Molecular Characterization of a Plasmid Specifying Ampicillin Resistance and its Relationship to other R Factors from *Haemophilus influenzae*

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The ampicillin-resistant isolate *Haemophilus influenzae* KRE5367 which produced a TEM type β -lactamase contained a 30 megadalton (Mdal) plasmid (pKRE5367) with a copy number of approximately 2 per chromosomal equivalent and a mole fraction guanine plus cytosine content of 0.39. Ampicillin resistance was transferred by cell-to-cell contact, and the transformation to ampicillin resistance of a sensitive *Escherichia coli* strain with isolated pKRE5367 DNA proved that the structural gene for the TEM type β -lactamase resided on the plasmid genome. It was shown by molecular hybridization that pKRE5367 probably contained the ampicillin translocation DNA segment (TnA) found on some R factors of enteric origin. The *H. influenzae* plasmid pKRE5367 had most of its polynucleotide sequences in common with other R factors recently isolated from *H. influenzae*. DNA-DNA hybridization studies indicated that pKRE5367 shared about 70% base sequence homology with the two tetracycline-resistance R plasmids pLU121 (31.5 Mdal) and pFR16017 (33 Mdal) recently isolated in W. Germany. In addition, pKRE5367 had nearly 100% of its polynucleotide sequences in common with the 30 Mdal ampicillin-resistance R plasmid RSF007, isolated in the U.S.A.; it shared 70% base sequence homology with the 31.5 Mdal tetracycline-resistance plasmid pUB701, isolated in the U.K.; and it had 65% of its polynucleotide sequences in common with the 38 Mdal tetracycline- and chloramphenicol-resistance R plasmid pR1234 isolated in the Netherlands. Despite the broad geographical range, the R plasmids of *H. influenzae* appeared to be closely related.

INTRODUCTION

The emergence of ampicillin resistance in *Haemophilus influenzae* is of great clinical concern. *Haemophilus influenzae* had, until recently, been regarded as uniformly susceptible to ampicillin, but resistant strains have been isolated from children infected with *H. influenzae* (Clymo & Harper, 1974; Tomeh *et al.*, 1974), including fatal meningitis (Khan *et al.*, 1974). Such reports have stimulated the search for R factors responsible for the resistance. Farrar & O'Dell (1974) and Saunders & Sykes (1977) have demonstrated that the ampicillin resistance is transferable by conjugation to other bacteria. Furthermore, in this organism, R factors carrying the genes specifying the production of a β -lactamase have been demonstrated, and they contain the same translocatable DNA segment (TnA) which codes for the β -lactamase carried on many enteric R factors (Elwell *et al.*, 1975).

This present study reports the isolation of a 30 megadalton (Mdal) plasmid from an ampicillin-resistant *H. influenzae* strain isolated in Germany. It has been shown by conjugation, by transformation and by DNA-DNA hybridization experiments that the resis-

Table 1. *Bacterial strains used*

Strain	Characteristics	Source/reference
<i>Haemophilus influenzae</i>		
KRE5367	Isolated in W. Germany	H. Finger, Hygiene-Institut Krefeld, W. Germany
LU121	Isolated in W. Germany	D. Fritsche, Hygiene-Institut Ludwigshafen, W. Germany
FR16017	Isolated in W. Germany	K. Pelz, Hygiene-Institut Freiburg, W. Germany
FR325	Isolated in W. Germany	K. Pelz, Hygiene-Institut Freiburg, W. Germany
HR-7	Isolated in the U.S.A.	Elwell <i>et al.</i> (1975)
G32	Isolated in the U.S.A.	Elwell <i>et al.</i> (1975)
UB2811	Isolated in the U.K.	Elwell <i>et al.</i> (1977)
HC234	Isolated in the Netherlands	van Klingeren <i>et al.</i> (1977)
<i>Escherichia coli</i> K12		
C600	<i>lac</i> , <i>thr</i> , <i>leu</i> , <i>thy</i>	Heffron <i>et al.</i> (1975)
1485-1	Nal ^R , <i>thy</i> , F ⁻	Heffron <i>et al.</i> (1975)

