Molecular Properties of Transmissible R Factors of 
*Haemophilus influenzae* Determining Tetracycline Resistance

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The tetracycline-resistant *Haemophilus influenzae* strains LU121 and FR16017, recently isolated in West Germany, each harbour a plasmid; that of the former (pLU121) has a mol. wt of $31.5 \times 10^6$ and that of the latter (pFR16017) has a mol. wt of $33 \times 10^6$. Conjugation and DNA–DNA hybridization studies have shown that both plasmids are self-transmissible and carry tetracycline-resistance genes. The purified plasmid DNA of *H. influenzae* strain LU121 transformed a sensitive *Escherichia coli* strain to tetracycline resistance. The two R factors are closely related to the *H. influenzae* plasmid specifying ampicillin resistance (pKRE5367). Electron microscope DNA heteroduplex analysis indicated that pLU121 and pFR16017 probably carry the tetracycline-resistance transposon TnD and that pKRE5367 probably carries the ampicillin-resistance transposon TnA. There is more than one integration site for the insertion which probably represents TnD in pFR16017. All three plasmids have a similar plasmid core and could have a common evolutionary origin.

**INTRODUCTION**

*Haemophilus influenzae* causes serious clinical infections including meningitis, epiglottitis, pneumonia and otitis media. Recently, plasmid-linked resistances to some antibiotics have appeared in *H. influenzae*, notably ampicillin resistance determined by transmissible R factors (Elwell *et al.*, 1975; Laufs & Kaulfers, 1977). Resistance to tetracycline alone or combined with chloramphenicol resistance has been observed in the United Kingdom (Elwell *et al.*, 1977) and in the Netherlands (van Klinger, van Embden & Dessens-Kroon, 1977). This study describes the properties of two *H. influenzae* plasmids, recently isolated in West Germany, which determine tetracycline resistance.

**METHODS**

**Bacterial strains.** The tetracycline-resistant *H. influenzae* strains LU121 and FR16017 were recently isolated in Ludwigshafen, West Germany, and Freiburg, West Germany, respectively. The other bacterial strains and plasmids used in this study have been described by Laufs & Kaulfers (1977).

**Media.** The liquid medium for *H. influenzae* contained 3.5% (w/v) brain heart infusion (BHI, Oxoid) supplemented with 10 μg haemin ml⁻¹, 10 μg of L-histidine ml⁻¹ and 10 μg NAD ml⁻¹. Chocolate agar was used as a solid medium, sometimes supplemented with 20 μg ampicillin ml⁻¹, or 10 or 20 μg tetracycline ml⁻¹. For DNA labelling, *H. influenzae* was grown in the minimal growth medium (M-IIg) described by Spencer & Herriott (1965). Cultures on solid media were incubated at 37 °C in a CO₂ incubator (CO₂ concentration, 3 to 8%, v/v). Cultures in broth were grown in a shaker in an air incubator at 36 °C.

**Isolation of plasmid DNA.** Plasmid DNA was isolated by a modification of the cleared lysate method (Clewell & Helinski, 1969), as recently described by Laufs & Kaulfers (1977).

**Preparation of labelled plasmid DNA and unlabelled whole-cell DNA.** Plasmid DNA was purified by the method of So, Crosa & Falkow (1975). DNA from *Escherichia coli* was labelled as described by Heffron *et al.*
For hybridization studies, whole-cell DNA from *H. influenzae* and *E. coli* strains was extracted according to the method of So et al. (1975).

**Conjugation.** Exponential-phase cultures of the donor strains LU121 and FR16017 were mixed 1:1 with the recipient *E. coli* strain 1485-1 and incubated overnight. In some experiments, pancreatic DNAase I (20 μg ml⁻¹) was included. A 1:10 dilution of the mating mixture was incubated in liquid media containing 0.5 μg tetracycline ml⁻¹ and then incubated overnight again before plating on agar supplemented with 20 μg tetracycline ml⁻¹.

