Plasmid Mediated Antimicrobial Resistance in Ontario Isolates of *Actinobacillus (Haemophilus) pleuropneumoniae*

Kimberley A. Gilbride, Søren Rosendal and James L. Brunton

ABSTRACT

The genetic basis of antimicrobial resistance in Ontario isolates of *Actinobacillus (Haemophilus) pleuropneumoniae* was studied. Two Ontario isolates of *A. pleuropneumoniae* were found to be resistant to sulfonamides (Su), streptomycin (Sm) and ampicillin (Amp). Resistance to Su and Sm was specified by a 2.3 megadalton (Mdal) plasmid which appeared to be identical to pVM104, which has been described in isolates of *A. pleuropneumoniae* from South Dakota. Southern hybridization showed that the 2.3 Mdal Su Sm plasmid was highly related to those *Hinc* II fragments of RSF1010 known to carry the Su Sm genes, but was unrelated to the remainder of this *Salmonella* resistance plasmid. Resistance to Su and Amp was specified by a 3.5 Mdal plasmid and appeared identical to pVM105 previously reported. The beta-lactamase enzyme had an isoelectric point of approximately 9.0. Southern hybridization showed no relationship to the TEM beta-lactamase determinant which is the most widespread plasmid mediated beta-lactamase. A third isolate of *A. pleuropneumoniae* was found to be resistant to chloramphenicol (Cm), Su and Sm by virtue of a 3.0 Mdal plasmid which specified a chloramphenicol acetyl transferase.

We conclude that resistance to Su, Sm, Amp and Cm is mediated by small plasmids in *A. pleuropneumoniae*. Although the Su and Sm resistance determinants are highly related to those found in Enterobacteriaceae, the plasmids themselves and the beta-lactamase determinant are different.

RÉSUMÉ

Cette expérience visait à étudier la base génétique de la résistance aux antibiotiques, de la part de souches d'*Actinobacillus pleuropneumoniae*, isolées en Ontario. Deux d'entre elles s'avérèrent résistantes aux sulfonamides, à la streptomycine et à l'ampicilline. La résistance aux sulfonamides et à la streptomycine dépendait d'un plasmide de 2,3 mégadaltons qui sembla identique au plasmide VM104, déjà décrit pour des souches d'*A. pleuropneumoniae* du Dakota du Sud. L'hybridation, d'après la méthode de Southern, révéla une relation étroite entre le plasmide de 2,3 mégadaltons et les fragments *Hinc* II du plasmide RSF1010, qui contiennent les gènes responsables de la résistance aux sulfonamides et à la streptomycine. Cette hybridation ne démontra cependant aucune relation entre le plasmide de 2,3 mégadaltons et le reste du plasmide de résistance RSF1010, provenant de salmonelles. La résistance aux sulfonamides et à l'ampicilline dépendait d'un plasmide de 3,5 mégadaltons qui sembla identique au plasmide VM105, déjà rapporté. L'enzyme bêta-lactamase afficha un point isoélectrique d'environ 9,0. L'hybridation précitée ne révéla aucun rapport entre le déterminant de la bêta-lactamase du type TEM, laquelle correspond à la bêta-lactamase à médiation plasmidique la plus répandue. Une troisième souche d'*A. pleuropneumoniae* se révéla résistante au chloramphénicol, aux sulfonamides et à la streptomycine, grâce à un plasmide de 3,0 mégadaltons qui déterminait une acétyltransférase du chloramphénicol.

Les auteurs concluent que la résistance aux sulfonamides, à la streptomycine, à l'ampicilline et au chloramphénicol est codée par des petits plasmides d'*A. pleuropneumoniae*. Même si les déterminants de la résistance aux sulfonamides et à la streptomycine affichent une relation étroite avec ceux qu'on retrouve chez les Enterobacteriaceae, les plasmides eux-mêmes et le déterminant de la bêta-lactamase sont différents.