Table 2. *Plasmids used*

Plasmid	Origin	Resistance pattern*	10 ⁻⁶ × Mol. wt	Source/reference
pKRE5367	<i>H. influenzae</i> KRE5367	Ap	30	This laboratory
RSF007	<i>H. influenzae</i> HR-7	Ap	30	Elwell <i>et al.</i> (1975)
pRI234	<i>H. influenzae</i> HC234	Tc, Cm	38	van Klingeren <i>et al.</i> (1977)
pLU121	<i>H. influenzae</i> LU121	Tc	31.5	This laboratory
pFR16017	<i>H. influenzae</i> FR16017	Tc	33	This laboratory
pUB701	<i>H. influenzae</i> UB2811	Tc	31.5	Elwell <i>et al.</i> (1977)
pMB8::Ap	<i>E. coli</i>	Ap	5	Heffron <i>et al.</i> (1975)
R1drd19	<i>Salmonella paratyphi</i>	Ap, Sm, Cm, Km, Su	62	Meyers <i>et al.</i> (1976)
RP4	<i>Pseudomonas aeruginosa</i>	Tc, Ap, Km	36	Barth <i>et al.</i> (1976)
Sa	<i>Shigella</i> sp.	Cm, Km, Sm, Su	25	Meyers <i>et al.</i> (1976)
RSF1010	By transformation from <i>Salmonella panama</i>	Su, Sm	5.5	Meyers <i>et al.</i> (1976)
ColE1	<i>E. coli</i>	—	4.2	Falkow (1975)

* Ap, Ampicillin; Tc, tetracycline; Sm, streptomycin; Cm, chloramphenicol; Km, kanamycin; Su, sulphonamide.

tance gene is located on this plasmid. The gene product was characterized as a β -lactamase of the TEM type (Heffron *et al.*, 1975). The relation between this plasmid isolated in Germany and other *H. influenzae* R factors was investigated.

METHODS

Bacterial strains. The bacterial strains and plasmids used are listed in Tables 1 and 2.

Media. The liquid medium for growth of *H. influenzae* was 3.5% (w/v) brain heart infusion (BHI, Oxoid) supplemented with 10 μ g haemin ml⁻¹, 10 μ g L-histidine ml⁻¹ and 10 μ g NAD ml⁻¹. Chocolate agar was used as the solid medium supplemented, when required, with ampicillin (10 μ g ml⁻¹) or tetracycline (10 μ g 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 unlabelled plasmid DNA. Bacteria were grown overnight to about 10⁹ cells ml⁻¹ in liquid medium, and then harvested. Plasmid DNA was isolated by a modification of the cleared lysate method described by Clewell & Helinski (1969) in which Triton X-100 [0.1% (v/v) final concentration] was used in place of Brij 58. The lysate was mixed with caesium chloride (0.84 g per ml lysate) and ethidium bromide [0.06 ml per ml lysate of a 10% (w/v) ethidium bromide solution in TES buffer (0.05 M-NaCl/0.005 M-EDTA/0.03 M-Tris, pH 8.0)]. The final density was adjusted to 1.7936 g cm⁻³ (refractive index 1.4085). The mixture was centrifuged for 40 to 48 h at 15 °C and 40000 rev. min⁻¹ in a Beckman fixed-angle type 50 rotor. DNA bands in the gradients were located by using a black-light lamp (UVL-22; Ultraviolet Products, San Gabriel, California, U.S.A.). Plasmid and chromosomal DNA bands were collected from the

bottom of the tube. The plasmid DNA was rebanded in a second CsCl-ethidium bromide gradient. Ethidium bromide was extracted from the dense satellite bands, which contained covalently closed circular DNA molecules, with caesium chloride-saturated 2-propanol. The plasmid DNA was dialysed against at least 200 volumes of TES buffer.

Preparation of labelled plasmid DNA. *Haemophilus influenzae* DNA was labelled by growing cells in M-IIg medium containing $6 \mu\text{Ci}$ [methyl- ^3H]thymidine (specific activity $2000 \text{ mCi mmol}^{-1}$; Amersham Buchler). Plasmid DNA was purified by the method of So, Crosa & Falkow (1975). DNA from *E. coli* was labelled as described by Heffron *et al.* (1975).

Conjugation. Exponential-phase cultures of the donor strain KRE5367 were mixed 1:10 with the recipient *E. coli* strain 1485-1 or *H. influenzae* strain FR325 and incubated overnight. Pancreatic DNAase I ($20 \mu\text{g ml}^{-1}$) was included in some mixtures.