**Transformation.** The purified plasmid DNA dissolved in TES buffer (0.05 M-NaCl/0.005 M-EDTA/0.03 M-Tris, pH 8.0) was incubated at 40 °C for 20 min with pronase (5 μg ml⁻¹). Transformation was carried out as described by Cohen, Chang & Hsu (1972). After the 42 °C heat pulse, the transformation mixture was diluted 1:5 in BHI and incubated overnight at 37 °C to allow phenotypic expression. Cells were cultured in BHI with 0.5 μg tetracycline ml⁻¹ for an additional day and then plated on chocolate agar supplemented with 10 μg tetracycline ml⁻¹.

**DNA-DNA duplex studies.** Before hybridization, labelled and unlabelled DNA were sheared by sonication (Branson sonicator model 140D for 4 min at 4 °C, output setting 3) and then dialysed against 0.4 M-NaCl. Hybridizations were carried out as described by Crossa, Brenner & Falkow (1973). Approximately 0.001 μg ³H-labelled, sheared plasmid DNA (specific activity 1.5 × 10⁶ c.p.m. μg⁻¹) was mixed in a glass vial with 150 μg unlabelled whole-cell DNA in a total volume of 1.5 ml 0.21 M-NaCl. The DNA was denatured by placing the vial in a boiling-water bath for 10 min, and renaturation was immediately started by transferring the vial to a 70 °C bath. The DNA was allowed to reanneal for 2 h using ³H-labelled pKKT007 plasmid DNA. Renaturation was stopped by placing the vial in an ice bath. The S1 endonuclease reaction was carried out as described by Crossa et al. (1973).

**Agarose gel electrophoresis of plasmid DNA.** The plasmid DNA was identified and characterized according to the method of Meyers et al. (1976).

**DNA contour length.** Spreading and staining of DNA were performed essentially as described by Kleinschmidt (1968) using parlodion-coated electron microscope grids. The true magnification of the electron microscope was determined by calibration with a diffraction grating, and the molecular weights of the plasmids were calculated assuming 1 μm DNA to be equivalent to 2.07 megadalton (Mdal).

**Electron microscope DNA heteroduplex analysis.** To detect similarities between the *H. influenzae* plasmids pKRE5367, pLU121 and pFR16017, the electron microscope DNA heteroduplex methods described by Davis, Simon & Davidson (1971) were followed. The covalently closed circular (CCC) plasmid DNA was incubated at 40 °C for 20 min with 5 μg pronase ml⁻¹ and rebanded. Before heteroduplexing, the CCC plasmid DNA was nicked with 200 kV X-rays under the protection of histidine (10 μl 0.1 M-histidine to 0.4 ml DNA). In the ethidium bromide-CsCl medium, a dose of 300 rad converted approximately 50 % closed circles to open circles. Approximately 0.2 μg X-ray-nicked plasmid DNA was mixed with 0.25 ml of a solution containing 0.1 M-NaOH and 0.01 M-EDTA. The DNA was allowed to denature at pH 12.5 for 10 min. The pH was then lowered to 8.5 with 2 M-Tris (pH 7.1) and 0.25 ml formamide (Fluka, Buchs, Switzerland). The DNA was allowed to reanneal under these conditions at room temperature for 5 h. Cytochrome c was added to a final concentration of 0.1 μg ml⁻¹, and the reannealed DNA was spread at the air-water interface on a glass slide on to a hypophase of a solution containing 10 % (v/v) formamide, 0.01 M-Tris and 0.001 M-EDTA (pH 8.5). After 1 to 2 min, the DNA was picked from the surface of the hypophase on a parlodion-coated electron microscope grid. The grid was stained with 50 μl uranyl acetate in 90 % (v/v) ethanol for 30 s and fixed in isopropanol for 10 s. Grids were shadowed with platinum/palladium and examined in a Siemens Elmiskop 101 electron microscope.

The method described for DNA preparation gives reduced values for the contour length of single-stranded DNA due to shrinkage (Davis et al., 1971). Therefore, single-stranded DNA derived from the 5.5 Mdal plasmid RSF1010 was used as a control to estimate the shrinkage (Heffron, Rubens & Falkow, 1977). We found in several different experiments that the single-stranded RSF1010 DNA molecules showed 19 to 36 % size reduction compared with the double-stranded RSF1010 DNA molecules. For an approximate estimation of the molecular size of the single-stranded DNA loops, the molecular weight values obtained from the formula for double-stranded DNA were considered to give only about 73 % of the real molecular size.

