ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1979, p. 294-299
0066-4804/79/02-0294/06$02.00/0

Vol. 15, No. 2

Plasmid-Mediated Ampicillin Resistance in *Haemophilus ducreyi*

JAMES L. BRUNTON,[1,2] IAN MACLEAN,[1] ALLAN R. RONALD,[1,2] AND WILLIAM L. ALBRITTON[1,3]*

Departments of Medical Microbiology,[1] Medicine,[2] and Pediatrics,[3] University of Manitoba, Winnipeg, Manitoba, Canada R3E 0Z3

Received for publication 21 November 1978

Three of 19 strains of *Haemophilus ducreyi*, isolated during a recent outbreak of chancroid, were found to produce β-lactamase and to harbor a 6.0×10^6-dalton plasmid. *Escherichia coli* tranformed with this plasmid acquired β-lactamase-mediated resistance to ampicillin. The guanine-plus-cytosine content of the plasmid was found to be 41 mol%. Restriction endonuclease digestion studies suggest that a relatively large portion of the Tn*1* translocon is carried by this plasmid. Whereas this plasmid could not be transferred to *H. influenzae* by mating on membrane filters, a strain of *H. ducreyi* was able to receive and donate a 30×10^6-dalton ampicillin resistance plasmid from *H. influenzae*. The ability of *H. ducreyi* to receive and donate conjugative plasmids may result in the appearance of multiply resistant strains.

Hammond et al. (13) reported the antibiotic susceptibility of 19 strains of *Haemophilus ducreyi* isolated during an outbreak of chancroid in Winnipeg, Manitoba, Canada. Three strains were resistant to ampicillin (minimal inhibitory concentration > 128 μg/ml) and produced β-lactamase as determined by hydrolysis of a chromogenic cephalosporin (23). Since plasmid-mediated β-lactamase production had been documented in *Haemophilus influenzae* (10) and *Neisseria gonorrhoeae* (2, 11, 26), we wondered if β-lactamase production might also be plasmid mediated in *H. ducreyi* and whether there might be any relationship between such a plasmid and the previously characterized plasmids of *N. gonorrhoeae* and *H. influenzae*.

We report here the demonstration and preliminary characterization of a 6.0×10^6-dalton nonconjugative plasmid (pJB1) which specifies β-lactamase production in these isolates of *H. ducreyi*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Sixteen clinical isolates of *H. ducreyi* previously reported with minimal inhibitory concentrations of ampicillin of ≤1 μg/ml are not listed (13). *H. ducreyi* strain 35000 Sm^r is a streptomycin-resistant mutant of strain 35000, which is a clinical isolate from the same study. *H. influenzae* strain B031 is a clinical isolate whose plasmid complement and ability to transfer antibiotic resistance have been characterized in this laboratory. All strains of *Escherichia coli* listed, with the exception of $C600r^-m^+$ (pJB1), were the kind gift of Stanley Falkow, University of Washington School of Medicine, Seattle. The plasmids used as molecular weight standards in agarose gel electrophoresis were R1*drd*19, RP4, Sa, RSF1010, and pMB8. They were donated by Dr. Falkow and have been previously described (20).

Media. Strains of *H. ducreyi* were grown on chocolate agar supplemented with 1% IsoVitaleX as previously described (13). β-Lactamase-producing strains were maintained on chocolate agar supplemented with 1 μg of ampicillin per ml. *E. coli* strains were grown in L-broth supplemented with 25 μg of ampicillin per ml for β-lactamase-producing strains. *E. coli* plasmid deoxyribonucleic acid (DNA) was labeled with [*methyl*-^{3}H]thymine as described previously (15).

Transfer experiments. Mating experiments were carried out on membrane filters as described by Thorne and Farrar (30). Transconjugants were selected on chocolate agar supplemented with 2 μg of ampicillin and 1,000 μg of streptomycin per ml. Single colonies of *H. ducreyi* growing on this selective medium were identified by colonial morphology and selected for futher study. Ampicillin-resistant isolates were checked for β-lactamase production, and their plasmid complement was determined by agarose gel electrophoresis.

Agarose gel electrophoresis. The growth of *H. ducreyi* from three plates of chocolate agar was suspended in TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris, pH 8.0) buffer and washed. Clear lysates were prepared and subjected to agarose gel electrophoresis as described by Meyers et al. (20).