Transformation. For transformation (Cohen, Chang & Hsu, 1972), the purified plasmid DNA dissolved in TES buffer was incubated at 40°C for 20 min with pronase ($5 \mu\text{g ml}^{-1}$) followed by a 42°C heat pulse. The mixture was then diluted 1:5 in BHI and incubated for 2 h at 37°C to allow gene expression. Cells were plated on chocolate agar supplemented with ampicillin ($10 \mu\text{g ml}^{-1}$).

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

Plasmid 'curing'. Organisms were passaged in liquid medium at 39°C over 12 days. Dilutions of the sixth passage were plated on drug-free chocolate agar. After incubation for 18 h at 37°C , suitable plates were replicated on to similar plates containing ampicillin ($10 \mu\text{g ml}^{-1}$).

Determination of minimum plasmid copy size. Portions (2 to 5 ml) of exponentially growing cultures of *H. influenzae* were labelled with [^3H]thymidine. Lysates were prepared by the method of Young & Sinsheimer (1967), except that 1.2 % sarkosyl was used instead of sodium lauryl sulphate; the DNA was examined as described by De Graaff, Elwell & Falkow (1976). The copy number was calculated, assuming the molecular weight of the chromosome (1.66×10^9 ; De Graaff *et al.*, 1976) and of the plasmid (30.6×10^6).

Determination of G+C content of DNA. The guanine plus cytosine (G+C) content of DNA was determined by the method of Mandel, Schildkraut & Marmur (1968). DNA was centrifuged to equilibrium in a CsCl density gradient in a Beckman type 50 rotor for 48 h, and the gradients were collected on filters (Whatman). The DNA was precipitated with trichloroacetic acid and counted in a Packard liquid scintillation counter. The reference standards were RSF007 plasmid DNA (mol fraction G+C, 0.39; De Graaff *et al.*, 1976) and DNA from *E. coli* (mol fraction G+C, 0.50).

DNA contour length. Spreading and staining of DNA were performed essentially by the method of Kleinschmidt (1968) using parlodian-coated electron microscope grids; the molecular weights of the plasmids were calculated assuming $1 \mu\text{m}$ DNA to be equivalent to 2.07 Mdal.

DNA-DNA duplex studies. Whole-cell DNA from *H. influenzae* and *E. coli* strains was extracted as described by So *et al.* (1975). 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 0.42 M-NaCl. Hybridizations were carried out as described by Crosa, Brenner & Falkow (1973). Approximately $0.001 \mu\text{g}$ ^3H -labelled, sheared plasmid DNA (specific activity $1.5 \times 10^6 \text{ c.p.m. } \mu\text{g}^{-1}$) was mixed in a glass vial with $150 \mu\text{g}$ 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 9 h when ^3H -labelled plasmid DNA from *H. influenzae* was used and for 4 h when ^3H -labelled pMB8::Ap DNA was used. Renaturation was stopped by placing the vial in an ice bath. The degree of duplex formation for the homologous reaction, by the S1 endonuclease assay (Crosa *et al.*, 1973), was 70 to 75 %, whereas that for the chromosomal control was 7 to 8 %. 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 %).

Agarose gel electrophoresis of plasmid DNA. The plasmid DNA was identified and characterized as described by Meyers *et al.* (1976). Plasmid-containing strains were grown overnight in 30 ml liquid medium and harvested by centrifugation. The cells were lysed and cleared lysates were digested with ribonuclease (Serva, W. Germany; 1 mg ml^{-1} in 50 mM-sodium acetate pH 5.0, heated for 10 min at 90°C). The DNA was extracted with phenol and precipitated with 95 % (v/v) ethanol overnight at -20°C . Ethanol-precipitated DNA was resuspended in $100 \mu\text{l}$ TES buffer, and 10 to $30 \mu\text{l}$ portions were electrophoresed in 0.7 % agarose dissolved in Tris/borate buffer (89 mM-Tris base/2.5 mM- Na_2EDTA /8.9 mM boric acid) in a vertical lucite gel apparatus. The gel was stained in a solution of ethidium bromide in water ($0.4 \mu\text{g ml}^{-1}$) for 10 min. The fluorescing DNA bands were photographed with a Polaroid camera under long-wavelength ultraviolet light on a transilluminator type C51 (Ultraviolet Products). The following plasmids were used as molecular weight markers: R1drd19 (62 Mdal, Meyers *et al.*, 1976), RP4 (36 Mdal, Barth *et al.*, 1976), Sa (25 Mdal, Meyers *et al.*, 1976), RSF1010 (5.5 Mdal, Meyers *et al.*, 1976) and ColE1 (4.2 Mdal, Falkow, 1975).