**RESULTS**

**Demonstration of a plasmid in each of the tetracycline-resistant *H. influenzae* strains LU121 and FR16017**

The tetracycline-resistant *H. influenzae* strains LU121 and FR16017 were examined by agarose gel electrophoresis for the presence of extrachromosomal DNA. The ampicillin-resistant *H. influenzae* strain KRE5367 containing a 30 Mdal plasmid (Laufs & Kaulfers,
Tetracycline resistance of *H. influenzae*

Fig. 1. Agarose gel electrophoresis of ethanol-precipitated DNA from bacterial lysates. (A, E) Standard plasmid DNAs ranging from 62 to 4.2 Mdal; chr indicates the band position of chromosomal DNA. (B) Lysate from *H. influenzae* strain KRE5367 Ap<sup>+</sup>; 30 ± 1 Mdal plasmid present. (C) Lysate from *H. influenzae* strain LU121 Tcr; 31 ± 1 Mdal plasmid present. (D) Lysate from *H. influenzae* strain FR16017 Tc<sup>+</sup>; 33 ± 1 Mdal plasmid present.

1977) was used as a control. The cleared lysates of the strains contained CCC DNA. Lysates of each of the two tetracycline-resistant *H. influenzae* isolates gave a single plasmid band in the gel and both plasmids showed a similar mobility. The estimation of plasmid mass from the extent of DNA migration revealed a molecular weight of about 30 x 10<sup>6</sup> to 40 x 10<sup>6</sup> (Fig. 1). The plasmid present in strain LU121 was named pLU121 and that in strain FR16017 was named pFR16017.

The cleared lysates of the *H. influenzae* strains LU121 and FR16017 centrifuged to equilibrium in a CsCl-ethidium bromide gradient showed a dense peak composed of CCC DNA and a less dense peak containing linear chromosomal DNA and open circular DNA (data not shown). The bands of CCC DNA in CsCl-ethidium bromide gradients were examined in the electron microscope. Only plasmids of similar molecular size were detectable in each preparation. Twelve well-isolated open circular DNA molecules of pLU121 and of pFR16017 were photographed and measured. The mean molecular weight of pLU121 was 31.5 x 10<sup>6</sup> and that of pFR16017 was 33 x 10<sup>6</sup>.  

*Transfer of tetracycline resistance of strains of LU121 and FR16017 to E. coli by conjugation*

Tetracycline resistance of strains LU121 and FR16017 was transmissible to *E. coli* strain 1485-1 by co-cultivation. The transfer frequency was not reduced by the addition of DNAase I to the mating mixture. After mating, the recipients were cultured overnight in medium containing 0.5 μg tetracycline ml<sup>-1</sup> to allow phenotypic expression of tetracycline resistance. The recipients were then plated on agar containing 20 μg tetracycline ml<sup>-1</sup>. The transfer frequency, determined by dividing the number of tetracycline-resistant recipients present at the end of mating by the number of donor cells present at the beginning of mating, was 2 x 10<sup>-4</sup> for both donors. Gel electrophoresis of the cleared lysate of the recipients revealed plasmids of the same molecular size as in the donors.
Transformation using purified plasmid DNA isolated from the tetracycline-resistant H. influenzae strain LU121

Preparations of pronase-treated, purified CCC DNA from H. influenzae strain LU121 were used for transformation. Exposure of the sensitive E. coli strain c600 to purified plasmid DNA resulted in the appearance of tetracycline-resistant clones which carried a plasmid of similar size to that carried by the parental strain LU121, as shown by agarose gel electrophoresis. The transformation frequency obtained in these experiments using 5 μg purified pLU121 DNA was 8 tetracycline-resistant transformants per 10⁸ cells.
Tetracycline resistance of H. influenzae

Demonstration of tetracycline-resistance genes in pLU121 and pFR16017 by molecular hybridization