Purification of plasmid DNA. Cleared lysates were prepared by a modification of the method of Clewell and Helinski in which Triton X-100 (0.1% final concentration) was substituted for Brij 58 (10). Covalently closed circular DNA was isolated by cesium

TABLE 1. *Bacterial strains and their plasmids*

Strain	Plasmid complement ($\times 10^6$ daltons)	Phenotype	Source
H. ducreyi			
54198 (pJB1)	6.0	Ap^r Tc^r	G. Hammond (13)
54207	6.0	Ap^r Tc^r	G. Hammond (13)
54211	6.0	Ap^r Tc^r	G. Hammond (13)
35000 Sm^r [a]	None	Sm^r	G. Hammond (13)
H. influenzae			
B031	30	Ap^r	W. Albritton (1)
B003 Nal^r [a]	None	Nal^r	W. Albritton (1)
E. coli			
C600r^-m^+	None	r^-m^+ Thr^- Leu^- Thi^-	R. Gill (14)
C600r^-m^+ (pJB1)	6.0	Ap^r	This study by transformation of *E. coli* C600r^-m^+ with plasmid DNA isolated from *H. ducreyi* 54198
C600 (RSF1010::Tn*1*) Ap101 [b]	8.7	Ap^r	R. Gill (14)
C600 (RSF1010::Tn*1*) Ap111 [b]	8.7	Ap^r Su^r	R. Gill (14)
C600 (RSF1010::Tn*3*) Ap230	8.7	Ap^r	R. Gill (14)
C600 (RSF1010)	5.5	Su^r Sm^r	S. Falkow (3)
W1485-1 (RSF1030)	5.5	Ap^r	R. Gill (4, 7)

[a] These strains were made resistant to streptomycin and nalidixic acid, respectively, by serial passage of clinical isolates on chocolate agar containing increasing concentrations of antibiotic.

[b] RSF1010::Tn*1* plasmids Ap101 and Ap111 have the Tn*1* translocon inserted at 8.5 and 92.5%, respectively, of the distance from the left-hand end generated by *Eco*RI cleavage of RSF1010 (14).

chloride (CsCl)-ethidium bromide equilibrium density gradient centrifugation.

Transformation of plasmid DNA. Purified plasmid DNA from *H. ducreyi* strain 54198 was transformed into *E. coli* C600r^-m^+ and C600 by the method of Cohen et al. (8). Transformants were selected on MacConkey agar supplemented with 25 μg of ampicillin per ml.

Electron microscopy of plasmid DNA. Covalently closed circular plasmid DNA was nicked by treatment with 5 ng of deoxyribonuclease I (Sigma Chemical Co.) for 6 min. φX174 RF-II DNA (New England Biolabs) was included as an internal standard for contour-length and molecular-weight measurements. The DNA was spread and shadowed as previously described (9, 17). The molecular weight of pJB1 was calculated assuming a molecular weight of 3.49×10^6 for φX174 RFII (27).

Restriction endonuclease digestion. *Pst*I, *Hin*cII, *Bam*HI, and *Hin*dIII were purchased from New England Biolabs. Reactions were carried out at 37°C for 90 min and were halted by adding a stop mix (33% glycerol, 7% sodium dodecyl sulfate, 0.05% bromophenol blue) to a final concentration of 17%. Reaction conditions were those recommended by the supplier. (Details of the exact reaction conditions may be obtained from us on request.) Phage lambda DNA (New England Biolabs) cleaved with *Hin*dIII was used as a molecular-weight standard (22). The digests were analyzed by electrophoresis on vertical slab gels of 0.7 to 1.5% agarose, using tris(hydroxymethyl)-aminomethane-borate buffer (20).

Determination of G+C content. ^3H-labeled pJB1 plasmid DNA and ^{14}C-labeled *E. coli* chromosomal DNA (50 mol% guanine plus cytosine [G+C]) were centrifuged to equilibrium in a neutral CsCl gradient. The G+C content was then calculated as described by Mandel et al. (18), using the *E. coli* chromosomal DNA as an internal standard.