INTRODUCTION

Actinobacillus pleuropneumoniae (previously known as *Haemophilus pleuropneumoniae*) causes hemorrhagic necrotizing lobar pneumonia in swine (1,2). Antibiotic treatment is often the only effective measure to decrease morbidity and mortality. Therefore, the appearance of antimicrobial resistant isolates in this species has important implications for the future management of this disease. Hirsh *et al* (3) described two *A. pleuropneumoniae* isolates, SD-1 and SD-2, from the lungs of feeder pigs from South Dakota. Isolate SD-1 carried two plasmids, pVM104 and pVM105. Plasmid pVM104 had a molecular weight of 2.3 megadaltons (Mdal) and specified resistance to sulfonamide (Su) and streptomycin (Sm). Plasmid pVM105 had a molecular weight of 3.5 Mdal and specified resistance to Su and ampicillin (Amp). The second isolate SD-2 carried only one plasmid pVM106 which appeared identical to pVM104. Hirsh *et al* (3) reported that the ampicillin resistance was due to a beta-lactamase with a substrate profile similar to that

Departments of Microbiology and Medicine, University of Toronto (Gilbride, Brunton) and Department of Veterinary Microbiology and Immunology, University of Guelph, Guelph, Ontario N1G 2W1 (Rosendal).

Reprint requests to K.A. Gilbride, Mount Sinai Hospital, Suite 435, 600 University Avenue, Toronto, Ontario M5G 1X5.

Submitted March 3, 1988.

of the TEM type enzyme found in *Haemophilus* species. Medeiros *et al* (4) reported that the beta-lactamase specified by pVM105 had an isoelectric point (pI) of 8.1 and was similar to the Rob-1 beta-lactamase found in rare isolates of *H. influenzae* type b.

We have previously reported that *A. pleuropneumoniae* isolates from Ontario were resistant to Amp, Sm, Su and chloramphenicol (Cm) (5). The purpose of this study was to further characterize the nature and genetic basis of the antimicrobial resistance in these isolates. We were interested in determining the relationship of the antimicrobial resistance between the Ontario isolates and the South Dakota isolates described by Hirsh *et al* (3). We were also interested in the nature of the beta-lactamase, produced by the *A. pleuropneumoniae* isolates, and its relationship at the DNA sequence level to the widely disseminated TEM beta-lactamase determinant which is widespread in Enterobacteriaceae, *Haemophilus* species and *Neisseria gonorrhoeae* (6,7).

TABLE I. Original Host, Molecular Mass and Antibiotic Resistance Phenotype of Plasmids

Plasmids	Host[a]	Size (Mdal)	Phenotype[b]	Source
pBr322	*E. coli*	2.6	Amp^rTc^r	Sutcliffe (21)
pFA3	*N. gonorrhoeae*	4.7	Amp^r	Brunton *et al* (2)
RSF1010	*S. panamq*	5.0	Su^rSm^r	Guerry *et al* (22)
pVM104	*A. plpn* SD-1	2.3	Su^rSm^r	Hirsh *et al* (3)
pVM105	*A. plpn* SD-1	3.5	Amp^rSu^r	Hirsh *et al* (3)
pVM106	*A. plpn* SD-2	2.3	Su^rSm^r	Hirsh *et al* (3)
pKG100	*A. plpn* WF83 *VSB113*	2.3	Su^rSm^r	This paper
pKG200	*A. plpn* WF83 VSB113	3.5	Amp^rSu^r	This paper
pKG300	*A. plpn* RH82	3.0	$Su^rSm^rCm^r$	This paper
pKG400	*A. plpn* RH82	1.8	NPA[c]	This paper

[a] *A. plpn* = *A. pleuropneumoniae*
[b] Amp^r = Ampicillin resistance, Tc^r = Tetracycline resistance, Su^r = Sulfonamide resistance, Sm^r = Streptomycin resistance, Cm^r = Chloramphenicol resistance
[c] NPA = No phenotype assigned to this plasmid

MATERIALS AND METHODS

BACTERIAL STRAINS, PLASMIDS AND MEDIA

The bacterial strains and plasmids used in this study are listed in Table I. The minimal inhibitory concentrations of antimicrobials for the Ontario *A. pleuropneumoniae* isolates, RH82, WF83 and VSB113, have been determined as previously described (5).

Actinobacillus pleuropneumoniae strains were grown in Luria broth supplemented with 0.4% glucose and 10 μg/mL of nicotinamide adenine dinucleotide (5).

