Distinct plasmid profiles of *Pasteurella haemolytica* serotypes and the characterization and amplification in *Escherichia coli* of ampicillin-resistance plasmids encoding ROB-1 β -lactamase

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Thirty-five isolates of *Pasteurella haemolytica* from cattle or sheep were screened for the presence of plasmids and for resistance to a range of antibiotics. Eight strains (four of serotype A1, three of serotype A2 and one untypable) contained plasmid DNA and isolates of the same serotype had similar plasmid profiles, which were different from those of the other serotypes. All but one of the plasmid-bearing strains were isolated from pneumonic animals or from animals in contact with pneumonic cattle or sheep. In A2 and untypable strains, there was no obvious correlation between antibiotic resistance and the presence of a specific plasmid. In contrast, all plasmid-bearing A1 strains exhibited ampicillin resistance (Ap^R), which was shown by transfer studies to be plasmid-mediated. Plasmid DNA prepared from *E. coli* transformants was not routinely detected on ethidium-bromide-stained agarose gels, but could be amplified to detectable levels by treatment of cultures with chloramphenicol (Cm) or by modifying the growth conditions. The Ap^R plasmids from *P. haemolytica* were identical by restriction enzyme analysis. Restriction analysis and hybridization data indicated that these plasmids were closely related to the prototype ROB-1 β -lactamase-encoding plasmid, originally isolated from *Haemophilus influenzae*. From substrate profiles and isoelectric focusing data, the β -lactamases encoded by the *P. haemolytica* plasmids were indistinguishable from the ROB-1 β -lactamase.

Introduction

Pasteurella haemolytica, a Gram-negative bacterium, is an important pathogen of domestic ruminants and causes a substantial economic loss to the cattle and sheep industries (Yates, 1982; Donachie, 1988). The presence of plasmids and their association with antimicrobial resistance phenotypes in this species has been described previously (Zimmerman & Hirsh, 1980; Boyce & Mortér, 1986; Chang *et al.*, 1987; Haghour *et al.*, 1987; Livrelli *et al.*, 1988; Schwarz *et al.*, 1989; Craig *et al.*, 1989), and some reports have correlated antibiotic resistance with transfer of plasmid DNA from *P. haemolytica* to *E. coli* by transformation (Zimmerman & Hirsh, 1980; Livrelli *et al.*, 1988; Schwarz *et al.*, 1989; Craig *et al.*, 1989) and back to *P. haemolytica* by conjugation (Craig *et al.*, 1989). Previously, we investigated the transfer of a small plasmid encoding Ap^R between *P. haemolytica* strains by electroporation (Craig *et al.*, 1989). Here we report the plasmid profiles exhibited by a range of *P. haemolytica*

isolates and the inter- and intraspecies transfer of four plasmids which encoded β -lactam resistance. This resistance was shown in all cases to be due to β -lactamase activity of the ROB-1 type. Plasmid DNA was not readily detected in *E. coli* transformants by a routine plasmid isolation procedure but detection and amplification of these plasmids in *E. coli* was achieved by manipulating the growth conditions.

Methods

Bacterial strains, plasmids and growth media. The *P. haemolytica* strains used in this study were: 18 serotype A1 strains, 15 of bovine origin (11 from pneumonic calves, 2 from animals in contact with pneumonic calves and 2 from healthy calves), 3 of ovine origin (from pneumonic sheep); 7 serotype A2 strains, 3 of bovine origin (2 from pneumonic calves and 1 from a healthy calf), 4 of ovine origin (from pneumonic sheep); 1 strain each from serotypes T4 and T10 from pneumonic sheep; 8 untypable strains (5 from pneumonic calves and 3 from pneumonic sheep). In addition, strain A2S was used, which was a spontaneously derived streptomycin (Sm)-resistant mutant of *P. haemolytica* strain FA2 (serotype A2). *E. coli* strains and plasmids used are given in Table 1.

All strains were stored frozen in 50% (v/v) glycerol in brain heart

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Table 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference
<i>E. coli</i>		
DH1/DH5	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> (1989)
JM83	<i>ara Δ(lac-proAB) rpsL φ80α lacZΔM15</i>	Vieira & Messing (1982)
JC3272	<i>his lys trp</i>	Achtman <i>et al.</i> (1971)
J53	<i>pro met</i>	Meynell & Datta (1966)
Plasmids		
pRK2013	48 kb, Km ^R <i>IncP</i> Tra ⁺ ColE1	Figurski & Helinski (1979)
R6K	40 kb, Ap ^R (TEM-1) Sm ^R Tn2660 <i>IncX</i> Tra ⁺	Kontomichalou <i>et al.</i> (1970)
RP4	54 kb, Ap ^R (TEM-2) Km ^R Tc ^R Tn1 <i>IncP</i> Tra ⁺	Datta <i>et al.</i> (1971)
pRM3022	4.4 kb, Ap ^R (ROB-1)	Rubin <i>et al.</i> (1981)
pPH2	4.3 kb, Ap ^R (ROB-1)	This study
pPH33	4.3 kb, Ap ^R (ROB-1)	This study
pPH821	4.3 kb, Ap ^R (ROB-1)	This study
pPH843	4.3 kb, Ap ^R (ROB-1)	This study

infusion (BHI) (Oxoid) broth at -70°C and subcultured routinely on BHI agar medium with or without the supplementation of 5% (v/v) defibrinated sheep blood (Becton Dickinson). Where necessary, antibiotic-resistant strains were grown on agar or in liquid media containing appropriate antibiotics. The concentrations of the antibiotics (Sigma) used in selective media were ($\mu\text{g ml}^{-1}$): Ap (50), Sm (200), nalidixic acid (Nal) (20), kanamycin (Km) (50), tetracycline (Tc) (10) and Cm (25).

