Molecular Characterization of a Small *Haemophilus influenzae* Plasmid Specifying β -Lactamase and its Relationship to R Factors from *Neisseria gonorrhoeae*

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The ampicillin-resistant *Haemophilus influenzae* strain Ve445 which caused purulent meningitis and septicaemia in a newborn child in Germany contained a 4.4 megadalton (Mdal) plasmid (pVe445) and produced a TEM type β -lactamase. The transformation to ampicillin resistance of a sensitive *Escherichia coli* strain with isolated pVe445 DNA proved that the structural gene for the β -lactamase resided on this plasmid genome. Molecular DNA–DNA hybridization studies and electron microscope DNA heteroduplex analysis indicated that pVe445 probably contained 38 to 41 % of the ampicillin translocation DNA segment (TnA) found on R factors of enteric origin. The TnA fragment present in pVe445 most likely does not contain both of the inverted repeat sequences of TnA. DNA–DNA polynucleotide sequence studies indicated that the 4.4 Mdal plasmid pVe445 was unrelated to the 30 to 38 Mdal *H. influenzae* R plasmids but was closely related to the 4.1 Mdal ampicillin resistance specifying *H. influenzae* plasmid RSF0885 isolated in the U.S.A. The *H. influenzae* plasmid pVe445 shared 91 % of its base sequences with the β -lactamase specifying *Neisseria gonorrhoeae* plasmid pMR0360 (4.4 Mdal) and had 85 % of its base sequences in common with the β -lactamase specifying *N. gonorrhoeae* plasmid pMR0200 (3.2 Mdal). All of the four 3.2 to 4.4 Mdal β -lactamase specifying R plasmids of *H. influenzae* and *N. gonorrhoeae* investigated probably have a common evolutionary origin.

INTRODUCTION

Recently we reported that ampicillin resistance and tetracycline resistance were plasmid-mediated in clinical isolates of *Haemophilus influenzae* type b from Germany (Laufs & Kaulfers, 1977). These *H. influenzae* R plasmids probably carry the ampicillin resistance transposon TnA or the tetracycline resistance transposon TnD (Kaulfers *et al.*, 1978). The plasmids were of 30 to 33 megadalton (Mdal) and were closely related to each other and to the 30 to 38 Mdal R plasmids found in *H. influenzae* isolates from other countries (Elwell *et al.*, 1977b; Van Klingeren *et al.*, 1977). Besides these 30 to 38 Mdal plasmids, small *H. influenzae* R plasmids which specify ampicillin resistance have been described (De Graaff *et al.*, 1976; Saunders & Sykes, 1977).

In this report we describe the molecular nature of a 4.4 Mdal plasmid of *H. influenzae* type b, which specifies a TEM type β -lactamase and which was isolated in Düsseldorf, West Germany. This small R factor is not related to the 30 to 38 Mdal *H. influenzae* plasmids. The *H. influenzae* strain which carries the plasmid had caused severe meningitis and septicaemia in a newborn child who was initially treated with ampicillin without success. The relationship of the plasmid with another small *H. influenzae* plasmid isolated in

the U.S.A. (De Graaff *et al.*, 1976) and with the ampicillin resistance specifying R factors from *Neisseria gonorrhoeae* isolated in the U.S.A. (Roberts *et al.*, 1977) and the U.K. (Phillips, 1976) is reported.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. Roberts *et al.* (1977) have recently reported that the molecular size of the plasmid RSF0885 is 4.1 Mdal and not 3.0 Mdal as reported earlier (De Graaff *et al.*, 1976) and that it was originally isolated from *Haemophilus parainfluenzae* and not from *H. influenzae* type b.

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 L-histidine ml⁻¹ and 10 µg NAD ml⁻¹. Chocolate agar was used as a solid medium, supplemented with 20 µg ampicillin ml⁻¹ when necessary. For DNA labelling, *H. influenzae* was grown in the minimal growth medium (M-IIg) described by Spencer & Herriott (1965). The media used for the growth of *N. gonorrhoeae* were those described by Roberts *et al.* (1977).