PLASMID DNA ISOLATION AND ANALYSIS

Cleared lysates were prepared as previously described using TritonX-100 (0.1% final concentration) (8). Plasmid DNA was isolated by cesium chloride (CsCl)/ethidium bromide (EtBr) equilibrium density gradient centrifugation. Plasmid DNA was electrophoresed through a 0.7% vertical agarose gel for 2 h at 100 volts in Tris-borate buffer (9). Gels were stained with EtBr and photographed under UV light with a polaroid camera (8). Restriction endonuclease enzymes were purchased from Boehringer Mannheim Corp. Reactions were carried out as recommended by the supplier.

Molecular weights of the covalently closed circular (CCC) plasmid species were calculated by comparison with the standard plasmids RP4, Sa, RSF1010, pBR322 and pMB8 as previously described (9).

To determine the antibiotic resistance phenotype, purified plasmid DNA from *A. pleuropneumoniae* strains was transformed into *Escherichia coli* J53 *lac*$^+$, *pro, gyrA* according to the method of Cohen *et al* (10). Transformants were selected on L-agar supplemented with either carbenicillin (30 μg/mL) or streptomycin (100 μg/mL) or chloramphenicol (30 μg/mL). Sulfonamide resistance was tested using sulfamethoxazole (300 μg/mL) in lysed horseblood agar.

SOUTHERN BLOTTING

Whole plasmid DNA and DNA fragments were transferred from agarose gels to nitrocellulose filters by the method of Southern (11). The DNA was labelled by nick translation using ^{32}P dCTP and the method of Rigby *et al* (12). Hybridization was carried out with 1 x 10^6 cpm of probe DNA in 2 x SSC, 50% formamide, and 10 x Denhardt's solution at 37°C for 18 h. After hybridization the filters were washed in 5 x SSC twice and 2 x SSC once. The latter wash was carried out at 65°C for 30 min. Radioautography was carried out using Kodak X-ray film.

ISOELECTRIC FOCUSING OF BETA-LACTAMASE

Isoelectric focusing was carried out as described by Matthew *et al* (13) using a 5% polyacrylamide gel polymerized with N,N,N′,N′-Tetramethyl ethylenediamine and ammonium persulfate. Ampholines pH 3.5-10 (Pharmacia Fine Chemicals) were used. The gels were run at increasing voltage for 3 h and the pH gradient of the gel was read at 0.5 cm intervals using a surface electrode. Gels were stained for beta-lactamase activity by using a chromogenic cephalosporin as substrate [3-(2,4-dinitro-styryl)-(6R,7R)7b (2-thienylacetamide-ceph-3-em-4-carboxylic acid, E-isomer] (14). Gels were photographed with Kodalith Ortho film (35 mm) using a Wratten 58 green filter.

SPECTROPHOTOMETRIC CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ACTIVITY

Supernatants of crude cell extracts were prepared by sonication of cells and centrifugation. The supernatants were assayed for CAT activity as described by Shaw (15). Chloramphenicol acetyl transferase catalyzes the transfer of an acetyl group from acetyl CoA to chloramphenicol. The free CoA sulfhydryl group reacts with 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) to form a mixed disulfide of CoA and thionitrobenzoic acid, and a molar equivalent of 5-thio-2-nitrobenzoate. Production of the latter was measured spectrophotometrically at 412 nm. Activity was expressed as μmol per min of Cm-dependent DTNB reacted as described (15).