Determination of antibiotic resistance pattern, minimum inhibitory concentration (MIC) and β -lactamase production. Antibiotic resistance patterns of the *P. haemolytica* strains were determined by the disk-diffusion method (Barry & Thornsberry, 1980) using cartridge-borne antibiotic disks and a multiple disk dispenser (Oxoid). Sixteen antibiotics were tested at the following concentrations per disk (μg): Ap (10), Cm (30), colistin sulphate (10), erythromycin (15), gentamicin (10), Km (30), lincomycin (Lm) (2), Nal (30), neomycin (30), nitrofurantoin (300), polymyxin B (300 I.U.), Sm (10), sulphonamide compound (Su) (3300), sulphamethoxazole-trimethoprim (SXT) (25), Tc (30) and trimethoprim (Tp) (1.25). MICs of β -lactam antibiotics were determined by the agar-dilution method (Washington & Sutter, 1980) using Iso-sensitest agar media (Oxoid). The β -lactam antibiotics used were: Ap, penicillin G (Pe), carbenicillin (Carb), ticarcillin (Tic), cephalixin (Cex), cephalothin (Cet) and cephaloridine (Cer) (all from Sigma, except Carb which was from Beecham). The production of β -lactamase was tested by β -lactamase detection papers (Oxoid).

Preparation, purification and standardization of plasmid DNA. Plasmid DNA was extracted by the alkaline-lysis method (Sambrook *et al.*, 1989). A single colony of *P. haemolytica* or *E. coli* from a fresh overnight-culture plate was inoculated into 10 ml BHI broth and incubated at 37°C on a rotary shaker for 18 h. One ml of this culture was processed for miniprep plasmid DNA isolation. Plasmid DNA preparations were suspended in Tris/EDTA (TE) buffer (g l^{-1} ; Tris, 1.21; Na₂EDTA, 0.37; pH 8.0) and treated with pancreatic ribonuclease (Sigma) ($20 \mu\text{g ml}^{-1}$ final concentration) at 37°C for 30 min before use. Plasmid DNA from large volumes of culture (500–1000 ml) was extracted by the same method and purified by precipitation with polyethylene glycol (mol. mass 8000; Sigma) (Sambrook *et al.*, 1989)

followed by ammonium acetate (2.5 M final concentration) precipitation. The covalently closed circular (ccc) form of the plasmid DNA was then further purified by the acid-phenol extraction method of Zasloff *et al.* (1978) with the exception that MgCl₂ (1.5 mM final concentration) was used instead of NaCl in the low ionic strength buffer. Gel electrophoresis was in 0.8% agarose (type II-A; Sigma) and Tris/borate/EDTA (TBE) buffer (g l^{-1} ; Tris, 10.78; boric acid, 5.5; Na₂EDTA, 0.744) in a horizontal submarine electrophoresis apparatus (GNA-100 or 200; Pharmacia). After electrophoresis, the gels were stained with ethidium bromide (Sigma) ($1 \mu\text{g ml}^{-1}$). Plasmid DNA size was estimated by reference to marker DNA (e.g. supercoiled DNA ladder from Gibco-BRL). The concentration of plasmid DNA was determined by comparison with known quantities of stained marker DNA (e.g. *Hind*III-digested λ DNA from Gibco-BRL).

Transformation and conjugation. *E. coli* strains were transformed with plasmid DNA by the standard CaCl₂-procedure (Sambrook *et al.*, 1989). Conjugation was by the plate-mating method as described previously (Craig *et al.*, 1989), with the exception that overnight cultures instead of exponential-phase cultures of donor, recipient and/or helper strains were used and with mating for 6 h (*E. coli* recipient) or 16 h (*P. haemolytica* recipient) instead of 1 h. Plasmid pRK2013 was used as the helper plasmid to supply *IncP* conjugative transfer functions. Transconjugant-selection plates were incubated for 18 h (*E. coli* recipient) or 48 h (*P. haemolytica* recipient).

Amplification of plasmid DNA. *E. coli* transformants were grown in BHI broth, nutrient broth (Oxoid), Luria-Bertani (LB) broth (g l^{-1} ; tryptone, 10; yeast extract, 5; NaCl, 10), Terrific broth (g l^{-1} ; tryptone, 12; yeast extract, 24; glycerol, 4 ml; KH₂PO₄, 2.31; K₂HPO₄, 12.54) (Tartof & Hobbs, 1987) or in Terrific broth supplemented with 1% (w/v) yeast nitrogen base (YNB) (Difco) for 6–7 h to late exponential phase ($\text{OD}_{600} \sim 0.6$). A 0.1 volume was inoculated into corresponding fresh broth and incubated with vigorous shaking at 37°C for 2.5–3.0 h ($\text{OD}_{600} \sim 0.4$). At this stage, Cm ($170 \mu\text{g ml}^{-1}$ final concentration) (Clewett, 1972) or Tc ($15 \mu\text{g ml}^{-1}$ final concentration) was added and incubation continued for a further 14–15 h. Ampicillin selection was maintained throughout growth and the OD_{600} noted before the cells were used for preparation of plasmid DNA.

Restriction endonuclease analysis. Restriction endonuclease enzymes were purchased from Gibco-BRL, Stratagene or Boehringer Mannheim and used according to the manufacturers' instructions. Restriction fragments were separated by electrophoresis on 1.8–2% agarose.

Southern hybridization. A *Sau*3AI digest of plasmid pPH843 was used as a probe for hybridization to *Sau*3AI-digested fragments of the prototype ROB-1 plasmid pRM3022 transferred to Hybond-N membrane (Amersham). DNA fragments were labelled to high specific activity with [α -³²P]dATP (Amersham) by the random priming technique (Feinberg & Vogelstein, 1984). Hybridizations were performed at 42°C in the presence of 50% (v/v) formamide and blots were washed in $1 \times \text{SSC}$, 0.1% SDS at 60°C for 2 h prior to autoradiography.