Fig. 1

Fig. 2

Fig. 1. Agarose gel electrophoresis of ethanol-precipitated DNA from bacterial lysates (A, F) Standard plasmid DNAs ranging from 62 to 4.2 Mdal; chr indicates the band position of chromosomal DNA. (B, C, D, E) Lysates from *H. influenzae* strains: (B) strain HC234 Tc^r and Cm^r, 38 Mdal plasmid present; (C) strain HR-7 Ap^r, 30 ± 1 Mdal plasmid present; (D) strain KRE5367 Ap^r, 30 ± 1 Mdal plasmid present; (E) strain UB2811 Tc^r, 31.5 Mdal plasmid present.

Fig. 2. Ethidium bromide-CsCl gradient of a cleared lysate of *H. influenzae* strain KRE5367 grown in medium M-IIg in the presence of [³H]thymidine (0.006 mCi ml⁻¹). Cleared lysates were prepared by the method of Clewell & Helinski (1969). CsCl and ethidium bromide were added, and the final density was adjusted to 1.7936 g cm⁻³ (refractive index 1.4085). The solution was centrifuged in a Beckman type 50 rotor for 48 h at 15 °C and 40000 rev. min⁻¹. The gradient was collected, and samples were spotted on Whatman filters and, after trichloroacetic acid precipitation and drying, counted in a Packard liquid scintillation counter.

RESULTS

Demonstration of a plasmid in the ampicillin-resistant *H. influenzae* strain KRE5367. The ampicillin-resistant *H. influenzae* strain KRE5367 was examined, together with strains HR-7, UB2811 and HC234, for the presence of extrachromosomal DNA by agarose gel electrophoresis. The cleared lysates of each resistant strain contained a single species of covalently closed circular DNA. The molecular weights of the plasmids of the ampicillin-resistant *Haemophilus* strains KRE5367 and HR-7 were 30 × 10⁶ to 40 × 10⁶ (Fig. 1).

A cleared lysate of [³H]thymidine-labelled *H. influenzae* strain KRE5367 centrifuged to equilibrium in a CsCl-ethidium bromide gradient showed a dense peak composed of covalently closed circular DNA and a less dense peak containing linear chromosomal and open circular DNA (Fig. 2). DNA was isolated from *H. influenzae* strain KRE5367 by direct vision of the covalently closed circular band in an ethidium bromide-CsCl gradient under long-wavelength ultraviolet light (data not shown). The size of the open circular DNA was found by electron microscopy to be 30.6 Mdal (mean of 21 molecules).

Production of β-lactamase by *H. influenzae* strain KRE5367. The ampicillin-resistant *H. influenzae* strain KRE5367, like strain H-7 and *E. coli* c600T (a transformant derived from the exposure of *E. coli* c600 to the plasmid DNA isolated from strain KRE5367), was

Table 3. *Relative rates of hydrolysis of various β -lactam antibiotics by extracts of sonically disrupted H. influenzae KRE5367, HR-7 and G32*

Strain	Substrate			
	Penicillin G*	Ampicillin	Cephalothin	Oxacillin
KRE5367	100 (50.69)	97	7.2	3.6
HR-7	100 (46.10)	95.9	12.3	6.8
G32	0	0	Not done	Not done

* Penicillin G = 100; numbers in parentheses indicate rates of hydrolysis relative to benzyl penicillin (specific activity expressed as units per mg bacterial protein).

resistant to 1000 μ g ampicillin ml⁻¹, whereas strain G32 was sensitive to 1 μ g ml⁻¹. This suggested that the resistance was due to a β -lactamase. The relative rates of hydrolysis of various β -lactam antibiotics by extracts of sonically disrupted *H. influenzae* strain KRE5367 were therefore measured and compared with those of the ampicillin-resistant reference strain HR-7 and of the ampicillin-sensitive strain G32. The β -lactamase produced by strain KRE5367 had a similar substrate profile to that of strain HR-7 (Elwell *et al.*, 1975) (Table 3). The high activity against ampicillin and penicillin G and the low activity against oxacillin are characteristic of the TEM type β -lactamase (Heffron *et al.*, 1975).