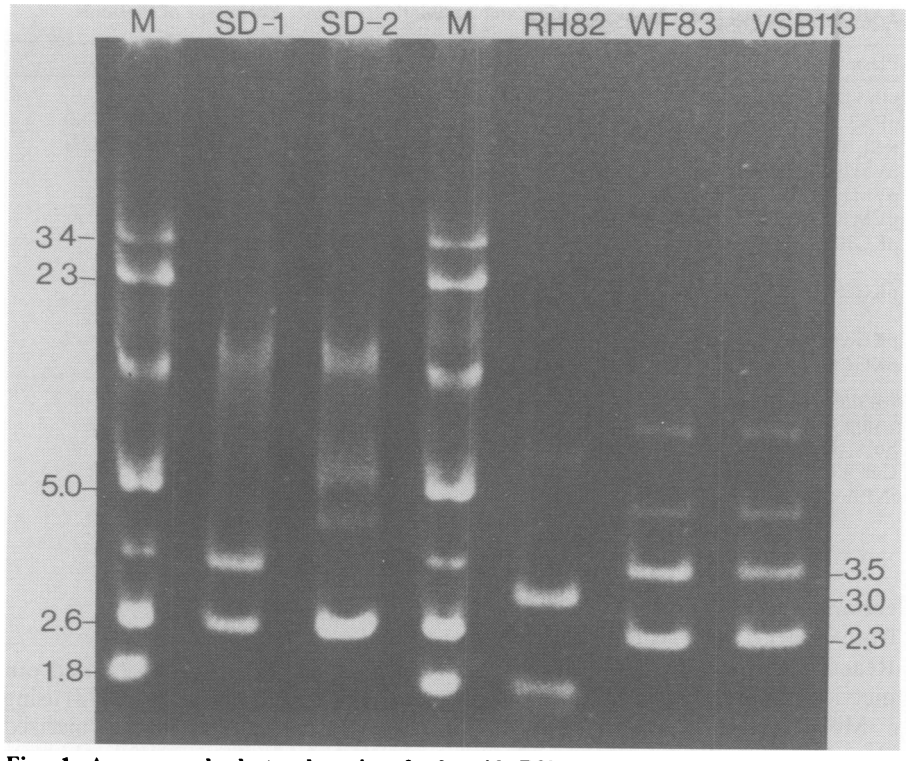

Fig. 1. Agarose gel electrophoresis of plasmid DNA extracted from strains of *A. pleuropneumoniae*. Lanes marked M contain plasmids used as molecular weight markers; RP4 (34 Mdal); Sa (23 Mdal); RSF1010 (5 Mdal); pBr322 (2.6 Mdal); pMB8 (1.8 Mdal); Lane SD-1 contains pVM104 (2.3 Mdal), pVM105 (3.5 Mdal); Lane SD-2 contains pVM106 (2.3 Mdal); Lane RH82 contains pKG300 (3.0 Mdal), pKG400 (1.8 Mdal); Lane WF83 contains pKG100 (2.3 Mdal), pKG200 (3.5 Mdal); Lane VSB113 contains pKG100 (2.3 Mdal), pKG200 (3.5 Mdal).

RESULTS

ANALYSIS OF *A. PLEUROPNEUMONIAE* PLASMIDS AND THEIR ASSOCIATED PHENOTYPE

Figure 1 shows the results of agarose gel electrophoresis of plasmid DNA extracted from isolates of *A. pleuropneumoniae*. Covalently closed circular (CCC) plasmid DNA species of 1.8 and 3.0 Mdal were found in strain RH82, while CCC plasmids of 2.3 and 3.5 Mdal were found in strains WF83 and VSB113. The other bands seen in these plasmid preparations were found to be open circular derivatives. This was demonstrated by comparison of the mobility with plasmid preparations nicked by partial DNaseI digestion (data not shown). The molecular weights of the plasmid species are summarized in Table I together with their antibiotic resistance phenotype. The latter was assigned by transformation of plasmid DNA into *E. coli* followed by determination of the resistance phenotype of the transformants and analysis of their plasmid DNA content. Linked resistance to Su and Sm was associated with the 2.3 Mdal plasmid, pKG100 while Su resistance linked with beta-lactamase production was associated with the 3.5 Mdal plasmid, pKG200. Linked resistance to Cm, Su and Sm was associated with the 3.0 Mdal plasmid, pKG300, found in strain RH82. We were unable to assign a resistance phenotype to the 1.8 Mdal plasmid, pKG400, in this strain. Transformation of *E. coli* with total plasmid DNA purified from strain RH82 did not yield beta-lactamase producing colonies. We were therefore unable to assign this property to either the 1.8 or 3.0 Mdal plasmid.

