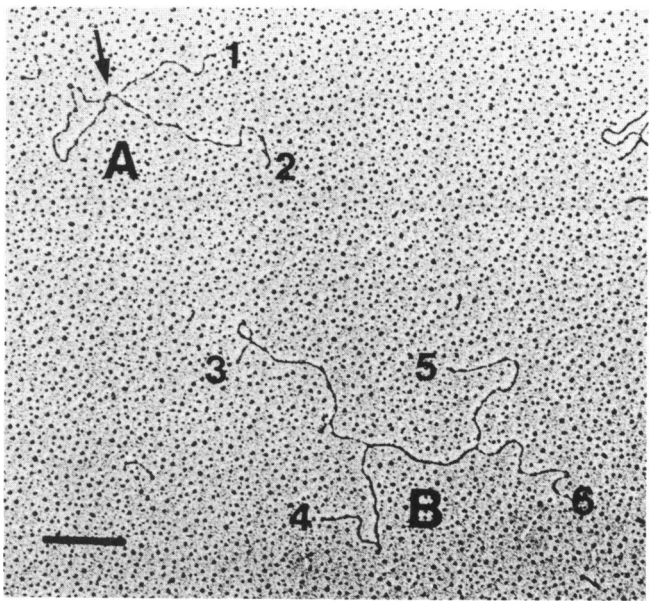

FIG. 1. Electron micrograph of a homoduplex of the plasmid pPJ301 linearized with *Eco*RI (A) and of a heteroduplex between pPJ301 and the gonococcal plasmid pPJ102 linearized with Eco*RI* and *Hin*dIII, respectively (B). In molecule A, note the 5.0-kb loop and the small double-stranded stem (arrow). In molecule B, the double-stranded region is 1.6 kb long. Single-stranded fragments 1, 2, 3, 4, 5, and 6 are 1.9, 3.0, 5.2, 2.7, 3.1, and 2.9 kb long, respectively. Bar is 1 kb.

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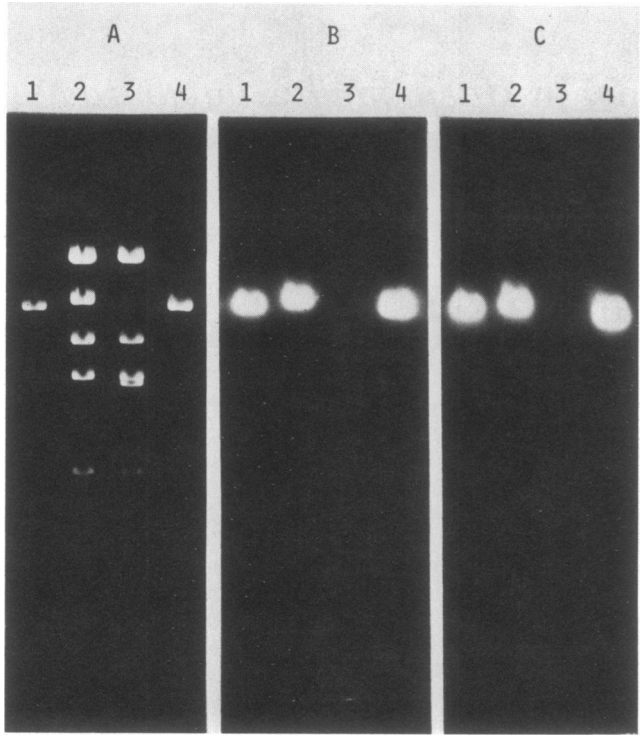

FIG. 2. (A) Agarose gel electrophoresis of *Eco*RI digestions of DNAs from the plasmids pPJ301 and pPJ302 (lanes 1 and 4, respectively) and the phages λ*b515b519*::Tn*3* and λ*b515b519* (lanes 2 and 3, respectively). (B and C) The corresponding autoradiographs after transfer onto nitrocellulose filters and hybridization with ^{32}P-labeled pPJ301 and pPJ302, respectively. Tn*3* is inserted in the second band of λ*b515b519*::Tn*3* (lane 2).

extremities (Fig. 1); the structure was joined to the remaining molecule by a small double-stranded stem. This suggested that pPJ301 and pPJ302 carry a Tn*2*-like transposon (which is 5.0 kb long), an hypothesis that was confirmed first by transposition of ampicillin resistance to the bacteriophage λ genome (4, 21). Lysates obtained from *E. coli* C600-(pPJ301) and C600(pPJ302) infected with phage λ were used to lysogenize C600-susceptible cells, and ampicillin-resistant clones were selected; 12 such clones were induced and shown to produce phages transducing ampicillin resistance at a frequency close to 100%; their DNA examined by restriction analysis was shown to have undergone an increase of 5.0 kb (data not shown). That pPJ301 and pPJ302 carry a transposon close to Tn*2* was further demonstrated by a Southern hybridization experiment (7, 22) between ^{32}P-labeled pPJ301 and pPJ302 and DNA containing a Tn*3* transposon insertion (Fig. 2; Tn*2* and Tn*3* are highly related transposons).

These data are supported by the restriction map presented in Fig. 3 which shows the *Pst*I, *Hin*dII, and *Bam*HI internal fragments characteristic of the Tn*2* elements (1, 6, 14); moreover, restriction analysis indicated that pPJ301 and pPJ302 cannot be distinguished from each other and thus are similar (see Fig. 4).