Plasmid 'curing'. A derivative of *H. influenzae* strain KRE5367 cured of ampicillin resistance by growth at 39 °C contained no extrachromosomal DNA detectable by agarose gel electrophoresis. The simultaneous loss of plasmid DNA and ampicillin resistance suggested that the β -lactamase gene was plasmid-determined.

Transfer of the resistance marker of KRE5367 to another H. influenzae strain and to E. coli by conjugation. Ampicillin resistance was transferred to *H. influenzae* strain FR325 with a frequency of 2×10^{-4} per donor cell and to *E. coli* 1485-1 with a frequency of 3×10^{-1} . The frequency of transfer was not reduced by the addition of DNAase I.

Transformation using plasmid DNA isolated from H. influenzae strain KRE5367. Preparations of pronase-treated, purified covalently closed circular DNA from *H. influenzae* KRE5367 were used for transformation. Exposure of the sensitive *E. coli* strain C600 to purified plasmid DNA resulted in the appearance of a significant number of ampicillin-resistant clones (C600r) which carried a plasmid of similar size to that carried by the parental strain KRE5367 (Fig. 3). The transformation frequency obtained in these experiments using purified pKRE5367 DNA was 17.3 ampicillin-resistant transformants per 10^8 cells. The transformed strains also synthesized a β -lactamase.

Molecular properties of pKRE5367. [³H]Thymidine-labelled plasmid DNA from *H. influenzae* KRE5367 (³H-labelled pKRE5367) was analysed in neutral CsCl gradients to determine its mol fraction G+C, using [¹⁴C]thymidine-labelled *E. coli* DNA as an internal marker. The R factor pKRE5367 had a mol fraction G+C of 0.39 (Fig. 4) which is similar to that of the *H. influenzae* chromosome with a mol fraction G+C of 0.38 (De Graaff *et al.*, 1976).

Whole-cell sarkosyl lysates were prepared to determine the number of pKRE5367 copies per chromosome equivalent. Assuming that the molecular weight of the *Haemophilus* chromosome is 1.66×10^9 and that of pKRE5367 is 30.6×10^6 , the minimum copy number of the plasmid in *H. influenzae* KRE5367 was estimated to be about 2 per chromosome equivalent. Thus this size of plasmid showed the anticipated few copies per chromosome (Clowes, 1972).

Relationship between pKRE5367 and plasmids of other resistant H. influenzae. The ampicillin-resistant *H. influenzae* strain KRE5367 contained a plasmid having similar molecular properties to the *H. influenzae* plasmid RSF007 (De Graaff *et al.*, 1976) carried by a β -lactamase-producing *H. influenzae* strain isolated in the U.S.A. The tetracycline-resistant

Fig. 3. Demonstration of transformation of *E. coli* strain c600 with pKRE5367 plasmid DNA by agarose gel electrophoresis. (A) Lysate from *H. influenzae* strain KRE5367 with plasmid DNA (30 ± 1 Mdal). (B) Purified pKRE5367 plasmid DNA. (C) Standard DNAs: 62, 36, 25, 5.5 and 4.2 Mdal. (D) Lysate from *E. coli* strain c600 after transformation (c600r) with the purified pKRE5367 DNA by the method of Cohen *et al.* (1972). 30 ± 1 Mdal plasmid present. (E) Lysate from *E. coli* strain c600 prior to transformation. No plasmid present.

Fig. 4. Determination of G+C content of *H. influenzae* strain KRE5367 plasmid DNA in CsCl gradients. ^{14}C -labelled chromosomal DNA of *E. coli* (mol fraction G+C, 0.50), used as an internal marker, was mixed with (a) ^3H -labelled pKRE5367 DNA or (b) ^3H -labelled RSF007 DNA (mol fraction G+C, 0.39; De Graaff *et al.*, 1976), which was used as a reference. The G+C content of pKRE5367 DNA was determined by the method of Mandel *et al.* (1968) as described in Methods.

Table 4. Hybridization between ^3H -labelled *H. influenzae* plasmid DNA and whole-cell DNA

The degree of DNA-DNA duplex formation was assayed by the S_1 endonuclease method as described in Methods. Each value is the average of three assays.