CHARACTERIZATION OF THE SU SM RESISTANCE PLASMID pKG100

Plasmid pKG100 was compared to the Su Sm resistance plasmid pVM104 found in South Dakota isolates of *A. pleuropneumoniae* by Hirsh *et al* (3) and the broad host range Su, Sm resistance plasmid RSF1010 (16). Plasmid pKG100 was cleaved twice by *Pst*I and once by *Hinc*II. The fragmentation pattern for pKG100 with the two enzymes was identical to that of pVM104 (data not shown). This suggests that the two plasmids are virtually identical. Southern blots of whole plasmid DNA confirmed that pVM104 and pKG100 are homologous (data not shown). Radiolabeled pKG100 was hybridized to a Southern blot of *Hinc*II cleaved RSF1010. Figure 2 shows that pKG100 hybridized to the 0.4 Mdal fragment known to carry the Su resistance gene and the 1.3 Mdal fragment which carries the Sm resistance gene (16). However there was little if any hybridization to the remaining 3.8 Mdal fragment which carries the origin of replication and broad host range determinants (19).

CHARACTERIZATION OF THE AMP RESISTANCE PLASMID pKG200

The Amp, Su resistance plasmids, pKG200 and pVM105 both had a single *Pst*I cleavage site but no *Hinc*II sites (data not shown). We were interested in determining whether there was any relationship between the *H. pleuropneumoniae* beta-lactamase determinant and the TEM beta-lactamase determinant which is widely disseminated in *Haemophilus* sp., *Neisseria gonorrhoeae* and Enterobacteriaceae isolated from both humans and animals (4). Plasmid pFA3, the prototype of small TEM beta-lactamase specifying plasmids found in *Haemophilus* species and *N. gonorrhoeae*, was used as a probe for Southern hybridizations. Figure 3 shows that pFA3 hybridized to itself and pBR322 which also contains the TEM beta-lactamase gene. There was no hybridization to the *A. pleuropneumoniae* beta-lactamase plasmids pVM105 and pKG200. Thus there appears to be no relationship at all between pFA3 and the *A. pleuropneumoniae* plasmids. Consequently, the *A. pleuropneumoniae* beta-lactamase gene is unrelated to the TEM gene.

Isoelectric focusing of crude extracts of *A. pleuropneumoniae* strains SD-1, SD-2 and WF83 is shown in Fig. 4. Control enzymes, TEM I extracted from an *E. coli* strain containing the plasmid RSF1030 and the *Enterobacter cloacae* P99 enzyme, focused at pI's of 5.4 and 8.0 re-

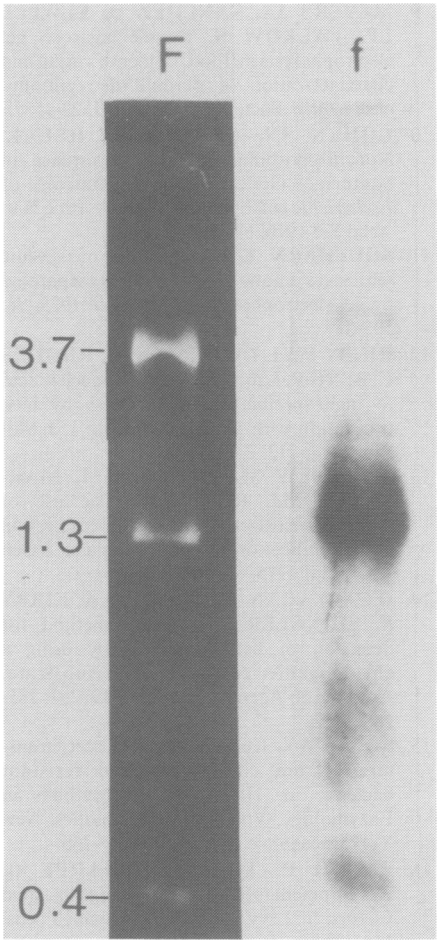

Fig. 2 Hybridization of pKG100 to RSF1010. Lane F, Agarose gel electrophoresis of *Hinc* II digested RSF1010. Lane f, Autoradiogram. Almost all hybridization occurs to the 0.4 and 1.3 Mdal fragments.

spectively. The enzyme from all the beta-lactamase producing strains of *A. pleuropneumoniae* strains had pI's of approximately 9.0. No enzyme band could be detected in extracts of the beta-lactamase negative strain SD2.