As a probe for tetracycline-resistance genes, we used the plasmid pKT007 kindly supplied by Dr K. Timmis. The size of the tetracycline-resistance determinant in this 9.9 Mdal enteric plasmid was estimated to be approximately 2.8 Mdal. The 3H-labelled pKT007 plasmid DNA was hybridized with unlabelled whole-cell DNA from each of the two tetracycline-resistant H. influenzae isolates LU121 and FR16017 as well as with unlabelled whole-cell DNA of three controls (H. influenzae strain G32, E. coli strain 1485-1 and H. influenzae strain KRE5367, Ap'). The 3H-labelled pKT007 DNA hybridized significantly with LU121 DNA (21 %) as well as with FR16017 DNA (28 %), but not with the DNA of the controls (0 to 3 %).

Electron microscope DNA heteroduplex analysis of base sequence homologies

The similarity between the molecular weights of the two H. influenzae plasmids specifying tetracycline resistance (pLU121 and pFR16017) and that of the H. influenzae plasmid specifying ampicillin resistance (pKRE5367; Laufs & Kaulfers, 1977) prompted us to question whether these Haemophilus plasmids have any polynucleotide sequences in common. We found that both the H. influenzae plasmids specifying tetracycline resistance (pLU121 and pFR16017) shared about 70 % of their base sequences with the plasmid pKRE5367 specifying ampicillin resistance. Therefore, it seemed possible that some of the differences in base homology are due to different transposons integrated in similar plasmid cores. This was investigated by electron microscope DNA-DNA heteroduplex analysis.

Eight well-isolated heteroduplex molecules between pLU121 and pKRE5367 were photographed and measured. All of them showed the insertion of a single-stranded DNA loop with an average contour length of 1.06 μm corresponding to about 3.0 Mdal (Table 1), which originated in some molecules from a very short double-stranded stalk (Fig. 2). In addition, the heteroduplex molecules showed a second somewhat larger single-stranded loop on a long double-stranded stalk (Fig. 2). The average molecular size of the double-stranded stalk was 0.92 Mdal, and the contour length of the single-stranded loop was 1.36 μm corresponding to about 3.9 Mdal (Table 1). The shortest distances between the insertion of the loop with the short stalk and the larger loop with the long stalk were measured, and these corresponded to between 10.39 and 11.86 Mdal. Only in one heteroduplex molecule was the measured distance of 5.07 Mdal markedly different (Table 1).

For studies of heteroduplex formation between the plasmid pFR16017 and plasmid pKRE5367, three different pFR16017 DNA preparations were used which were obtained from cloned FR16017 cells that were passaged in vitro after cloning for 4 to 6 months. Ten well-isolated heteroduplex molecules were photographed and measured. Again, the molecules carried two single-stranded loops, one with a very short stalk and the other with a long stalk. The average contour length of the single-stranded loops on the long double-stranded stalks was 1.4 μm corresponding to about 3.22 Mdal and the molecular size of the stalks was 1.02 Mdal. The average contour length of the single-stranded loops with the short stalks was 1.27 μm corresponding to about 3.60 Mdal. The shortest distances measured between the insertions varied considerably and ranged between 1.53 and 11.59 Mdal (Table 2). One heteroduplex molecule was found which carried two loops on long stalks (Fig. 3).

The heteroduplexes between pLU121 and pKRE5367 and those between pFR16017 and pKRE5367 showed, in addition, small insertions and/or deletion loops indicating differences in the base sequences in the cores of the plasmids.
Table 1. Molecular weights of the insertion loops in heteroduplex molecules between pLU121 (Tc') and pKRE5367 (Ap) plasmid DNAs

For the conversion of contour length of double-stranded DNA to molecular weight the formula $1 \mu m = 2.07$ Mdal was used. The values obtained for single-stranded DNA when using this formula were considered to give only 73% of the real molecular weights and were corrected accordingly. Numbers in parentheses indicate contour length ($\mu m$) of single-stranded DNA.