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

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

Assay of β -lactamase activity. R plasmid-specified β -lactamase was characterized by the acidimetric method, with phenol red as indicator (Rubin & Smith, 1973).

Agarose gel electrophoresis of plasmid DNA. The plasmid DNA was identified and characterized according to the method of Meyers *et al.* (1976). The plasmids used as internal molecular weight standards were those described by Laufs & Kaulfers (1977).

Transformation. *Escherichia coli* C600 was transformed with purified pVe445 DNA by a modified method of Cohen *et al.* (1972). After a 42 °C heat pulse for 2 min, the bacteria were chilled, and the transformation mixture was diluted 1:10 in broth and incubated for 90 min at 37 °C to permit phenotypic expression of ampicillin resistance. Bacteria were then plated on solid medium containing ampicillin (20 µg ml⁻¹).

DNA-DNA duplex studies. Before hybridization, the labelled plasmid DNA and the unlabelled whole-cell DNA were sheared by sonication and then dialysed against 0.42 M-NaCl. Approximately 0.01 µg ³H-labelled, sheared, denatured plasmid DNA (3000 to 4000 c.p.m.) was incubated with 150 µg unlabelled, sheared, denatured whole-cell DNA and allowed to reassociate in 0.21 M-NaCl at 70 °C for 120 min (total vol. 1.5 ml). Renaturation was stopped by placing the vial in an ice bath. The S1 endonuclease reaction was carried out as described by Crosa *et al.* (1973).

DNA contour length. Spreading and staining of DNA were performed essentially as described by Kleinschmidt (1968) using parlodian-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 Mdal.

Electron microscope DNA heteroduplex analysis. To detect the ampicillin resistance transposon TnA, or parts of it, in pVe445, electron microscope DNA heteroduplex analysis of base sequence homologies between pVe445 and pUWH (RSF1010::TnA) was done as described by Davis *et al.* (1971). The pUWH DNA was cut with the restriction endonuclease *EcoRI*, and the pVe445 DNA was converted to open circles with X-rays (200 kV), using a dose of 800 rad and the protection of histidine (10 µl 0.1 M-histidine to 0.4 ml DNA). The denaturation, reannealing and spreading of DNA was done as described by Kaulfers *et al.* (1978). Under the conditions used, the shrinkage of the single-stranded DNA as compared with double-stranded DNA is about 27 % (Kaulfers *et al.*, 1978).

RESULTS

Demonstration of a 4.4 Mdal plasmid in H. influenzae strain Ve445

The ampicillin-resistant *H. influenzae* strain Ve445 was examined, together with two *E. coli* HB101 strains containing the *N. gonorrhoeae* plasmids pMR0360 and pMR0200 (Roberts *et al.*, 1977) and *H. influenzae* strain G32 with plasmid RSF0885 (De Graaff *et al.*, 1976), for the presence of extrachromosomal DNA by agarose gel electrophoresis. The cleared lysates of these four ampicillin-resistant strains each gave a single plasmid DNA

Table 1. *Bacterial strains and their plasmids*

Strain	Plasmid	10 ⁻⁶ × Mol. wt	Resistance pattern*	Source/reference
<i>Haemophilus influenzae</i> Ve445	pVe445	4.4	Ap	This study
<i>H. influenzae</i> FR16017	pFR16017	33	Tc	Kaulfers <i>et al.</i> (1978)
<i>H. influenzae</i> G32	RSF0885	4.1	Ap	De Graaff <i>et al.</i> (1976)
<i>H. influenzae</i> KRE5367	pKRE5367	30	Ap	Laufs <i>et al.</i> (1977)
<i>Neisseria gonorrhoeae</i>	pMR0200	3.2	Ap	Phillips (1976)
<i>N. gonorrhoeae</i>	pMR0360	4.4	Ap	Roberts <i>et al.</i> (1977)
<i>Escherichia coli</i> C600	pUWH (RSF1010::TnA)	8.7	Ap	Kaulfers <i>et al.</i> (1978)
<i>E. coli</i> C600	RSF1010	5.5	Su, Sm	Guerrey <i>et al.</i> (1974)
<i>E. coli</i> C600	RSF1030	5.5	Ap	Heffron <i>et al.</i> (1975)
<i>E. coli</i> HB101	pMR0360	4.4	Ap	Roberts <i>et al.</i> (1977)
<i>E. coli</i> HB101	pMR0200	3.2	Ap	Roberts <i>et al.</i> (1977)