RESULTS

All three β-lactamase-producing strains of *H. ducreyi* were found to harbor a single plasmid species. Figure 1 shows the migration in a 0.7% agarose gel of plasmid DNA purified from *H. ducreyi* strain 54198. The plasmid DNA found in the two other strains had an identical mobility. The molecular weight of this plasmid calculated from its electrophoretic mobility is 6.0×10^6. We have designated this plasmid pJB1. None of the 16 ampicillin-susceptible strains of *H. ducreyi* we examined by agarose gel electrophoresis were found to harbor any plasmid DNA. The molecular weight estimated by comparison of the contour length of nicked pJB1 DNA with that of ØX174 RFII DNA was 5.9×10^6. Figure 2 is an electron photomicrograph showing one molecule of pJB1 and three molecules of ØX174 RFII.

Figure 3 shows the results of a typical neutral CsCl gradient containing ^3H-labeled pJB1 plasmid DNA and ^{14}C-labeled *E. coli* chromosomal DNA. It can be seen that the *E. coli* DNA equilibrates at a lower point in the gradient than does pJB1 DNA. The G+C content was calculated to be 41 mol%.

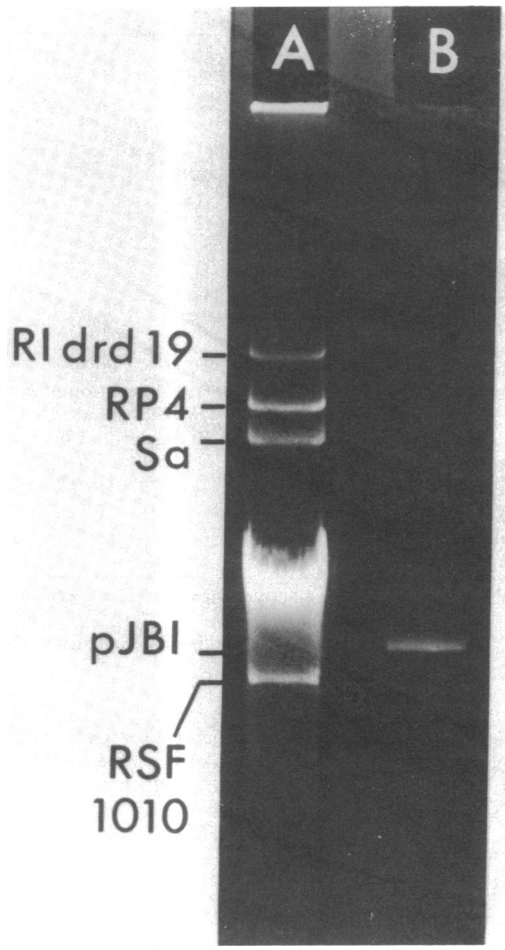

FIG. 1. *Agarose gel electrophoresis of purified pBJ1 plasmid DNA. (A) Molecular-weight standards: R1drd19, 62 × 10⁶; RP4, 34 × 10⁶; Sa, 23 × 10⁶; RSF1010, 5.5 × 10⁶. (B) pJB1 plasmid DNA isolated by CsCl density gradient centrifugation from H. ducreyi strain 54198. The calculated molecular weight is 6.0 × 10⁶.*

Plasmid DNA from the three β-lactamase-producing strains of *H. ducreyi* was purified and analyzed by electrophoresis of fragments produced by restriction endonuclease digestion. Digestion with *Bam*HI yielded fragments with molecular weights of 4.8×10^6 and 1.6×10^6. *Pst*I digestion yielded fragments of 3.8×10^6, 1.8×10^6, and 0.4×10^6 daltons. The digestion fragmentation patterns produced by each enzyme were identical for the plasmid DNA isolated from all three strains.

Figure 4 shows the pattern produced by *Hinc*II digestion of pJB1 DNA compared with that given by digestion of RSF1010:Tn*1* Ap101, RSF10::Tn*1* Ap111, RSF1010, and RSF1010::Tn*3* Ap230. The RSF1010::Tn*1* plamids differ from RSF1010 only in having the Tn*1* translocon inserted in TSF1010 (14). The Ap101 translocation differs from Ap111 only in the location of insertion of the Tn*1* translocon (14). It can be seen from Fig. 4 that the two fragments labeled 1 and 2 are present in pJB1 and in the two RSF1010::Tn*1* plasmids, as well as in RSF1030, which carries the Tn*2* translocon (6), but are absent from RSF1010 and RSF1010:Tn*3*. Molecular weight estimates of fragments 1 and 2 are 1.0×10^6 and 0.7×10^6, respectively. Furthermore, there are three more fragments generated from RSF1010::Tn*1* than from RSF1010. We interpret this as indicating that there are three *Hinc*II cleavage sites in Tn*1* and Tn*2* and that these sites are present in pJB1.