<table>
<thead>
<tr>
<th>No. of molecule</th>
<th>Loop possibly representing IR* (Mdal)</th>
<th>Loop possibly representing insertion loops (Mdal)</th>
<th>Distance between insertion loops (Mdal)</th>
<th>$10^{-6} \times$ mol. wt of molecule without insertion loops</th>
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<td><strong>Average</strong></td>
<td>3.88 (1.36)</td>
<td>0.92</td>
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<td>28.71</td>
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</tbody>
</table>

* Stalk formed by inverted repeat sequences (IS 3).

Table 2. Molecular weights of the insertion loops in heteroduplex molecules between pFR16017 (Tc') and pKRE5367 (Ap) plasmid DNAs

See Table 1 legend for details.

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<tr>
<th>No. of molecule</th>
<th>Loop possibly representing IR* (Mdal)</th>
<th>Loop possibly representing insertion loops (Mdal)</th>
<th>Distance between insertion loops (Mdal)</th>
<th>$10^{-6} \times$ mol. wt of molecule without insertion loops</th>
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<td>3.60 (1.27)</td>
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* Stalk formed by inverted repeat sequences (IS 3).

DISCUSSION

The tetracycline-resistant H. influenzae strains LU121 and FR16017, recently isolated in West Germany, each harbour a plasmid of similar size. Plasmid inheritance of tetracycline resistance was established by isolation of CCC DNA after conjugation with a sensitive recipient. In addition, purified LU121 plasmid DNA transformed E. coli to tetracycline resistance, and DNA-DNA hybridization studies indicated that the plasmids of the two H. influenzae strains carried tetracycline-resistance genes. The two plasmids pLU121 and pFR16017 are self-transmissible and closely related to the ampicillin R factor pKRE5367 isolated from H. influenzae in West Germany at the same time (Laufs & Kaulfers, 1977). pKRE5367 is also self-transmissible, and its molecular characterization, as well as DNA heteroduplex studies of this plasmid specifying ampicillin resistance and the two plasmids specifying tetracycline resistance, indicated that they all have a largely identical plasmid
Fig. 3. (a) Heteroduplex of pFR16017 (Tc') and pKRE5367 (Ap') plasmid DNA. (b) Diagram of the same heteroduplex: symbols as in Fig. 2. Bar marker represents 1 nm.
core and that parts of the non-homologous regions are due to different base sequences in the DNA regions of the plasmids conferring resistance.

Elwell et al. (1977) described a plasmid specifying tetracycline resistance isolated in the United Kingdom. This plasmid (pUB701) showed the same base sequence homology with an ampicillin R factor (RSF007) isolated from *H. influenzae* in the U.S.A., as did the two tetracycline R factors (pLU121 and pFR16017) with the ampicillin R factor (pKRE5367), all isolated in West Germany. In addition to the findings with pUB701, we could demonstrate the self-transmissibility of pLU121 and pFR16017 and the transformation ability of pLU121 DNA. There was no indication here that the plasmids of *H. influenzae* specifying tetracycline resistance are particularly fragile or too large to be transformed.

The DNA heteroduplexes between pKRE5367 (Ap') and pFR16017 (TcP) showed two types of insertion loops. One type had an average molecular size of about 3·0 to 3·6 Mdal and in some molecules showed a very short double-stranded stalk. We assume that these loops represent the ampicillin-resistance transposon TnA. However, this has not yet been proven positively. The size of TnA is 2·7 to 4·0 Mdal with very small (0·1 Mdal) inverted repetitions at the ends (Hedges & Jacob, 1974; Heffron et al., 1975; Bennett & Richmond, 1976; Starlinger & Saedler, 1976). The values we measured are similar to those reported. We have been able to transpose a DNA segment specifying ampicillin resistance from pKRE5367 to the 5·5 Mdal plasmid RSF1010 (Heffron et al., 1977). Gel electrophoresis showed that the hybrid plasmid RSF1010::Ap was 3·2 Mdal larger than RSF1010 (unpublished data), indicating that pKRE5367 probably contains the whole TnA.