* Ap, Ampicillin; Tc, tetracycline; Su, sulphonamide; Sm, streptomycin.

Fig. 1. Agarose gel electrophoresis of ethanol-precipitated DNA from bacterial lysates. (A) Standard plasmid DNAs ranging from 62 to 4.2 Mdal; chr indicates the band position of chromosomal DNA. (B) Lysate from *H. influenzae* strain Ve445 Ap^r; 4.4 Mdal plasmid present. (C) Lysate from *E. coli* strain HB101 containing the *N. gonorrhoeae* plasmid pMR0360 Ap^r; 4.4 Mdal plasmid present.

band in the gel. The plasmid present in strain Ve445 was named pVe445. Plasmids pVe445 and pMR0360 showed similar mobility in the gel (Fig. 1). The estimation of plasmid mass from the extent of DNA migration revealed that pVe445 and pMR0360 were about 4 to 5 Mdal and RSF0885 and pMR0200 were about 3 to 4 Mdal.

The cleared lysate of the ³H-labelled *H. influenzae* strain Ve445 centrifuged to equilibrium in a CsCl-ethidium bromide gradient showed a dense peak of covalently closed circular (CCC) DNA and a less dense peak containing linear chromosomal DNA and open circular DNA (data not shown). Plasmid DNA was located by visualization of the CCC band under long-wavelength ultraviolet light. The size of the open circular DNA was found by electron microscopy to be 4.4 Mdal (mean of 29 molecules measured).

Production of a TEM type β-lactamase by H. influenzae strain Ve445

Haemophilus influenzae strain Ve445 was resistant to 1000 µg ampicillin ml⁻¹ whereas *H. influenzae* strain G32 was sensitive to 1 µg ml⁻¹. The relative rates of hydrolysis of various

β -lactam antibiotics by extracts of sonically disrupted *H. influenzae* strain Ve445 were measured and compared with those of the ampicillin-resistant *H. influenzae* reference strain KRE5367 and of the ampicillin-sensitive strain G32 (specific activity expressed as units per mg bacterial protein.) The β -lactamase produced by strain Ve445 had a similar substrate profile to that of strain KRE5367 (Laufs & Kaulfers, 1977). The high activities against penicillin G (336 units) and ampicillin (380 units) and the low activities against cephalothin (57 units) and oxacillin (37 units) indicated the presence of a TEM type β -lactamase (Heffron *et al.*, 1975).

*Transformation using purified plasmid DNA isolated from
H. influenzae strain Ve445*

Preparations of purified CCC pVe445 plasmid DNA were used for transformation. Exposure of the sensitive *E. coli* strain C600 to 5 μ g purified plasmid DNA resulted in the appearance of one ampicillin-resistant transformant per 2×10^8 bacteria. The transformants synthesized a β -lactamase, and the cleared lysates obtained from them showed the same DNA band in an agarose gel as did the cleared lysate of *H. influenzae* strain Ve445. The plasmid pVe445 was stably maintained in *E. coli* only so long as ampicillin was maintained in the nutrient medium. On plates without ampicillin, pVe445 was lost in *E. coli* after 2 to 5 passages while pVe445 was stably maintained in *H. influenzae* in all 20 passages tested. It was not possible to transfer the resistance marker from Ve445 to *E. coli* by conjugation.