CHARACTERIZATION OF THE CM RESISTANCE PLASMID pKG300

The Cm resistance plasmid pKG300 had a mass of 3.0 Mdal and also specified resistance to Sm and Su (Table I). When pKG300 was cleaved with *Alu*I many fragments were identical in size to those produced from pKG100 suggesting that the two plasmids were highly related (data not shown). The basis of the chloramphenicol resistance of strain RH82 was studied by assaying a crude extract (prepared as described in methods) for chloramphenicol acetyl transferase activity. Extracts of RH82 reacted 0.002 μmol of DTNB per min in the presence of Cm but less than 0.0001 μmol per min in the absence of Cm. Extracts of the Cm sensitive strain WF83 reacted less than 0.0001 μmol DTNB per minute in the presence of chloramphenicol. These results show that strain RH82 possesses chloramphenicol acetyl transferase activity which is most likely the cause of its chloramphenicol resistance.

DISCUSSION

In this study we demonstrated that the resistance to sulfonamides, streptomycin, ampicillin and chloramphenicol previously reported in Ontario isolates of *H. pleuropneumoniae* is mediated by plasmids. Moreover the 2.3 Mdal Su Sm resistance plasmids pVM104 and pKG100 found in isolates from South Dakota and Ontario respectively, were identical in size, resistance phenotype and restriction endonuclease digestion profile. Similarly, the Su resistance, beta-lactamase specifying plasmids pVM105 and pKG200, appeared identical. It is impossible to determine whether this similarity is the result of importation of hogs carrying resistant strains and their resistance plasmids from the United States or whether the resistance plasmids evolved separately in the two regions. However the findings suggest that the strains could be epidemiologically related.

Chloramphenicol resistance in *Haemophilus* species is usually specified by plasmids of 30-40 Mdal which may or may not be conjugative (6). Thus plasmid pKG300 is the first small chloramphenicol resistance plasmid to be reported in *Haemophilus* species. This is also the first report to demonstrate that Cm resistance in *A. pleuropneumoniae* is plasmid mediated. We are aware that many strains of chloramphenicol resistant *A. pleuropneumoniae* have been isolated in Quebec and Western Canada recently (17,18) but we are not aware of any reports on the basis of this resistance. Nevertheless it seems very likely that these strains will prove to have plasmids similar or identical to pKG300.

RSF1010 is a small, nonconjugative broad host range plasmid, originally found in *Salmonella panama* which specifies resistance to Sm and Su (16). The 0.4 and 1.3 Mdal *Hinc*II fragments of RSF1010 carry the sulfonamide and streptomycin resistance determinants respectively (16). The hybridization between pKG100 and these fragments suggests that the resistance genes of pKG100 and RSF1010 are homologous while the remainder of the plasmid functions including the origin of

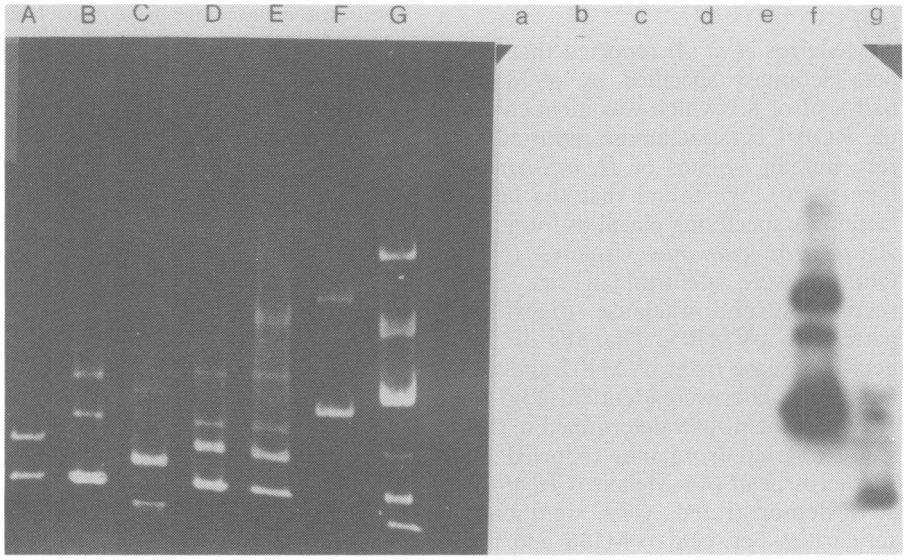

Fig. 3. Hybridization of pFA3 to the *A. pleuropneumoniae* plasmids.
Left: Agrose gel electrophoresis; Right: Autoradiogram;
Lane A(a): SD-1; Lane B(b): SD-2; Lane C(c): RH82; Lane D(d): WF83; Lane E(e): VSB113; Lane F(f): pFA3; Lane G(g): Molecular weight standards RP4, Sa, RSF1010, pBR322, pMB8.
Hybridization is seen to the homologous control in lane f including its CCC, OC and dimer CCC form. Hybridization in lane g is seen to pBR322 and its OC form. No hybridization occurred to any of the *A. pleuropneumoniae* plasmids.