β -Lactamase assay. β -Lactamase activity was assayed by the macroiodometric method of Ross & O'Callaghan (1975) on crude sonic extracts of *E. coli* cells grown overnight with shaking in 1 litre of BHI broth in the presence of Ap ($50 \mu\text{g ml}^{-1}$). Cells were pelleted, washed in potassium phosphate buffer (pH 7.0), and sonicated in the same buffer (4 $\times$ 30 s with 30 s intervals on ice) with an ultrasonic disintegrator (MSE). Supernates were collected after centrifugation at $140\,000 \text{ g}$ for 1 h at 4°C , dialysed overnight at 4°C against deionized distilled water, and concentrated by freeze-drying. Freeze-dried samples were reconstituted with phosphate buffer before use. Penicillin G, Ap, Carb, oxacillin (Oxa), cloxacillin (Clox), Cer, Cet and Cex were used as substrates to measure the rate of β -lactam hydrolysis. Inhibition of β -lactamase activity, either by 100 mM-NaCl or 0.1 mM-Clox, was carried

Fig. 1. Agarose gel (0.8%) electrophoresis of plasmid DNA preparations from *P. haemolytica* serotype A1 strains: B2070 (lane 1), CVL33 (lane 2), S/C 82/1 (lane 3), S/C 84/3 (lane 4); serotype A2 strains: B664 (lane 5), D/I 85/1 (lane 6), G/T 85/15 (lane 7) and untypable strain T1368 (lane 8). Supercoiled DNA ladder was used as the marker DNA (lane M). The figure is a composite of three separate gels run under identical conditions with the marker DNA in each. The two smaller plasmids of strain T1368 (lane 8) are not clearly separated in this figure. The diffuse staining regions in lanes 4, 5, 7 and 8 are assumed to represent chromosomal DNA.

out with Pe as substrate. Inhibitors were added to enzyme suspensions before addition of Pe. Protein content of the enzyme preparations was estimated by the Bradford assay (Bradford, 1976) using bovine serum albumin as standard, and specific activity of the β -lactamases was expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

Analytical isoelectric focusing. Analytical isoelectric focusing was performed at a constant power of 1 W for 4 h on Ampholine PAG plates (pH 3.5–9.5) (Pharmacia LKB) with an LKB Multiphor apparatus. The test samples were sonic extracts of *E. coli* containing either the β -lactamase-encoding plasmids from *P. haemolytica* or the known TEM- and ROB-type β -lactamase-encoding plasmids. The gel was stained with nitrocefin (0.5 mg ml^{-1}) (Oxoid) for detection of β -lactamase activity and photographed as described previously (Matthew *et al.*, 1975). Isoelectric points (pIs) of unknown β -lactamases were determined by reference to those of known β -lactamases.

Results

Antibiotic resistance and plasmid profiles of P. haemolytica strains

All 35 *P. haemolytica* strains showed resistance to Lm, Sm and Su. In addition, some strains were resistant to SXT, Tp, Tc, Ap and Cm. Plasmid analysis revealed the presence of plasmid DNA in eight of the strains (Fig. 1). The plasmid contents of these strains and their antibiotic resistance phenotypes are given in Table 2. A 4.3 kb plasmid was present in each of the four serotype A1 strains. Strain S/C 84/3 additionally contained two other plasmids of approximately 20 kb and 2.8 kb. The three

serotype A2 strains each appeared to contain two plasmids; two strains had similar plasmid profiles (~ 4.0 and 2.7 kb) whilst in the third strain the 2.7 kb plasmid was replaced by a 7.6 kb plasmid. The untypable strain had three plasmids, all apparently different from those of the A1 and A2 strains. Neither the T4 nor the T10 serotype strain appeared to contain plasmid DNA. Since the Lm, Sm and Su resistances were common to all the strains of *P. haemolytica*, it is unlikely that they are plasmid-mediated. Since these were the only resistances exhibited by the plasmid-carrying A2 and untypable strains, no resistance phenotype can be ascribed to their plasmids. The plasmid-carrying A1 strains were Ap^R and Tc^R , and also Cm^R in the case of strain S/C 84/3, and these were candidate resistances for carriage by these plasmids. However, Tc^R was also exhibited by two plasmid-less A1 strains.

Transformation of E. coli with P. haemolytica A1 plasmid DNA

Plasmid DNA from A1 strains B2070, CVL33, S/C 82/1 and S/C 84/3 was used to transform *E. coli* strain DH1 selecting separately for Ap^R , Tc^R and, in the case of S/C 84/3, Cm^R transformants. Only Ap^R transformants were obtained. Transformation frequencies were similar with plasmid DNA preparations from strains B2070, CVL33 and S/C 82/1, $0.7\text{--}1.3 \times 10^6$ transformants per μg plas-

Table 2. Plasmid profiles of *P. haemolytica* strains and their antibiotic resistance phenotypes

Serotype (<i>n</i> = no. of strains tested)	Animal source (health status)	Strain no. (positive for plasmid)	Antibiotic resistance phenotypes*	Plasmid (kb)
A1 (<i>n</i> = 18)	Bovine (pneumonic)	B2070	Ap, Tc	4.3
	Bovine (pneumonic)	CVL33	Ap, Tc	4.3
	Bovine (pneumonic)	S/C 82/1	Ap, Tc	4.3
	Bovine (in-contact)	S/C 84/3	Ap,† Tc, Cm	~20, 4.3, 2.8
	Ovine (pneumonic)	B664	None	7.6, 4.0
A2 (<i>n</i> = 7)	Bovine (pneumonic)	D/I 85/1	None	3.9, 2.7
	Bovine (healthy)	G/T 85/15	None	3.9, 2.7
	Ovine (pneumonic)	T1368	None	5.9, 3.1, 2.9

* All 35 strains (including the two T4 and T10 strains) tested by the disk-diffusion method were resistant to Lm, Sm and Su.

† Partially resistant, as observed by partial inhibition of growth.

Table 3. Mobilization frequencies of *P. haemolytica* Ap^R plasmids from *E. coli* transformants to *P. haemolytica* and *E. coli* recipients by conjugation

<i>E. coli</i> donor strain	Helper plasmid*	Recipient strain†	Mobilization frequency (transconjugants per recipient)‡
DH1(pPH2)	pRK2013	A2S Ph	$1.2(\pm 0.3) \times 10^{-5}$
		JC3272 Ec	$2.8(\pm 0.5) \times 10^{-4}$
DH1(pPH33)	pRK2013	A2S Ph	$1.3(\pm 0.5) \times 10^{-5}$
		JC3272 Ec	$3.3(\pm 0.6) \times 10^{-4}$
DH1(pPH821)	pRK2013	A2S Ph	$1.3(\pm 0.4) \times 10^{-5}$
		JC3272 Ec	$3.4(\pm 0.7) \times 10^{-4}$
DH1(pPH843)	pRK2013	A2S Ph	$4.4(\pm 0.4) \times 10^{-4}$
		JC3272 Ec	$1.3(\pm 0.2) \times 10^{-1}$

* *E. coli* DH5 (Nal^R) was used as the carrier of the helper plasmid when Sm^R recipient strains (A2S and JC3272) were used. Transconjugants were selected on BHI agar with Ap + Sm. Recipient cell numbers were calculated by plating appropriate dilutions on BHI agar containing Sm.