The second type of insertion was characterized by a loop with an average size of 3·2 to 3·9 Mdal on a double-stranded stalk of 0·9 to 1·0 Mdal. This insertion most probably represents the tetracycline-resistance transposon TnD, but again the identity of these insertion loops is deduced and not proven. The size of TnD is 5·5 Mdal for the total length (Kleckner et al., 1975) and 0·9 Mdal for the stalk which consists of double-stranded DNA formed by insertion sequences (IS 3) at the ends of TnD (Starlinger & Saedler, 1976). Our values for the single-stranded part of TnD (3·2 to 3·9 Mdal) are close to those reported (3·6 Mdal), and the length of the double-stranded stalk measured by us is in close agreement with other published values (Ptashne & Cohen, 1975). We have been able to transpose a DNA segment specifying tetracycline resistance from pLU121 as well as from pFR16017 on to the 5·5 Mdal plasmid RSF1010 (Heffron et al., 1977). In the gels, the hybrid plasmid RSF1010::Tc was about 5·5 Mdal larger than RSF1010 (unpublished data), indicating that pLU121 and pFR16017 probably carry the whole TnD.

The plasmids pLU121 and pFR16017 are not replicate isolates of one plasmid. They not only have different molecular weights (3·15 × 10⁶ and 3·30 × 10⁶, respectively) but also differ in their base sequences. Molecular DNA–DNA hybridization studies showed that they are not identical, although 70 % of each base sequences is common to both (Laufs & Kaulfers, 1977). The heteroduplex analysis revealed another difference between the two plasmids. Whereas the measured distances between the sites of the insertions were similar in the DNA heteroduplexes between pLU121 and pKRE5367 – in seven out of eight measured molecules the distance between the insertion loops was 10·39 to 11·86 Mdal – such clustering was not apparent in the DNA heteroduplex molecules between pFR16017 and pKRE5367. The non-uniformity of the heteroduplexes formed between pFR16017 and pKRE5367 was observed when using three different pFR16017 DNA preparations obtained from cloned FR16017 cells which were passaged *in vitro* for 4 to 6 months. The findings indicate that the association of the insertion loop, which probably represents TnD, with pFR16017 is not stable and that rearrangement occurs. The demonstration of a heteroduplex molecule between pFR16017 and pKRE5367 with two insertion loops representing, most likely, TnD indicates that there is more than one integration site for the presumptive TnD in the plasmid pFR16017. Our findings are in good agreement with the hypothesis (Kleckner et al., 1975) that TnD is able to integrate into many different places on a DNA molecule.
Tetracycline resistance of H. influenzae

The similarity between the cores of the three H. influenzae R factors that we have examined and their close relationship to R factors of H. influenzae isolated in other countries (Elwell et al., 1977; Laufs & Kaulfers, 1977) is compatible with the so far unproven idea that these R factors arose as a result of the transposition of different resistance genes on to closely related cryptic H. influenzae plasmids which may have a common evolutionary origin. The isolation frequency of indigenous H. influenzae plasmids closely related to the described R factors is low. Among 699 H. influenzae and H. parainfluenzae strains isolated during 1976 and 1977 we found only one 27 Mdal cryptic H. influenzae plasmid (pW266) which showed 82% base homology with pKRE5367. However, the frequency of R factors was similarly low. Only one out of these 699 H. influenzae and H. parainfluenzae strains carried a 30 Mdal plasmid specifying ampicillin resistance (unpublished data). The low frequency of cryptic plasmids does not exclude the possibility that the R factors have arisen as a result of the transposition of resistance genes from anonymous donor R plasmids on to such closely related cryptic plasmids. We have shown by molecular hybridization studies using pMB8::Ap (Laufs & Kaulfers, 1977) and pKT007 as probes that the ampicillin- and tetracycline-resistance genes on the R factors of H. influenzae are most likely the same as those on R factors prevalent in Enterobacteriaceae. Van Klinger et al. (1977) have shown that a single H. influenzae plasmid (pR1234) carries tetracycline plus chloramphenicol-resistance genes which are both known to appear on transposons (Starlinger & Saedler, 1976). This plasmid pR1234 shares 63% of its base sequences with pKRE5367 (Laufs & Kaulfers, 1977).

An alternative hypothesis is that closely related R factors from the same incompatibility group with different resistance genes have now infected H. influenzae strains throughout the world. Experiments in our laboratory are in progress to examine whether the R factors of H. influenzae belong to one of the known incompatibility groups.

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