Transfer experiments. Attempts to transfer the ampicillin resistance from *H. ducreyi* strain 45198 to *H. influenzae* strain B003 Nal^r by mating on membrane filters failed. This was not unexpected in view of the low molecular weight of the plasmid. Nevertheless, to ensure that the failure of transfer was not simply due to an inability of *H. ducreyi* to act as a plasmid donor, the following control experiments were performed. First, *H. ducreyi* strain 35000 Sm^r, containing no plasmid DNA, was shown to be able to receive a 30×10^6-dalton ampicillin resistance plasmid from *H. influenzae* strain B031. Second, the ampicillin-resistant *H. ducreyi* transconjugant was then able to donate the 30×10^6-dalton plasmid to *H. influenzae* strain B003 Nal^r.

Plasmid DNA isolated from *H. ducreyi* strains 54198 and 54207 was successfully transformed into *E. coli* C600 and C600r$^-$m$^+$. Transformants were resistant to ampicillin by virture of β-lactamase production and were found to harbor a 6.0×10^6-dalton plasmid species. Tetracycline resistance was not cotransformed even when putative transformants were selected on MacConkey agar supplemented with 8 μg of tetracycline per ml. Electrophoresis of *Hinc*II, *Bam*HI, and *Pst*I digests of plasmid DNA purified from the transformants gave fragmentation patterns identical to the digests of plasmid DNA purified from *H. ducreyi* strain 54198.

In preliminary experiments, we were unable to cure the drug resistance from *H. ducreyi* strain 54198 by ultraviolet irradiation or three passages on chocolate agar supplemented with ethidium bromide (3 μg/ml; 5). Fifteen colonies tested for β-lactamase production after 50 passages on drug-free chocolate agar were positive.

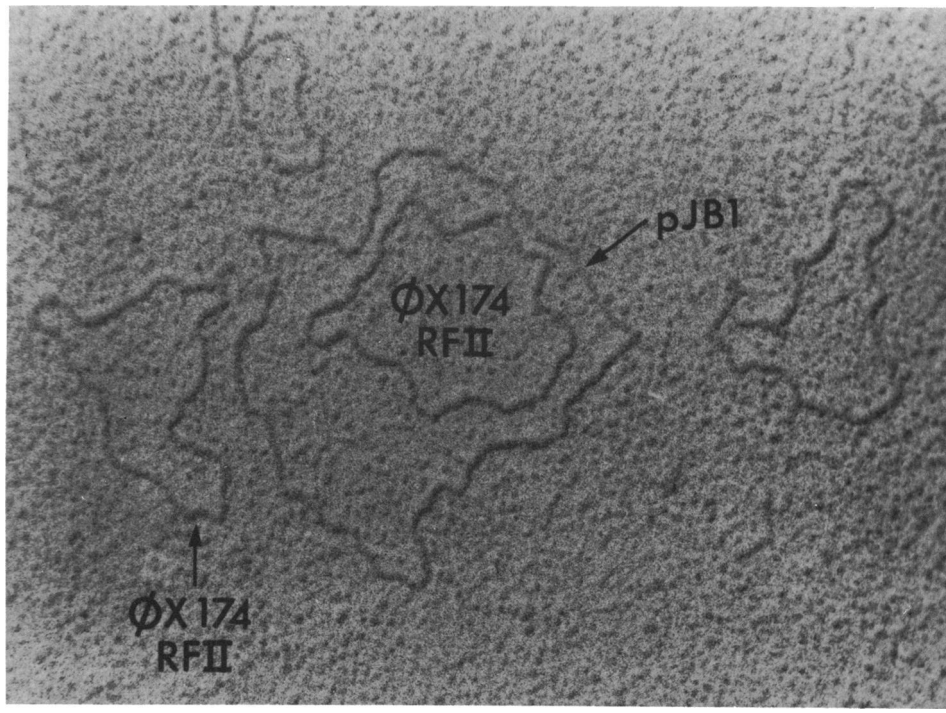

FIG. 2. *Electron micrograph of plasmid DNA. One molecule of pJB1 and three molecules of nicked φX174 RFII are shown. The molecular weight of pJB1 calculated by contour-length measurement of eight molecules is 5.9 × 10⁶, assuming that the molecular weight of φX174 RFII is 3.49 × 10⁶.*