† Ph, *P. haemolytica*; Ec, *E. coli*.

‡ Each value shown is the mean ($\pm$ SEM) of observations from three separate experiments.

mid DNA, but were noticeably lower with that from strain S/C 84/3, presumably because the presence of other species of plasmid DNA reduced the apparent transformation frequency. Similar results were obtained with *E. coli* JM83 as recipient. Plasmid DNA was not detected in *E. coli* transformants and their presence was only confirmed by transformation of *E. coli* again to Ap^R with the plasmid DNA preparations. The Ap^R-encoding plasmids from *P. haemolytica* strains B2070, CVL33,

S/C 82/1 and S/C 84/3 were designated pPH2, pPH33, pPH821 and pPH843 respectively.

Mobilization of *P. haemolytica* plasmids from *E. coli* to *P. haemolytica* and *E. coli* by conjugation

Plasmid transfer from *P. haemolytica* strains B2070, CVL33, S/C 82/1 or S/C 84/3 to either *P. haemolytica* or

Fig. 2. Agarose gel (0.8%) electrophoresis of plasmid DNA preparations from *P. haemolytica* and *E. coli* Ap^R transconjugants. *P. haemolytica* A1 Ap^R plasmids (all 4.3 kb) pPH2 (lane 1), pPH33 (lane 2), pPH821 (lane 3) and pPH843 (lane 4) are seen in *P. haemolytica* A2S transconjugants (group A), but only pPH843 is visible in *E. coli* JC3272 transconjugants (group B). The helper plasmid pRK2013 (48.0 kb) used in conjugation is shown by *E. coli* transconjugants, but not by *P. haemolytica* transconjugants. Lane M represents a supercoiled DNA ladder. The upper faint band present in all lanes of group A probably represents an open circular (oc) form of the 4.3 kb plasmid.

Table 4. β -Lactamase profile and MICs of β -lactam antibiotics for plasmid-containing Ap^R strains

For abbreviations of β -lactam antibiotics, see Methods.

Strain	β - Lactamase	MIC ($\mu\text{g ml}^{-1}$)							
		Pe	Ap	Carb	Tic	Cex	Cet	Cer	
<i>P. haemolytica</i>									
B2070	+	64	64	64	16	2	4	2	
CVL33	+	128	128	128	32	2	4	2	
S/C 82/1	+	64	64	64	16	2	4	2	
S/C 84/3	—	2	1	0.5	0.5	2	4	1	
A2S (plasmid-free)	—	<0.25	<0.25	<0.25	<0.25	1	<0.25	<0.25	
A2S(pPH2), A2S(pPH33) and A2S(pPH821)	} *	+	128	64	256	32	2	4	2
A2S(pPH843)*		+	256	128	256	64	2	4	2
<i>E. coli</i>									
DH1 (plasmid-free)	—	4	2	2	1	4	2	1	
DH1(pPH2), DH1(pPH33) and DH1(pPH821)	} †	$\pm$ ‡	2048	1024	> 2048	2048	16	1024	16
DH1(pPH843)†		+	> 2048	> 2048	> 2048	> 2048	128	2048	128
DH1(RP4)	+	> 2048	> 2048	> 2048	> 2048	8	32	32	
DH1(pRM3022)	+	2048	2048	> 2048	> 2048	32	64	64	

* Transconjugants; † transformants; ‡ weakly positive.

E. coli by conjugation was unsuccessful, either in the presence or absence of the broad-host-range helper plasmid pRK2013. The large plasmid present in S/C 84/3 was therefore not able to mobilize either of the two smaller plasmids present in that strain. However, the plasmids could be mobilized from *E. coli* DH1 into both *P. haemolytica* A2S and *E. coli* JC3272 by using the transfer functions of pRK2013 (Table 3). No transconju-

gants were obtained in the absence of the helper plasmid. The mobilization frequency into *E. coli* was noticeably higher than into *P. haemolytica*.

Interestingly, plasmid pPH843 was mobilized with the highest frequency into *E. coli* JC3272 by conjugation, yet showed the lowest transformation frequency into *E. coli* DH1. Plasmid profiles of all transconjugants of *P. haemolytica* A2S (Fig. 2) showed the presence of a

Table 5. Substrate and inhibition profiles, and specific activities of *E. coli* extracts containing β -lactamases encoded by plasmids pPH2, pPH33, pPH821 and pPH843 compared with those of TEM- and ROB-type β -lactamases

For abbreviations of β -lactam antibiotics, see Methods. Values are the means of two independent determinations.

Plasmid (β -lactamase type)	Relative rate of hydrolysis (%)*							Specific activity†	Percentage activity in presence of:‡	
	Ap	Carb	Oxa	Clox	Cer	Cet	Cex		NaCl (100 mM)	Clox (0.1 mM)
R6K (TEM-1)	115	13	7	2	168	15	11	6.8	98	80
RP4 (TEM-2)	117	14	5	<2	166	15	10	49.6	98	75
pRM3022 (ROB-1)	189	29	2	0	42	4	37	13.0	100	32
pPH2	178	31	2	0	40	6	38	0.8	100	41
pPH33	185	32	2	0	43	4	33	0.76	100	36
pPH821	187	32	2	0	42	5	33	0.6	100	38
pPH843	196	27	2	0	38	4	30	10.0	100	28

* Activity relative to activity against penicillin G (defined as 100%).

† Expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ with penicillin G as substrate.

‡ Expressed as a percentage of the activity against penicillin G in the presence of inhibitor.

predominant 4.3 kb band, but a band of similar size was visible only in transconjugants of *E. coli* JC3272 containing plasmid pPH843. This confirms that the 4.3 kb plasmid present in the original strain S/C 84/3 (Table 2) encoded Ap^R. *E. coli* transconjugants showed the presence of plasmid pRK2013, which was not shown by *P. haemolytica* transconjugants (Fig. 2) as pRK2013 is a ColE1-based replicon which does not replicate in *P. haemolytica*.