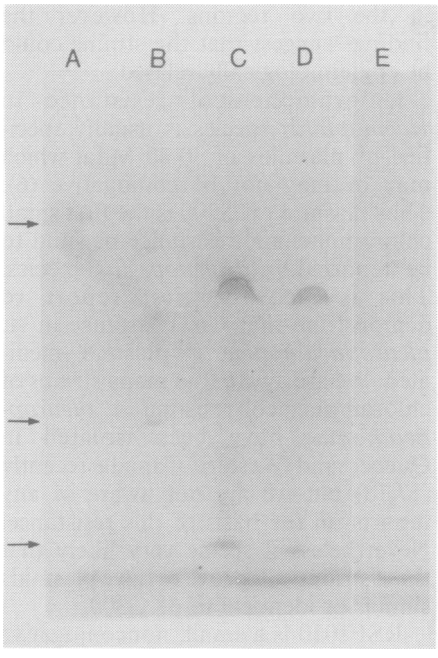

Fig. 4. Isoelectric focusing of beta-lactamase enzymes.
Lane A, *E. coli* (RSF1030) TEM type 1 enzyme, pI 5.4; Lane B, *Enterobacter cloacae* p99 enzyme, pI 8.0; Lane C, *A. pleuropneumoniae* WF83, pI 9.0; Lane D, *A. pleuropneumoniae* SD-1, pI 9.0; Lane E, *A. pleuropneumoniae* SD-2, no beta-lactamase is produced by this strain. Large bands are seen for WF83 and SD-1 at the point of application, however, when the point of application was changed the pH at which these spots appeared changed accordingly.

replication, mobilization genes and the genes required for replication are not (19).

Medeiros *et al* (4) reported that the beta-lactamase specified by pVM105 had a pI of 8.1 which was identical to the ROB-1 beta-lactamase reported in rare human isolates of *H. influenzae* type b (20). We found that the beta-lactamase specifying plasmids found in *A. pleuropneumoniae* isolates from Ontario were identical in size and restriction endonuclease digestion pattern to pVM105. The pI's of the beta-lactamase from pVM105 and the Ontario isolates were identical (Fig. 4). However the pI of 9 determined in this study differed from that reported by Medeiros *et al* (4). Medeiros *et al* (4) also demonstrated very significant similarities between pVM105 and the entire Rob plasmid. Our study shows that there is no relationship between the *A. pleuropneumoniae* beta-lactamase plasmid and the TEM beta-lactamase specifying plasmid pFA3 which is representative of the small beta-lactamase specifying plasmids commonly found in *Haemophilus* species and *N. gonorrhoeae*. We have shown that pFA3 is likely a derivative of a recombinant plasmid produced by transposition of TnA sequences introduced from enteric bacteria into a phenotypically cryptic plasmid found in *H. parainfluenzae* (6). It would appear that the ROB-1 beta lactamase and the pVM105 beta-lactamase were not derived from an enteric source. Whether human *H. influenzae* share a resistance plasmid pool with porcine *A. pleuropneumoniae* strains or whether the plasmids, Rob, pVM105 and pKG200, evolved separately in the respective species is not clear. *Actinobacillus pleuropneumoniae* has recently been reclassified to the genus *Actinobacillus* (2). It is therefore possible that further studies will show that the *A. pleuropneumoniae* plasmid pool is related to that of other *Actionobacillus* species.