Source of unlabelled <i>H. influenzae</i> DNA	% relative DNA sequence homology with ^3H -labelled plasmid DNA		
	pKRE5367	RSF007	pLU121
KRE5367(pKRE5367)	100	99	61
HR-7(RSF007)	103	100	68
LUI21(pLU121)	69	72	100
FR16017(pFR16017)	71	67	70
UB2811(pUB701)	71	69	Not done
HC234(pRI234)	63	65	Not done

H. influenzae strain UB2811 isolated in the U.K. (Elwell *et al.*, 1977), the tetracycline- and chloramphenicol-resistant *H. influenzae* strain HC234 isolated in the Netherlands (van Klingeren, van Embden & Dessens-Kroon, 1977), and the two tetracycline-resistant *H. influenzae* strains LUI21 and FR16017 recently isolated in W. Germany also harbour single plasmid species with molecular weights similar to pKRE5367, as shown by agarose gel electrophoresis (data not shown). These findings raised the question as to whether the *Haemophilus* plasmids in isolates from different countries shared any polynucleotide base sequences in common other than those determining ampicillin and tetracycline resistance. Therefore bacterial and plasmid DNA homo- and heteroduplexes were analysed using the single-strand specific endonuclease, S_1 , of *Aspergillus oryzae* (Crosa *et al.*, 1973). The [^3H]thymidine-labelled plasmids of *H. influenzae* strains KRE5367(pKRE5367), HR-7(RSF007) and LUI21(pLU121) were hybridized with unlabelled whole-cell DNA from each of the resistant *H. influenzae* strains and appropriate control strains. The plasmid pKRE5367 shared 100% of its polynucleotide sequence with RSF007, and the reciprocal hybridization value of 99% was in very close agreement. The plasmid pKRE5367 was 69 to 71% related to the tetracycline-resistant R plasmid pUB701 (31.5 Mdal) as well as to pLU121 (31.5 Mdal), and pFR16017 (33 Mdal). The base sequence relationship between pKRE5367 and pRI234 was 63%. The reciprocal hybridizations of the ^3H -labelled RSF007 plasmid DNA and the ^3H -labelled pLU121 plasmid DNA with unlabelled KRE5367 DNA gave similar values (Table 4).

Demonstration of the ampicillin transposon TnA in pKRE5367 by molecular hybridization. As a probe for the ampicillin transposon we used the plasmid pMB8::Ap which is a 5.5 Mdal plasmid containing the whole of TnA (Kleckner *et al.*, 1975). Hybridization of [^3H]thymine-labelled pMB8::Ap with *H. influenzae* strain KRE5367 showed a significant homology of 26%, but less than 1% with the *H. influenzae* control strain G32.

DISCUSSION

The sudden emergence in different parts of the world of strains of *H. influenzae* resistant to high levels of ampicillin and which carry transmissible R factors may be of great clinical significance, because, for over a decade, ampicillin has been an important therapy for *H. influenzae* infections. The finding that the R plasmid in the German isolate probably contained the same translocatable DNA segment (TnA) that is found on many enteric R factors and that most of its polynucleotide sequences are closely related to other *H. influenzae* R plasmids isolated in W. Germany and other countries is of considerable interest.

The ampicillin resistance of our *H. influenzae* isolate KRE5367 was mediated by a β -lactamase of the TEM type determined by a 30 Mdal plasmid species (pKRE5367). Thus

a derivative of KRE5367 cured of ampicillin resistance also lost its extrachromosomal DNA. Two copies, at least, of the plasmid pKRE5367 were present per chromosome and pKRE5367 had a mol fraction G+C content of 0.39 which is similar to that of the chromosomal DNA of *H. influenzae* (De Graaff *et al.*, 1976). A 3.0 Mdal *H. influenzae* plasmid (RSFo885) which was previously found to specify ampicillin resistance had a mol fraction G+C content of 0.40 which is close to that of pKRE5367 (De Graaff *et al.*, 1976). Since RSFo885 is unrelated to RSFoo7 (Elwell *et al.*, 1975) it can be concluded that it is also unrelated to pKRE5367 which is almost identical with RSFoo7. Ampicillin resistance could be transferred by cell-to-cell contact, and 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. It was shown by DNA-DNA hybridization analysis that pKRE5367 probably contains the sequence of DNA characteristic of the ampicillin-resistance transposon TnA (Heffron *et al.*, 1975) which is often found in Enterobacteriaceae.