MICs of β -lactam antibiotics for plasmid-bearing *P. haemolytica* and *E. coli* strains

The MICs of β -lactam antibiotics correlated well for the original *P. haemolytica* isolates and the Ap^R transconjugant strains of *P. haemolytica* A2S (Table 4). Wild-type isolate S/C 84/3 was β -lactamase-negative and showed much lower MIC values for those antibiotics to which the other three original isolates were clearly resistant. However, strain A2S(pPH843), which contains plasmid pPH843 from strain S/C 84/3, exhibited a MIC profile similar to that of the other transconjugants. The MICs for both the penicillin and cephalosporin groups of antibiotics were markedly increased in each of the *E. coli* DH1 transformants, representing an 8- to 256-fold increase for transformants with plasmids pPH2, pPH33 and pPH821, and a 64- to >4096-fold increase for transformants with plasmid pPH843, over those values obtained with the original *P. haemolytica* hosts. The TEM-type β -lactamase plasmid RP4 and the ROB-type β -lactamase plasmid pRM3022 contained in *E. coli* DH1

were used for a comparison with the *P. haemolytica* plasmids in the same *E. coli* strain. Similar MIC profiles for the penicillin group and somewhat different profiles for the cephalosporin group were obtained when *E. coli* containing either type of the known β -lactamase plasmids was compared with *E. coli* containing the *P. haemolytica* plasmids.

β -Lactamase characterization

Table 5 shows the substrate and inhibition profiles, and specific activities of the plasmid-mediated *P. haemolytica* β -lactamases extracted from *E. coli* DH1 transformants. The prototype TEM-1 from *E. coli* strain J53(R6K), TEM-2 from DH1(RP4) and ROB-1 from DH1(pRM3022) were used as the reference β -lactamases. The TEM- and ROB-type enzymes can be distinguished by their different activities against penicillins and cephalosporins as well as by their pI values (Rubin *et al.*, 1981; Medeiros *et al.*, 1986). The substrate and inhibition profiles of β -lactamases produced by *P. haemolytica* plasmids pPH2, pPH33, pPH821 and pPH843 were very similar to those exhibited by the prototype ROB-1 (Table 5). As analysed by isoelectric focusing, the main bands of β -lactamase activity produced by these plasmids (Fig. 3, lanes 4–7) focused at the same pI (8.1) as the prototype ROB-1 activity (lane 3), while the TEM-1 and TEM-2 enzymes focused at pIs 5.4 and 5.6 respectively (lanes 1 and 2). The accompanying bands in lanes 1 and 2, and an additional band (pI 8.0) common to all enzyme preparations, probably represent

Fig. 3. Comparative analytical isoelectric focusing patterns of *E. coli* extracts containing known β -lactamases and those encoded by the *P. haemolytica* plasmids. Lane 1, TEM-1 (pI 5.4); lane 2, TEM-2 (pI 5.6); lane 3, ROB-1 (pI 8.1) (Medeiros *et al.*, 1985). The β -lactamases produced by plasmids pPH2 (lane 4), pPH33 (lane 5), pPH821 (lane 6) and pPH843 (lane 7) are seen at the same pI as the ROB-1 enzyme. A common additional band (pI 8.0) representing the chromosomally determined β -lactamase of *E. coli* is seen in all lanes. At least 10 times concentrated enzyme preparations from strains containing pPH2, pPH33 and pPH821 compared to the preparation from the strain containing pPH843 were loaded onto the gel.

Fig. 4. Agarose gel (0.8%) showing the yields of *P. haemolytica* Ap^R plasmids pPH2 (lane 1), pPH33 (lane 2), pPH821 (lane 3), and pPH843 (lane 4) from *E. coli* DH1 transformants after amplification procedures. The *E. coli* transformants were grown in Terrific broth alone (group A), or Terrific broth + Cm (group B) or Terrific broth + 1% YNB + Cm (group C). M, supercoiled DNA ladder. The additional upper band(s) (groups B and C) probably represent oc forms and/or dimers of the 4.3 kb plasmid.

Table 6. *Effects of amplification procedures on P. haemolytica plasmid DNA in E. coli DH1*

Ap ^R plasmid	Extent of amplification under different growth conditions* (OD ₆₀₀ of culture at time of harvest)					
	Broth medium alone†	BHI broth + Cm	Nutrient broth + Cm	LB broth + Cm	Terrific broth + Cm	Terrific broth + 1% YNB + Cm
pPH2	— (≤2.48)	— (1.3)	— (0.84)	— (0.97)	++ (1.4)	+++ (1.53)
pPH33	— (≤2.48)	— (1.4)	— (0.84)	— (0.96)	++ (1.32)	+++ (1.35)
pPH821	— (≤2.46)	— (1.3)	— (0.83)	— (0.94)	++ (1.3)	+++ (1.4)
pPH843	+/++‡ (≤2.49)	+++ (1.68)	+++ (0.92)	++++ (1.0)	++++ (1.7)	++++ (1.8)

* Grading of plasmid DNA amplification was done according to the relative intensity of ethidium bromide staining of plasmid DNA in agarose gels, ranging from a detectable level (+) to the maximum amount (+++++) obtained; — indicates 'not detectable'.

† BHI broth, Nutrient broth, LB broth or Terrific broth.

‡ Detectable in LB broth only and amplified in Terrific broth.

variant, satellite bands and the chromosomal β -lactamase of *E. coli*, respectively, as reported by Matthew *et al.* (1975) and Medeiros *et al.* (1985). The β -lactamase produced by the plasmid pPH843 showed a higher specific activity in *E. coli* than those produced by the other three *P. haemolytica* plasmids (Table 5). This is probably related to plasmid copy number as pPH843 was the only plasmid DNA visible in preparations from *E. coli* (Fig. 2B lane 4).

Amplification of P. haemolytica plasmid DNA in E. coli

As each Ap^R plasmid apparently had a low copy number in *E. coli*, attempts were made to amplify the plasmid DNA in *E. coli* transformants. DH1 transformants were grown in various nutrient media with or without the addition of Cm and the influence of culture conditions on the yield of the Ap^R plasmids was observed (Table 6). Changes in growth media alone affected the yield of plasmid pPH843, which was detected in LB broth and amplified in Terrific broth. This plasmid was, in addition, further amplified when exposed to Cm in any broth medium used, although Terrific broth gave the highest yield (Table 6; Fig. 4). Amplification was only evident with the other three plasmids (pPH2, pPH33 and pPH821) when the host strains were grown in LB or Terrific broth in the presence of Cm. Terrific broth supplemented with 1% YNB gave the highest yield (Table 6; Fig. 4). Under these conditions, plasmid DNA could be recovered from *E. coli* in amounts similar to

those obtained from the original *P. haemolytica* isolates or *P. haemolytica* transconjugants. A similar but slightly less pronounced amplification was achieved when Cm was replaced by Tc (data not shown).