The relations between pPJ301 or pPJ302 and the resistant genetic elements known to occur in *H. influenzae* were analyzed in the Southern hybridization experiments presented in Fig. 4: pW266 (the core plasmid related to the resistant elements larger than 45 kb) showed no homology with pPJ301 or pPJ302, whereas the gonococcal plasmid pPJ102 (12, 13), used as a representative of the 7.1-kb resistance elements, appeared to share common sequences with pPJ301 and pPJ302. However, the *H. influenzae* bands hybridizing to the gonococcal labeled plasmid are those containing the Tn*2* fragment present in pPJ102 (Fig. 3). This was confirmed by heteroduplex analysis. An example of a structure obtained from an *Eco*RI digest of pPJ301 and a *Hin*dIII digest of pPJ102 is shown in Fig. 1; the double-stranded region, indicating homology, was 1.6 kb long and was flanked at one side by two single-stranded fragments of 5.2 and 2.7 kb belonging to the *H. influenzae* and *N. gonorrhoeae* genetic elements, respectively, and at the other side by two single-stranded fragments of 2.9 and 3.1 kb originating from both of the reacting plasmids. The lengths found were similar to those expected if the homologous regions of pPJ301 and pPJ102 corresponded to the residual moiety of Tn*2* present on the gonococcal genetic element (see Fig. 3 for the pPJ301 restriction map and references 12 and 13 for that of pPJ102). Thus, pPJ301 or pPJ302 did not show any relationship with the other resistance plasmids now known to occur in *H. influenzae*, except for the Tn*2* common sequence present in the plasmid type similar to the gonococcal resistance element.

From an epidemiological point of view, the finding of this novel type of resistance plasmid in *H. influenzae* is important, particularly because this bacterial species can cause in infants such dramatic diseases as meningitis and epiglottitis, which need empiric early antibiotic treatment. Because pPJ301 and pPJ302 have been isolated from patients living in very different areas and because these plasmids are mobilizable by conjugative helper plasmids, it is expected that they

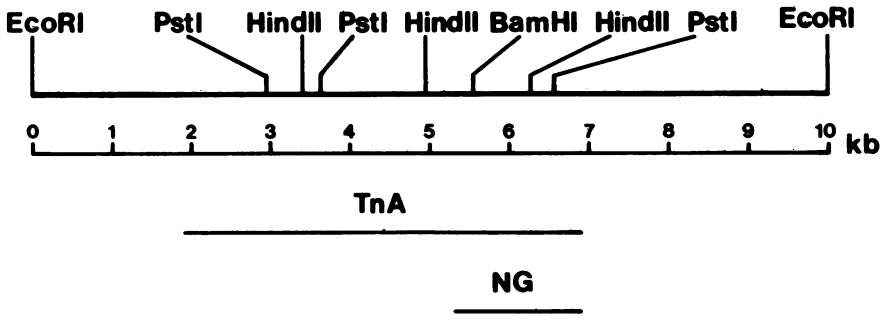

FIG. 3. Restriction map of the plasmids pPJ301 and pPJ302. The bar labeled TnA shows where the Tn*2* element is inserted; the bar labeled NG shows the portion of Tn*2* present in the gonococcal plasmid pPJ102.

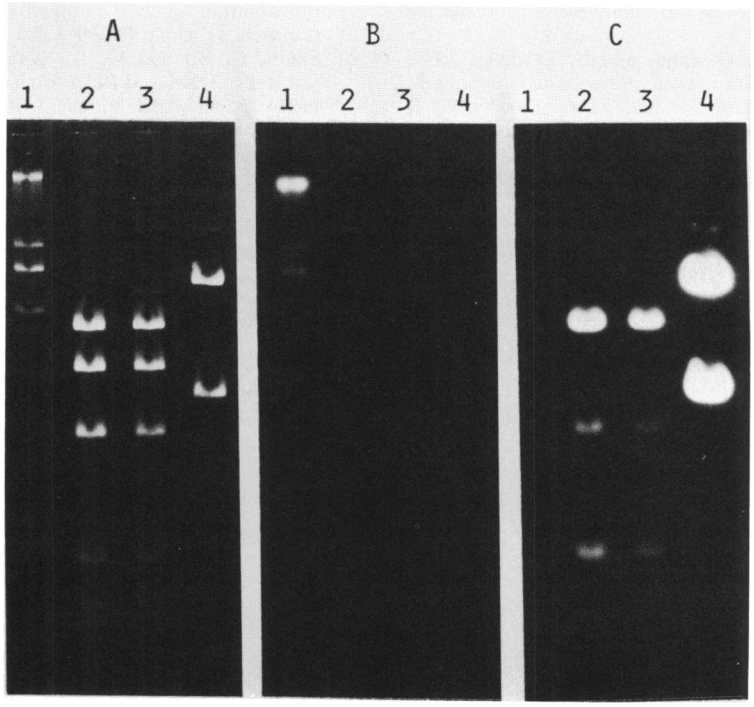

FIG. 4. Southern hybridization experiments between the plasmid pW266 (*H. influenzae* core plasmid), gonococcal plasmid pPJ102, and plasmids pPJ301 and pPJ302. (A) Agarose gel electrophoresis of pW266 digested with *Eco*RI (lane 1), pPJ301 and pPJ302 digested with *Eco*RI, *Bam*HI, and *Pst*I (lanes 2 and 3, respectively), and pPJ102 digested with *Bam*HI (lane 4). (B and C) The corresponding autoradiographs after transfer onto nitrocellulose filters and hybridization with ^{32}P-labeled pW266 and pPJ102, respectively. The reacting bands of pPJ301 and pPJ302 (panel C, lanes 2 and 3) are due to the portion of the Tn*2* transposon inserted in pPJ102.

will occur more and more frequently in *H. influenzae* clinical isolates.

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