It seems of considerable epidemiological interest that pKRE5367 shared about 69 to 71 % of its polynucleotide sequences with two *H. influenzae* plasmids which were isolated at the same time as pKRE5367 in W. Germany and which specify tetracycline resistance (Kaulfers & Laufs, unpublished results). Furthermore, DNA-DNA hybridization studies revealed that pKRE5367 is indistinguishable from the *H. influenzae* R plasmid RSFoo7 which was isolated in the U.S.A. and has similar molecular properties (De Graaff *et al.*, 1976). In addition, not only does pKRE5367 share 71 % of its base sequences with a plasmid specifying tetracycline resistance in *H. influenzae* (pUB701), isolated in the U.K. (Elwell *et al.*, 1977), but it also shares 63 % of its base sequences with a 38 Mdal conjugative *H. influenzae* R plasmid (pR1234) isolated in the Netherlands (van Klingerén *et al.*, 1977), which specifies resistance to tetracycline and chloramphenicol. It was recently shown that the *H. influenzae* R plasmids RSFoo7, pUB701 and pR1234 are closely related to each other (Elwell *et al.*, 1977), as probably are the *H. influenzae* R plasmids pKRE5367, pLU121 and pFR16017. All the *H. influenzae* R factors with molecular weights between 30×10^6 and 38×10^6 , recently isolated in different parts of the world, which were analysed in this study for base homology had 61 % or more of their polynucleotide sequences in common.

The question of whether these R factors have arisen independently from a common ancestral source or whether a world-wide distributed indigenous Haemophilus plasmid became the recipient of ampicillin-, tetracycline- or chloramphenicol-resistance genes transposed from anonymous donor R plasmids cannot be decided as yet. We are currently investigating whether a resident *H. influenzae* plasmid with a high degree of base homology with the R factors is demonstrable in antibiotic sensitive *H. influenzae* or *H. parainfluenzae* isolates. In 699 fresh clinical isolates so far examined by agarose gel electrophoresis, we found 12 indigenous plasmids with molecular weights of about 20×10^6 to 30×10^6 . One indigenous plasmid (pW266) with a molecular weight of 27×10^6 showed 82 % base homology with pKRE5367 (Laufs & Kaulfers, unpublished results) and would have been an ideal candidate as an acceptor plasmid for TnA (Ptashne & Cohen, 1975). This indigenous plasmid proved to be very unstable under *in vitro* conditions. However, since the G+C content of pKRE5367 is similar to that of the *H. influenzae* chromosome, the idea that an ubiquitous autochthonous *H. influenzae* plasmid might have acquired the resistance genes from R factors which were not stable in *H. influenzae* seems to be one of the possibilities for the origin of *H. influenzae* R factors. Another possibility is that the R factors evolved once and have spread efficiently with relatively few modifications around the world. In this case *H. influenzae* might also have acquired the R factors *in toto* from other species.

The isolation of *H. influenzae* strains with R factors which specify ampicillin resistance or tetracycline resistance with or without chloramphenicol resistance, suggests that the resistance genes prevalent in the Enterobacteriaceae may have spread to *H. influenzae* which can no longer be considered to be reliably sensitive to these antibiotics. The possi-

bility that a relationship exists between the increase of the R plasmid pool of enteric bacteria (Anderson, 1968) and the appearance of R factors in *H. influenzae* has serious epidemiological aspects in regard to the treatment of acute *H. influenzae* infections.