Higher yields of plasmids pPH2, pPH33 and pPH821, but less of plasmid pPH843, were achieved from *E. coli* JM83 transformants than from their DH1 counterparts following identical amplification procedures (data not shown). *H. influenzae* plasmid pRM3022 (4.4 kb) was amplified to a similar extent to that of *P. haemolytica* plasmid pPH843 in *E. coli* strain JM83 (Fig. 5).

Restriction enzyme analysis of plasmid DNA

Of 42 restriction enzymes tested, 12 were found to cut *P. haemolytica* plasmids pPH2, pPH33, pPH821 and pPH843 giving fragments of identical size. These enzymes were *AluI* (10 fragments), *Sau3AI*, *TaqI*, *CfoI* (5 fragments each), *DraI*, *ThaI* (3 fragments each), *HinfI*, *ScaI* (2 fragments each), *PstI*, *ApaLI*, *AvaI* and *Bsp1286I* (1 fragment each). Representative restriction digests of plasmid pPH843 are shown in Fig. 6A; the unique sites for the latter four enzymes were confirmed by double digestion (lanes 7–9). The prototype ROB-1 plasmid pRM3022 produced identical restriction patterns to those of pPH843 with all the enzymes except *AluI*, *Sau3AI* and *DraI*. With these three enzymes slight differences were found (Fig. 6B) whereby pRM3022 had two extra *AluI* sites and an additional *Sau3AI* and *DraI* site.

Fig. 5. Agarose gel (0.8%) showing the yields of *P. haemolytica* Ap^R plasmid pPH843 (4.3 kb) (lane 1) and *H. influenzae* ROB-type β -lactamase plasmid pRM3022 (4.4 kb) (lane 2) from *E. coli* JM83 transformants after amplification procedures. The *E. coli* transformants were grown in Terrific broth alone (group A), or Terrific broth + Cm (group B) or Terrific broth + 1% YNB + Cm (group C). M, supercoiled DNA ladder. The upper bands (lane 2; group C) probably represent a 'step ladder' of ccc molecules with different numbers of supercoil turns (Kieser, 1984).

Fig. 6. Restriction enzyme digestion patterns of ROB-1 β -lactamase-producing plasmids of *P. haemolytica* and *H. influenzae* origin. A: Restriction fragment(s) of plasmid pPH843 generated by the enzymes *Taq*I (lane 1), *Cfo*I (lane 2), *Tha*I (lane 3), *Hinf*I (lane 4), *Sca*I (lane 5), *Pst*I or *Apa*LI or *Ava*I or *Bsp*1286I (lane 6), *Apa*LI + *Pst*I (lane 7), *Ava*I + *Pst*I (lane 8) and *Bsp*1286I + *Pst*I (lane 9). B: restriction fragments of plasmids pPH33 (lane a), pRM3022 (lane b) and pPH843 (lane c) generated by enzymes *Alu*I (group 10), *Sau*3AI (group 11) and *Dra*I (group 12). Lane M: 1 kb DNA ladder as molecular size standard. The faint higher molecular mass bands in lanes 1, 7, 10a and 11c are presumed to be partially digested products.

Fig. 7. Homology between the ROB-1-producing plasmids. A: ethidium-bromide-stained agarose gel (1.8%) showing *Sau*3AI-digested fragments of plasmids pPH843 (lane 1) and pRM3022 (lane 2). B: autoradiograph of a Southern blot of the gel shown in panel A probed with *Sau*3AI-digested pPH843. Arrow (panel B, lane 2) shows a band exhibiting only faint hybridization, equivalent to ~600 bp fragment of pRM3022 (arrow, panel A, lane 2). M, 1 kb DNA ladder as molecular size standard.

Plasmid DNA hybridization

Hybridization experiments were carried out to assess the degree of homology between the ROB-1 plasmid from *P. haemolytica* (pPH843) and that from *H. influenzae* (pRM3022). All fragments of pRM3022 obtained by *Sau*3AI digestion hybridized to *Sau*3AI-digested ³²P-labelled pPH843, except one fragment (Fig. 7A, lane 2, arrowed), which showed only weak hybridization (Fig. 7B, lane 2, arrowed). This indicated a high degree of homology between the two ROB-1 plasmids, but suggested that a part of the DNA sequence from the *Haemophilus* plasmid was not present in the *Pasteurella* plasmid.

Discussion

Plasmid DNA was detected in four serotype A1 strains, three A2 strains and one untypable strain of *P. haemolytica*. The plasmid-bearing isolates of the same serotype showed similar plasmid profiles which clearly differed from the profiles exhibited by the other serotypes. This was noted previously by Boyce & Morter

(1986). Serotype A1 is predominant in bovine pasteurellosis and accounts for over 80% of *P. haemolytica* isolates (Frank, 1989). In our study, plasmid-mediated Ap resistance was found only in A1 strains of bovine origin obtained from different sources. This may well be due to selection pressure created by routine antibiotic treatment of cattle. All of these strains were isolated from pneumonic or in-contact calves (Table 2), but the significance of this is difficult to assess without further examination of a larger number of isolates. Small, non-conjugative plasmids associated with resistance to β -lactam antibiotics, particularly penicillins, have also been isolated from *P. haemolytica* A1 strains in the USA (Chang *et al.*, 1987), France (Livrelli *et al.*, 1988) and Germany (Schwarz *et al.*, 1989). In the UK, multiple resistance to antibiotics, including Ap and Pe, has been reported (Wray & Morrison, 1983; Allan *et al.*, 1985; Shoo, 1989), but a detailed analysis of transfer of an Ap^R plasmid between *E. coli* and *P. haemolytica* has been described only for one strain (Craig *et al.*, 1989). This plasmid, pPH33, was one of the four Ap^R plasmids of similar size (4.3 kb) examined here. Plasmids pPH2, pPH33 and pPH821 were essentially indistinguishable with respect to their transfer efficiencies, amplification properties and MICs with various β -lactam antibiotics. Plasmid pPH843 had a similar size (4.3 kb) to the other three, but had a lower copy number in the wild-type *P. haemolytica* strain S/C 84/3 (Fig. 1, lane 4) which was reflected in a β -lactamase-negative phenotype on test strips and low MIC values. Plasmid DNA preparations from strain S/C 84/3 consistently yielded a low number of Ap^R transformants. In contrast, an *E. coli* DH1 transformant containing pPH843 was strongly β -lactamase positive on a test strip, showed a strikingly high increase in MIC values for β -lactams and yielded the highest transfer frequency by conjugation. It is possible that the co-existing plasmids in *P. haemolytica* strain S/C 84/3 suppress the copy number of pPH843. This was substantiated by the observation that when the plasmid was transferred from the original host strain to another *P. haemolytica* strain, A2S, the β -lactamase phenotype was positive and the MIC values were comparable to those of the other Ap^R plasmids in *P. haemolytica*. It is interesting in this respect to note that each of the plasmids expressed Ap^R in the *P. haemolytica* A2 strain, yet no natural antibiotic resistance associated with plasmid DNA was found in three plasmid-bearing A2 isolates (Table 2). This is in keeping with the observations that each serotype appears to maintain a distinct plasmid profile and that transfer of plasmids between serotypes may not readily occur in nature.