REFERENCES

1. **SHOPE RE.** Porcine contagious pleuropneumoniae. I. Experimental transmission, etiology and pathology. J Exp Med 1964; 119: 357-368.
2. **POHL S, BERTSCHINGER HU, FREDERIKSEN W, MANNHEIM W.** Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus (Actinobacillus pleuropneumoniae* comb. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. Int J Syst Bacteriol 1983; 33: 510-514.
3. **HIRSH DC, MARTIN LD, LIBAL MC.** Plasmid mediated antimicrobial resistance in *Haemophilus pleuropneumoniae*. Am J Vet Res 1982; 43: 269-272.
4. **MEDEIROS AA, LEVESQUE R, JACOBY GA.** An animal source for the ROB-1 beta-lactamase of *Haemophilus influenzae* type-b. Antimicrob Agents Chemother 1986; 29: 212-215.
5. **GILBRIDE KA, ROSENDAL S.** Antimicrobial susceptibility of 51 strains of *Haemophilus pleuropneumoniae*. Can J Comp Med 1984; 48: 47-50.
6. **BRUNTON J, CLARE D, MEIER M.** Molecular epidemiology of antibiotic resistance plasmids of *Haemophilus* species and *Neisseria gonorrhoeae*. Rev Infect Dis 1986; 8: 713-724.
7. **BRUNTON JL, BENNETT P, GRINSTED J.** Molecular nature of a plasmid specifying beta-lactamase production in *Haemophilus ducreyi*. J Bacteriol 1981; 148: 788-795.
8. **ELWELL LP, deGRAAFF J, SEIBERT D, FALKOW S.** Plasmid linked ampicillin resistance in *Haemophilus influenzae* type b. Infect Immun 1975; 12: 404-410.
9. **MEYERS JA, SANCHEZ D, ELWELL LP, FALKOW S.** Simple agarose gel electrophoresis method for identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol 1976; 127: 1529-1537.
10. **COHEN SN, CHAN ACY, HSU L.** Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci 1976; 69: 2110-2114.
11. **SOUTHERN EM.** Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; 98: 503-517.
12. **RIGBY PWJ, DIECKMAN M, RHODES C, BERG P.** Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 1977; 113: 237-251.
13. **MATTHEW M, HARRIS AM, MARSHALL MJ, ROSS GW.** The use of analytical isoelectric focusing for detection and identification of beta-lactamase. J Gen Microbiol 1975; 88: 169-178.
14. **O'CALLAGAN CH, MORRIS A, KIRBY S, SHINGLER AH.** Novel method for detection of beta-lactamases by using a chromogenic cephalosporin substrate. Antimicrob Agents Chemother 1972; 1: 283-288.
15. **SHAW WV.** Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. In: Hash JH, ed. Methods in Enzymology. Vol. XLIII, Antibiotics. New York: Academic Press, 1975: 737-755.
16. **BARTH P, TOBIN L, SHARPE G.** Development of broad host-range plasmid vectors. In: Levy S, Clowes R, Koenig E, eds. Molecular Biology Pathogenicity and Ecology of Bacterial Plasmids. New York: Plenum Press, 1981: 439-448.
17. **HIGGINS R, LARIVIÈRE S, MITTAL KR, MARTINEAU GP.** Antimicrobial sensitivity patterns of *Haemophilus pleuropneumoniae* strains isolated in 1980, 1981 and 1982. Med Vet Quebec 1983; 13: 41-43.
18. **SEBUNYA TNK, SAUNDERS JR, OSBORNE AD.** Characteristics of *Haemophilus* pleuropneumoniae isolates and some epidemiological findings on porcine *Haemophilus* pleuropneumonia in Saskatchewan. Can Vet J 1982; 23: 224-228.
19. **SCHOLZ P, HARTING V, SCHERZINGER E, LURUZ R, BAGDASARIAN M, SCHUSTER H, BAGDASARIAN M.** Replication determinants of the broad host range plasmid RSF1010. In: Helinski D, Cohen S, Clewell D, Jackson D, Hollander A, eds. Plasmids in Bacteria. New York: Plenum Press, 1985: 243-259.
20. **RUBIN LJ, MEDEIROS AA, YOLKEN RH, MOXON ER.** Ampicillin treatment, failure of apparently beta-lactamase negative *Haemophilus influenzae* type b meningitis due to a novel beta-lactamase. Lancet 1981; ii: 1008.
21. **SUTCLIFFE JG.** pBR322 restriction map marked from the DNA sequence: Accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acid Res 1978; 5: 2721.
22. **GUERRY P, VAN EMBDEN J, FALKOW S.** Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J Bacteriol 1974; 117: 619-630.