*Demonstration of base sequence homology between pVe445 and the
 β -lactamase specifying plasmid RSF1030 by molecular hybridization*

As a probe for the ampicillin transposon we used the plasmid RSF1030 which is a 5.5 Mdal multicopy plasmid containing the whole of TnA (Tn2) which codes for a TEM type β -lactamase (Heffron *et al.*, 1975). RSF1030 DNA, in all cases studied, only forms DNA-DNA duplexes with plasmids carrying the structural gene for the TEM β -lactamase (Heffron *et al.*, 1975; Laufs & Fock, 1979). The ^3H -labelled RSF1030 DNA showed 24% homology with unlabelled pVe445 DNA using the single-strand specific nuclease S1 for analysis of the plasmid DNA-DNA duplexes, and the reciprocal experiment gave similar results. The degree of base homology between ^3H -labelled RSF1030 plasmid DNA and the unlabelled whole-cell DNA of the two ampicillin-resistant *N. gonorrhoeae* strains harbouring plasmids pMR0360 and pMR0200 was similar to that with pVe445 (Table 2).

*Electron microscope DNA heteroduplex analysis of base sequence homologies
between pVe445 and RSF1010::TnA.*

The finding that the 5.5 Mdal plasmid RSF1030 which contains the 3.2 Mdal TnA sequence (Heffron *et al.*, 1975) had only 24% of its base sequences in common with the 4.4 Mdal plasmid pVe445 indicated that pVe445 contains only a part of the 3.2 Mdal TnA sequence and that the homology is probably restricted to those DNA sequences in TnA which code for the TEM β -lactamase. It can be calculated from the degree of duplex formation (24%) that pVe445 contains about 41% of the TnA sequence. To examine further the possibility that only a portion of TnA is present in the *H. influenzae* plasmid pVe445, the DNA of this plasmid was hybridized with plasmid DNA of pUWH (RSF1010::TnA). This 8.7 Mdal plasmid was obtained by the transposition of TnA from the *H. influenzae* plasmid KRE5367 on to the 5.5 Mdal plasmid RSF1010 (Kaulfers *et al.*, 1978). Figures 2 and 3 show heteroduplex molecules between pUWH (RSF1010::TnA) and RSF1010. Prior to hybridization, these molecules were cut with *EcoRI*. The TnA region is identified by the inverted repeat base sequences on both ends that annealed to a short double-stranded stalk from which a single-stranded loop originates, which most likely represents TnA. The insertion of TnA in RSF1010 results in a short and a long arm of DNA flanking the TnA insertion as is seen in the heteroduplex molecules (Figs 2 and 3).

Table 2. Hybridization between ³H-labelled pVe445, pMR0360 and RSF1030 plasmid DNA and unlabelled whole-cell DNA

The degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method (Crosa *et al.*, 1973). Normalized values (recorded as percentage homology) were obtained from raw data by subtracting the chromosomal control (7 to 8 %) and dividing by the homologous reaction (70 to 75 %).

Source of unlabelled DNA	Relative DNA sequence homology with ³ H-labelled plasmid DNA (%)		
	pVe445	pMR0360	RSF1030
<i>H. influenzae</i> (pVe445)	100	93	24
<i>H. influenzae</i> (RSF0885)	92	85	21
<i>H. influenzae</i> (Pen ^s)	1	1	2
<i>N. gonorrhoeae</i> (pMR0360)	91	100	17
<i>N. gonorrhoeae</i> (pMR0200)	85	73	20
<i>N. gonorrhoeae</i> (Pen ^s)	0	1	1
<i>E. coli</i> (RSF1030)	28	Not done	100

Using the single-strand specific nuclease S1 for the analysis of DNA-DNA duplexes we found that the plasmid RSF1010 without TnA did not show common base sequences with pVe445. A heteroduplex molecule between DNA strands of *Eco*RI-cut pUWH (RSF1010::TnA) and circular pVe445 is shown in Fig. 4. It is likely that the double-stranded region corresponds to the TnA sequence, since it is orientated to the short arm of pUWH, as was the TnA loop in the heteroduplex molecules between RSF1010 and pUWH (Figs 2 and 3). The average molecular size of the double-stranded DNA region was 1.2 Mdal in 12 heteroduplex molecules measured, representing about 38 % of TnA. When single-stranded pVe445 DNA was self-annealed, double-stranded stalks were never observed, indicating that the inverted repeats of TnA are not both present in pVe445.