Plasmids pPH2, pPH33 and pPH821 were very unstable in *E. coli* and were rapidly lost after passage in the absence of antibiotic selection. Plasmid pPH843, on

the other hand, was stable even after 10 passages in the absence of selection (data not shown). The lower specific activities of β -lactamases specified by the former three plasmids (Table 5) may be due to their instability in *E. coli*. Interestingly, pPH843 was detectable in plasmid DNA preparations from *E. coli* JC3272 transconjugants (Fig. 2), but not from *E. coli* DH1 or JM83 transformants, suggesting host strain differences in terms of plasmid content.

The MIC values for all the plasmids in *E. coli* were noticeably increased over the values obtained with *P. haemolytica* hosts (Table 4). The differences are comparable to or higher in most cases than those reported by Livrelli *et al.* (1988) for a ROB-type Ap^R plasmid of similar size isolated from bovine *Pasteurella* strains. Differences in MICs between the plasmid-free *P. haemolytica* and *E. coli* strains (Table 4) could be due to differences in outer membrane permeability or the native chromosomally encoded β -lactamase present in *E. coli* K12 (Matthew *et al.*, 1975). Zimmerman & Hirsh (1980) described a bovine isolate of *P. haemolytica* with a plasmid-associated β -lactamase which had high activity against cephaloridine in cell extracts. Our data indicate little resistance to the cephalosporin class of β -lactams by the original and transconjugant *P. haemolytica* isolates, but higher resistance of *E. coli* transformants containing the Ap^R plasmids, particularly pPH843. The sensitivity and substrate profiles of the ROB- and TEM-type β -lactamases have been reported to be similar (Guttmann *et al.*, 1988) and this was confirmed in the present study (Tables 4 and 5). The ROB-1 β -lactamase has been differentiated from the TEM-1 enzyme by its higher pI and its ability to hydrolyse ampicillin more rapidly and cephaloridine more slowly (Rubin *et al.*, 1981). Our results (Table 5; Fig. 3) confirmed that all four Ap^R plasmids encode enzymes of the ROB-1 type. The ROB-1-encoding plasmid was first identified in *H. influenzae* of human origin (Rubin *et al.*, 1981) and later in *Actinobacillus pleuropneumoniae* of animal origin (Medeiros *et al.*, 1986).

A *P. haemolytica* isolate harbouring the ROB-1 plasmid was first reported in France (Livrelli *et al.*, 1988). Our findings confirm the widespread nature of ROB-1 β -lactamase-producing strains among the isolates of *P. haemolytica* A1 serotype. The fact that the ROB-1 plasmids examined here could be easily mobilized by conjugation (from *E. coli*) may explain the spread of such plasmids, although it does not explain the apparent absence of these plasmids in serotypes other than A1.

Previous investigators have reported that the recovery of *P. haemolytica* Ap^R plasmids from *E. coli* transformants was either poor (Livrelli *et al.*, 1988; Craig *et al.*, 1989) or non-existent (Zimmerman & Hirsh, 1980). Similar results were obtained in this study, but we found

that the *P. haemolytica* plasmids in *E. coli* could be amplified by manipulation of the growth conditions. The amplification of ColE1-type plasmids in *E. coli* by inhibition of protein synthesis with Cm (Clewell, 1972) or amino acid starvation (Schroeter *et al.*, 1988) is well established. More recently, amplification of plasmid pBR322 in *E. coli* was reported without blocking protein synthesis by using amino acid and Fe³⁺ supplements (Angelov & Ivanov, 1989). In all these cases, the extent of amplification depended on the growth medium used. In our work, LB broth and Terrific broth proved most useful, probably due to the absence in these media of glucose which influences the formation of DNA-protein relaxation complexes during plasmid DNA replication in *E. coli* (Clewell & Helinski, 1972). We observed highest amplification in Terrific broth, which was specifically formulated for plasmid and cosmid production (Tartof & Hobbs, 1987). The reason for obtaining the best yield of plasmids from YNB-supplemented Terrific broth is not clear, but might be due to the presence of multivitamins, amino acids and metal ions, including Fe³⁺. However, the further addition of amino acids and/or Fe³⁺ to YNB-supplemented Terrific broth reduced the amplification rate for plasmids (data not shown), which contrasts with the findings of Angelov & Ivanov (1989).

Restriction enzyme analysis and hybridization experiments demonstrated that the prototype ROB-1 β -lactamase-encoding plasmid pRM3022 from *H. influenzae* and those isolated from the *P. haemolytica* strains shared multiple fragments, but that they were not identical. Plasmid pRM3022 was slightly larger (4.4 kb) than the *P. haemolytica* plasmids (4.3 kb) and this was reflected in the presence of extra *AluI*, *Sau3AI* and *DraI* sites (Fig. 6B) and only weak hybridization of one ~600 bp *Sau3AI* fragment to pPH843 (Fig. 7, arrowed). As the hybridization was performed under high stringency conditions, this suggests that a major portion of the DNA sequence of this fragment is not present in pPH843. However, the data suggest a close relationship between the ROB-1-producing plasmids from the two different species and that one plasmid might have evolved from the other either by deletion or by recombination with pre-existing cryptic plasmids in the respective strains. Livrelli *et al.* (1991) have also recently reported homology between ROB-1 β -lactamase-encoding plasmids from *Pasteurella* isolates in France and *Haemophilus* strains. Since the restriction profiles of the four *P. haemolytica* Ap^R plasmids examined in this study were identical, they offered no explanation for the greater stability and higher amplification in *E. coli* of pPH843 compared to the other three plasmids.