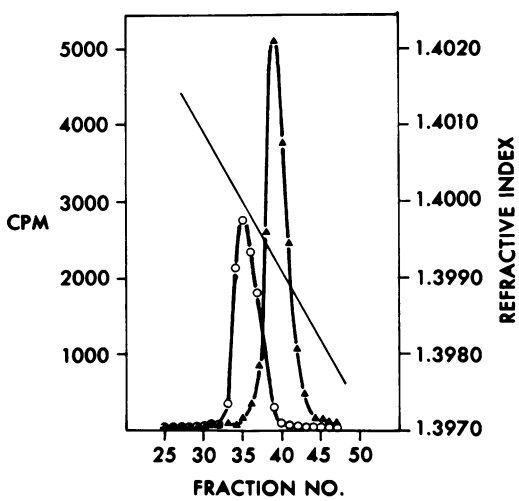

FIG. 3. *CsCl density gradient. A neutral CsCl density gradient containing ^{14}C-labeled E. coli chromosomal DNA and ^{3}H-labeled pJB1 plasmid DNA was centrifuged to equilibrium and fractionated. Fractions were counted for ^{14}C and ^{3}H, and the refractive index of every third fraction was determined. Symbols: (○) ^{14}C counts; (▲) ^{3}H counts; (——) refractive index. Note that the plasmid DNA was less dense than the E. coli chromosomal DNA. The G+C content of pJB1 was calculated as 41 mol%, assuming a G+C content of 50 mol% for the E. coli chromosome.*

DISCUSSION

The three β-lactamase-producing strains of *H. ducreyi* harbor a 6.0 × 10⁶-dalton nonconjugative plasmid. The plasmid from strain 54198 has been designated pJB1, although all three plasmids appear to be identical. This plasmid is absent from all of 16 β-lactamase-negative strains. The transformation experiments indicate that β-lactamase production is mediated by this plasmid. We noted that the minimal inhibitory concentrations of tetracycline for the three β-lactamase-producing strains were ≥8 μg/ml and were concerned that this resistance might also be plasmid mediated. The fact that tetracycline resistance could not be transformed into *E. coli* is evidence against this. The mediation of tetracycline resistance by insertion of the tetracycline resistance transposon into the *H. ducreyi* chromosome could be verified by DNA

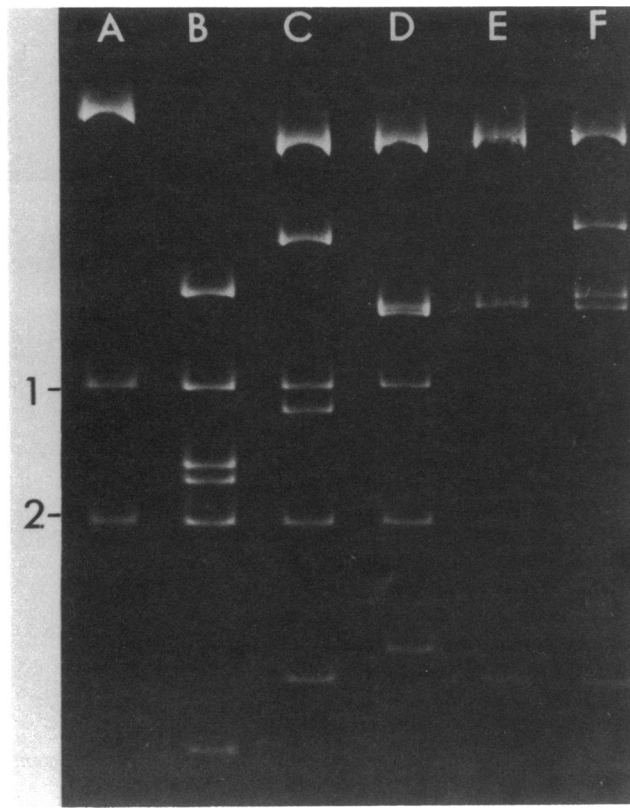

FIG. 4. *HincII endonuclease digests. Approximately 0.75 to 1 μg of purified plasmid DNA was digested with HincII as described in the text. Digests were subjected to electrophoresis on a 1.5% (wt/vol) agarose vertical slab gel. (A) pJB1; (B) RSF1030; (C) RSF1010::Tn1 (Ap111); (D) RSF1010::Tn1 (Ap101); (E) RSF1010; (F) RSF1010::Tn3 (Ap230). Molecular-weight estimates of fragments 1 and 2 are 1.0 × 10⁶ and 0.7 × 10⁶, respectively.*

hybridization experiments, but we have not examined this possibility.