Relationship between the H. influenzae plasmid pVe445 and R plasmids from N. gonorrhoeae and H. influenzae

The reported relationship between a small *H. influenzae* plasmid and R factors from *N. gonorrhoeae* (Roberts *et al.*, 1977) prompted us to question whether the *H. influenzae* plasmid isolated in Germany is related to the ampicillin resistance specifying *N. gonorrhoeae* plasmids pMR0200, isolated in Liverpool (Phillips, 1976), and pMR0360, isolated in the U.S.A. (Roberts *et al.*, 1977). Using the single-strand specific endonuclease S1 for the analysis of DNA-DNA duplexes, a 91 % base homology was demonstrated between the *H. influenzae* plasmid pVe445 and the *N. gonorrhoeae* plasmid pMR0360 and a 85 % base homology between pVe445 and the *N. gonorrhoeae* plasmid pMR0200. These results were confirmed by using ³H-labelled *N. gonorrhoeae* plasmid pMR0360 in the reciprocal experiment (Table 2).

The 4.4 Mdal *H. influenzae* plasmid pVe445 did not share common base sequences with the 33 Mdal *H. influenzae* plasmid pFR16017 which is closely related to all of the other 30 to 38 Mdal R plasmids isolated from *H. influenzae* in different countries (Laufs & Kaulfers, 1977). However, pVe445 is closely related (92 % base homology, Table 2) to the 4.1 Mdal *H. influenzae* R plasmid RSF0885 isolated in the U.S.A. (De Graaff *et al.*, 1976).

Figs 2 and 3. Heteroduplex of linear (*EcoRI*-cut) RSF1010 and linear (*EcoRI*-cut) pUWH (RSF1010::TnA) plasmid DNA: ss, single-stranded DNA; ds, double-stranded DNA; Ap, probable ampicillin resistance transposon TnA; IR, inverted repeat.

Fig. 4. Heteroduplex of single-stranded (ss), circular pVe445 and single-stranded, linear (*EcoRI*-cut) pUWH plasmid DNA. The double-stranded (ds) region has a molecular size of 1.2 Mdal and probably represents 38 to 41 % of TnA.

DISCUSSION

The ampicillin resistance of *H. influenzae* strain Ve445 was mediated by a β -lactamase of the TEM type determined by the 4.4 Mdal plasmid pVe445. The transformation of a sensitive *E. coli* strain to ampicillin resistance with isolated plasmid DNA demonstrated that the structural gene for the β -lactamase resided on the plasmid genome. DNA-DNA hybridization analysis, using the R plasmid RSF1030 which contains the whole TnA (Heffron *et al.*, 1975) as a probe, indicated that pVe445 probably contains 41% of the sequence of DNA characteristic of the ampicillin resistance transposon TnA (Hedges & Jacob, 1974; Heffron *et al.*, 1975; Bennett & Richmond, 1976) which is often found in Enterobacteriaceae. This finding was supported by electron microscope DNA heteroduplex analysis of base sequence homologies between pVe445 and pUWH (RSF1010::TnA) (Kaulfers *et al.*, 1978). It is likely that the 1.2 Mdal double-stranded region demonstrated corresponds to the TnA sequence which codes for the β -lactamase. The DNA sequence of TnA present in pVe445 probably does not contain both of the inverted repeat base sequences of TnA, as shown by self-annealing experiments, and is probably located in the right third of the TnA map proposed by Heffron *et al.* (1977).