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REFERENCES

- ANDERSON, E. S. (1968). The ecology of transferable drug resistance in the *Enterobacteriaceae*. *Annual Review of Microbiology* **22**, 132-180.
- BARTH, P. T., DATTA, N., HEDGES, R. W. & GRINTER, N. J. (1976). Transposition of a deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistances from R483 to other replicons. *Journal of Bacteriology* **125**, 800-810.
- CLEWELL, D. B. & HELINSKI, D. R. (1969). Supercoiled circular DNA-protein complexes in *Escherichia coli*: purification and induced conversion to open circular DNA form. *Proceedings of the National Academy of Sciences of the United States of America* **62**, 1159-1166.
- CLOWES, R. C. (1962). Molecular structure of bacterial plasmids. *Bacteriological Reviews* **36**, 261-305.
- CLYMO, A. B. & HARPER, I. A. (1974). Ampicillin-resistant *Haemophilus influenzae* meningitis. *Lancet* **i**, 453-454.
- COHEN, S. N., CHANG, A. C. Y. & HSU, L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 2110-2114.
- CROSA, J. H., BRENNER, J. & FALKOW, S. (1973). Use of a single-strand specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *Journal of Bacteriology* **115**, 904-911.
- DE GRAAFF, J., ELWELL, L. P. & FALKOW, S. (1976). Molecular nature of two beta-lactamase-specifying plasmids isolated from *Haemophilus influenzae* type B. *Journal of Bacteriology* **126**, 439-446.
- ELWELL, L. P., DE GRAAFF, J., SEIBERT, D. & FALKOW, S. (1975). Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type B. *Infection and Immunity* **12**, 404-410.
- ELWELL, L. P., SAUNDERS, J. R., RICHMOND, M. H. & FALKOW, S. (1977). Relationships between some R-plasmids found in *Haemophilus influenzae*. *Journal of Bacteriology* **131**, 356-362.
- FALKOW, S. (1975). In *Infectious Multiple Drug Resistance*, pp. 47-48. Edited by J. R. Lagnado. London: Pion.
- FARRAR, W. E. & O'DELL, N. (1974). β -Lactamase activity in ampicillin-resistant *Haemophilus influenzae*. *Antimicrobial Agents and Chemotherapy* **6**, 625-629.
- HEFFRON, F., SUBLETT, R., HEDGES, R. W., JACOB, A. & FALKOW, S. (1975). Origin of the TEM beta-lactamase gene found on plasmids. *Journal of Bacteriology* **122**, 250-256.
- KHAN, W., ROSS, S., RODRIGUEZ, W., CONTRORI, G. & SAZ, A. K. (1974). *Haemophilus influenzae* type B resistant to ampicillin. A report of two cases. *Journal of the American Medical Association* **229**, 298-301.
- KLECKNER, N., CHAN, R. K., TYE, B. K. & BOSTEIN, D. (1975). Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. *Journal of Molecular Biology* **97**, 561-575.
- KLEINSCHMIDT, A. K. (1968). Monolayer techniques in electron microscopy of nucleic acid molecules. *Methods in Enzymology* **12**, 361-377.
- MANDEL, M., SCHILDKRAUT, C. L. & MARMUR, J. (1968). Use of CsCl density gradient analysis for determining the guanine plus cytosine content of DNA. *Methods in Enzymology* **12B**, 184-195.
- MEYERS, J. A., SANCHEZ, D., ELWELL, L. P. & FALKOW, S. (1976). Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **127**, 1529-1537.
- PTASHNE, K. & COHEN, S. N. (1975). Occurrence of insertion sequence (IS) regions in plasmid deoxyribonucleic acid as direct or inverted nucleotide sequence duplication. *Journal of Bacteriology* **122**, 776-787.
- RUBIN, F. A. & SMITH, D. H. (1973). Characterization of R-factor β -lactamases by the acidimetric method. *Antimicrobial Agents and Chemotherapy* **3**, 68-73.
- SAUNDERS, J. R. & SYKES, R. B. (1977). Transfer of a plasmid-specified beta-lactamase gene from *Haemophilus influenzae*. *Antimicrobial Agents and Chemotherapy* (in the Press).
- SO, M., CROSA, J. H. & FALKOW, S. (1975). Polynucleotide sequence relationships among Ent plasmids and the relationship between Ent and other plasmids. *Journal of Bacteriology* **121**, 234-238.
- SPENCER, H. T. & HERRIOTT, R. (1965). Development of competence of *Haemophilus influenzae*. *Journal of Bacteriology* **90**, 911-920.
- TOMEH, M. O., STARR, S. E., MCGOWAN, J. E., TERRY, P. M. & NAHMIA, A. J. (1974). Ampicillin-resistant *Haemophilus influenzae* type B infection. *Journal of the American Medical Association* **229**, 295-297.

VAN KLINGEREN, B. J., VAN EMBDEN, D. A. & DESSENS-KROON, M. (1977). Plasmid-mediated chloramphenicol resistance in *Haemophilus influenzae*. *Antimicrobial Agents and Chemotherapy* **11**, 383-387.

YOUNG, E. T. & SINSHEIMER, R. L. (1967). Vegetative bacteriophage lambda DNA. II. Physical characterization and replication. *Journal of Molecular Biology* **30**, 165-200.