The possibility that the clinical use of penicillin could select for β-lactamase-producing strains of *H. ducreyi* was considered. One of the strains (54198) was isolated from a patient who had received penicillin for treatment of gonorrhea 2 weeks before chancroid was diagnosed. There was no history of penicillin treatment in the patients harboring the other two strains of β-lactamase-producing *H. ducreyi*. Because the Winnipeg outbreak occurred in a relatively closed population, we were unable to determine how many different strains were actually involved. Therefore, it is impossible to know whether the occurrence of plasmid-mediated β-lactamase production in our three strains represents more than one genetic event.

The origin of pJB1 is unknown. The *Hinc*II fragments labeled 1 and 2 in Fig. 4, present in pJB1, are of the same molecular weight as the fragments generated from Tn*1* and Tn*2*. Although this does not constitute absolute evidence that fragments 1 and 2 generated by *Hinc*II digestion of pJB1 are fragments cut from the related transposons Tn*1* and Tn*2*, it strongly suggests that this is so.

The G+C content of 41 mol% is close to that of *H. influenzae* and *H. ducreyi* chromosomal DNA (38 to 39) (16) and to that of the *H. influenzae* plasmid RSF0885 (40) and the *N. gonorrhoeae* plasmid pMR0360 (41) (25). Thus, there is a possibility that Tn*1* or Tn*2* translocated onto an indigenous cryptic plasmid previously resident in rare *H. ducreyi* strains, although we were unable to demonstrate such a plasmid in the limited number of strains available to us. It is also possible that pJB1 is simply an extension of the enteric plasmid pool, as has been suggested for the β-lactamase-specifying plasmids pMR0360 and pMR0200 found in *N. gonorrhoeae* (25).

We are unable to say whether the finding of three ampicillin-resistant strains in the Winnipeg outbreak actually represents the emergence of ampicillin resistance in *H. ducreyi*, since there

are only 42 strains reported in the literature which have been tested for penicillin susceptibility (21, 24, 28, 29, 31). Clinical chancroid resistant to treatment with ampicillin, tetracycline, and sulfonamides has been reported from Viet Nam (19). The demonstration that *H. ducreyi* can act as a recipient of conjugative plasmids suggests that such multiple drug resistance might be plasmid mediated.

ACKNOWLEDGMENTS

We express our sincere appreciation for the time, information, and bacterial strains so generously given by S. Falkow, P. Shipley, and L. Elwell, as well as by M. Roberts and R. Gill.

This work was supported in part by grants from the Medical Research Council of Canada (MA-6368), the Manitoba Medical Services Fund, Inc., and the Children's Hospital Research Foundation. J.L.B. is the recipient of a Fellowship from the Medical Research Council of Canada.