There are obviously at least two groups of R factors in *H. influenzae*: the 30 to 38 Mdal R plasmids (Elwell *et al.*, 1977*b*; Van Klinger *et al.*, 1977; Laufs & Kaulfers, 1977) and the 2.5 to 4.4 Mdal R plasmids (De Graaff *et al.*, 1976; Saunders & Sykes, 1977). The large 30 to 38 Mdal R factors which specify ampicillin, tetracycline or chloramphenicol resistance are all closely related to each other. Irrespective of their geographical origin they have 60% or more of their base sequences in common (Elwell *et al.*, 1977*b*; Laufs & Kaulfers, 1977). This paper indicates that at least two of the small 2.5 to 4.4 Mdal *H. influenzae* R plasmids which all specify ampicillin resistance are as closely related to each other as are the large *H. influenzae* R plasmids. The 4.4 Mdal *H. influenzae* plasmid pVe445 which was isolated in Germany shares 92% of its base sequences with the 4.1 Mdal *H. influenzae* plasmid RSF0885 isolated in the U.S.A. (De Graaff *et al.*, 1976). DNA-DNA hybridization studies indicated that pVe445 is not related to the 33 Mdal *H. influenzae* plasmid pFR16017 (Kaulfers *et al.*, 1978) and this finding is consistent with the report of De Graaff *et al.* (1976) that one of the other small *H. influenzae* plasmids, RSF0885, is not related to the 30 Mdal *H. influenzae* plasmid RSF007. The fact that only a limited variety of R plasmids are found in clinical isolates of *H. influenzae* may be due to the restriction and modification systems present in *H. influenzae*.

It seems of great epidemiological interest that the *H. influenzae* plasmid pVe445 isolated in Germany is closely related to the R plasmid found in β -lactamase-producing *N. gonorrhoeae* strains isolated in the U.S.A. (Roberts *et al.*, 1977) and the United Kingdom (Phillips, 1976). The base sequence homology between pVe445 and the *N. gonorrhoeae* R factor pMR0360 was 91% and with pMR0200 it was 85%. This close relationship indicates a common evolutionary origin of the small R plasmid of *H. influenzae* and those of *N. gonorrhoeae*. A similar relationship between the *H. influenzae* R plasmid RSF0885 and the R plasmids of *N. gonorrhoeae* was recently reported (Roberts *et al.*, 1977). The pVe445 plasmid is not conjugative and *H. influenzae* strain Ve445 did not contain an additional plasmid which might have mobilized pVe445. If one speculates that pVe445 was fed in by another species, e.g. *N. gonorrhoeae* or a member of the Enterobacteriaceae, this could have happened with the help of a self-transmissible plasmid which then was not stably maintained in *H. influenzae*. About 50% of the β -lactamase-producing *N. gonorrhoeae* strains harboured a 24.5 Mdal self-transmissible plasmid which could have mobilized the small R plasmids (Elwell *et al.*, 1977*a*). The child infected with *H. influenzae* strain Ve445 had developed purulent meningitis and septicaemia by 2 d after its birth but our clinical data gave no hint of where the *H. influenzae* strain Ve445 or its R plasmid may have come from. The finding that the *H. influenzae* plasmid pVe445 has the same molecular size as the

N. gonorrhoeae plasmid pMR0360 and that both plasmids have almost all of their base sequences in common would, of course, also be compatible with the hypothesis that the *N. gonorrhoeae* strains received their R plasmids from *H. influenzae*. Indeed, the appearance of R plasmids in *H. influenzae* preceded the appearance of R factors in *N. gonorrhoeae*. The finding that the small *H. influenzae* and *N. gonorrhoeae* R plasmids do not have all of their base sequences in common and some differ somewhat in size speaks against the dissemination of a single plasmid clone. The sudden appearance of ampicillin-resistant *H. influenzae* strains with small R plasmids in Germany in patients who had no contact with similar cases abroad may be followed by the appearance of ampicillin-resistant *N. gonorrhoeae* strains. The disquieting appearance of R plasmids in *H. influenzae* and *N. gonorrhoeae* is probably a result of the wide use of ampicillin throughout the world which is followed by the increase of β -lactamase R plasmids.

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