LITERATURE CITED

1. **Albritton, W. L., S. Penner, L. Slaney, and J. Brunton.** 1978. Biochemical characteristics of *Haemophilus influenzae* in relationship to source of isolation and antibiotic resistance. J. Clin. Microbiol. **7**:519-523.
2. **Baron, E. S., A. K. Saz, D. J. Kopecko, and J. Wohlhieter.** 1977. Transfer of plasmid-borne beta-lactamase in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **12**:270-280.
3. **Barth, P. T., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618-630.
4. **Bouanchaud, D. H., and Y. A. Chabbert.** 1969. Stable coexistence of three resistance factors (fi^-) in *Salmonella panama* and *Escherichia coli* K12. J. Gen. Microbiol. **58**:107-113.
5. **Bouanchaud, D. H., M. R. Scavizzi, and Y. A. Chabbert.** 1969. Elimination by ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. J. Gen. Microbiol. **54**:417-425.
6. **Campbell, A., D. Berg, D. Botstein, E. Lederberg, R. Novick, P. Starlinger, and W. Szybalski.** 1977. Nomenclature of transposable elements in prokaryotes, p. 15-22. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
7. **Chabbert, Y. A., M. R. Scavizzi, J. L. Witchitz, G. R. Gerband, and D. H. Bouanchaud.** 1972. Incompatibility groups and the classification of fi^- resistance factors. J. Bacteriol. **112**:666-675.
8. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110-2114.
9. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope hetero-duplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **21**:413-427.
10. **Elwell, L. P., J. De Graaff, D. Seibert, and S. Falkow.** 1975. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type *b*. Infect. Immun. **12**:404-410.
11. **Elwell, L. P., M. Roberts, L. W. Mayer, and S. Falkow.** 1977. Plasmid-mediated beta-lactamase production in *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **11**:528-533.
12. **Elwell, L. P., J. R. Saunders, M. H. Richmond, and S. Falkow.** 1977. Relationships among some R plasmids found in *Haemophilus influenzae*. J. Bacteriol. **131**: 356-362.
13. **Hammond, G. W., C. Lian, J. C. Wilt, and A. R. Ronald.** 1978. Antimicrobial susceptibility of *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **13**: 608-612.
14. **Heffron, F., C. Rubens, and S. Falkow.** 1975. Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. U.S.A. **72**:3623-3627.
15. **Heffron, F., R. Sublett, R. W. Hedges, A. Jacob, and S. Falkow.** 1975. Origin of the TEM beta-lactamase gene found on plasmids. J. Bacteriol. **122**:250-256.
16. **Kilian, M.** 1976. A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. J. Gen. Microbiol. **93**:9-62.
17. **Kleinschmidt, A. K.** 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. Methods Enzymol. **12B**:361-377.
18. **Mandel, M., C. L. Schildkraut, and J. Marmur.** 1969. Use of CsCl density gradient analysis for determining the guanine plus cytosine content of DNA. Methods Enzymol. **12B**:184-195.
19. **Marmar, K. L.** 1972. Management of resistant chancroid in Viet Nam. J. Urol. **107**:807-808.
20. **Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.
21. **Mortara, F., R. R. Feinis, and E. Levenkron.** 1944. Activity of penicillin against *Haemophilus ducreyi* in vitro. Proc. R. Soc. Exp. Biol. Med. **56**:163-166.
22. **Murray, K., and N. E. Murray.** 75. Phage lambda receptor chromosomes for DNA fragments made with restriction endonuclease III of *Haemophilus influenzae* and restriction endonuclease I of *Escherichia coli*. J. Mol. Biol. **98**:55564.
23. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and S. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283-288.
24. **Reymann, F.** 1949. Sensitivity of *Haemophilus ducreyi* to penicillin, streptomycin and sulfathiazole. Acta Pathol. Microbiol. Scand. **26**:309-318.
25. **Roberts, M., L. P. Elwell, and S. Falkow.** 1977. Molecular characterization of two beta-lactamase-specifying plasmids isolated from *Neisseria gonorrhoeae*. J. Bacteriol. **131**:557-563.
26. **Roberts, M., and S. Falkow.** 1977. Conjugal transfer of R plamids in *Neisseria gonorrhoeae*. Nature (London) **266**:630-631.
27. **Sanger, F., G. M. Air, B. G. Barrell, N. L. Brown, A. R. Coulson, J. C. Fiddes, C. A. Hutchinson III, P. M. Slocombe, and M. Smith.** 1977. Nucleotide sequence of bacteriophage ØX 174 DNA. Nature (London) **265**:687-695.
28. **Singer, S., and W. E. Deacon.** 1956. Effects of penicillin G in vitro on *Haemophilus ducreyi*. Public Health Rep. **71**:1112-1114.
29. **Thayer, J. D., F. W. Field, and M. I. Parry.** 1955. In vitro sensitivity of *Haemophilus ducreyi* to several antibiotics. Antibiot. Chemother. **5**:132-134.
30. **Thorne, G. M., and W. E. Farrar, Jr.** 1975. Transfer of ampicillin resistance between strains of *Haemophilus influenzae* type B. J. Infect. Dis. **132**:276-281.
31. **Wetherbee, D. G., M. A. Henke, R. I. Anderson, E. J. Pulaski, and D. M. Kuhus.** 1949. In vitro antibiotic effects on *Haemophilus ducreyi*. J. Vener. Dis. Inf. **33**: 462-472.