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References

- ACHTMAN, M., WILLETS, N. & CLARK, A. J. (1971). Beginning a genetic analysis of conjugational transfer determined by the F factor in *Escherichia coli* by isolation and characterization of transfer-deficient mutants. *Journal of Bacteriology* **106**, 529–538.
- ALLAN, E. M., WISEMAN, A., GIBBS, H. A. & SELMAN, I. E. (1985). *Pasteurella* species isolated from the bovine respiratory tract and their antimicrobial sensitivity pattern. *Veterinary Record* **117**, 629–631.
- ANGELOV, I. & IVANOV, I. (1989). Amplification of bacterial plasmids without blocking protein biosynthesis. *Plasmid* **22**, 160–162.
- BARRY, A. L. & THORNSBERRY, C. (1980). Susceptibility tests: diffusion test procedures. In *Manual of Clinical Microbiology*, pp. 463–474. Edited by E. H. Lennette, A. Balows, W. J. Hausler & J. P. Truant. Washington, DC: American Society for Microbiology.
- BOYCE, J. R. & MORTER, R. L. (1986). Plasmid profile analysis of bovine isolates of *Pasteurella haemolytica*. *American Journal of Veterinary Research* **47**, 1205–1207.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CHANG, Y. F., RENSHAW, H. W. & YOUNG, R. (1987). Pneumonic pasteurellosis: examination of typable and untypable *Pasteurella haemolytica* strains for leukotoxin production, plasmid content, and antimicrobial susceptibility. *American Journal of Veterinary Research* **48**, 378–384.
- CLEWELL, D. B. (1972). Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. *Journal of Bacteriology* **110**, 667–676.
- CLEWELL, D. B. & HELINSKI, D. R. (1972). Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. *Journal of Bacteriology* **110**, 1135–1146.
- CRAIG, F. F., COOTE, J. G., PARTON, R., FREER, J. H. & GILMOUR, N. J. L. (1989). A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. *Journal of General Microbiology* **135**, 2885–2890.
- DATTA, N., HEDGES, R. W., SHAW, E. J., SYKES, R. B. & RICHMOND, M. H. (1971). Properties of R factor from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **108**, 1244–1249.
- DONACHIE, W. (1988). *Pasteurella haemolytica* as a respiratory tract pathogen of sheep. In *Bacterial Infections of Respiratory and Gastrointestinal Mucosae*, Special Publications of the Society for General Microbiology vol. 27, pp. 57–66. Edited by W. Donachie, E. Griffith & J. Stephen. Oxford: IRL Press.
- FEINBERG, A. P. & VOGELSTEIN, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **137**, 266–267.
- FIGURSKI, D. H. & HELINSKI, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 1648–1652.
- FRANK, G. H. (1989). Pasteurellosis of cattle. In *Pasteurella and Pasteurellosis*, pp. 197–222. Edited by C. Adlam & J. M. Rutter. London: Academic Press.
- GUTTMANN, L., WILLIAMSON, R., COLLATZ, E. & ACAR, J. F. (1988). Mechanisms of beta-lactam resistance in *Haemophilus influenzae*. *European Journal of Clinical Microbiology and Infectious Disease* **7**, 610–615.
- HAGHOUR, R., HELLMANN, E. & SCHMIDT, J. (1987). Plasmids and resistance to 9 chemotherapeutic agents of *Pasteurella multocida* and *Pasteurella haemolytica*. *Journal of Veterinary Medicine* **B34**, 509–518.
- KIESER, T. (1984). Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 19–36.
- KONTOMICHALOU, P., MITANI, M. & CLOWES, R. C. (1970). Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under either relaxed or stringent control. *Journal of Bacteriology* **104**, 34–44.
- LIVRELLI, V. O., DARFEUILLE-RICHAUD, A., RICH, C. D., JOLY, B. H. & MARTEL, J.-L. (1988). Genetic determinant of the ROB-1 β -lactamase in bovine and porcine *Pasteurella* strains. *Antimicrobial Agents and Chemotherapy* **32**, 1282–1284.
- LIVRELLI, V., PEDUZZI, J. & JOLY, B. (1991). Sequence and molecular characterization of the ROB-1 β -lactamase gene from *Pasteurella haemolytica*. *Antimicrobial Agents and Chemotherapy* **35**, 242–251.
- MATTHEW, M., HARRIS, A. M., MARSHALL, M. J. & ROSS, G. W. (1975). The use of analytical isoelectric focusing for detection and identification of β -lactamases. *Journal of General Microbiology* **88**, 169–178.
- MEDEIROS, A. A., COHENFORD, M. & JACOBY, G. A. (1985). Five novel plasmid-determined β -lactamases. *Antimicrobial Agents and Chemotherapy* **27**, 715–719.
- MEDEIROS, A. A., LEVESQUE, R. & JACOBY, G. A. (1986). An animal source for the ROB-1 β -lactamase of *Haemophilus influenzae* type b. *Antimicrobial Agents and Chemotherapy* **29**, 212–215.
- MEYNELL, E. & DATTA, N. (1966). The relation of resistance transfer factors to the F-factor (sex-factor) of *Escherichia coli* K12. *Genetical Research* **7**, 134–140.
- ROSS, G. W. & O'CALLAGHAN, C. H. (1975). β -Lactamase assays. *Methods in Enzymology* **43**, 69–85.
- RUBIN, L. G., MEDEIROS, A. A., YOLKEN, R. H. & MOXON, E. R. (1981). Ampicillin treatment failure of apparently β -lactamase-negative *Haemophilus influenzae* type b meningitis due to novel β -lactamase. *Lancet* **ii**, 1008–1010.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SCHROETER, A., RIETHDORF, S. & HECKER, M. (1988). Amplification of different ColE1 plasmids in *Escherichia coli* *relA* strain. *Journal of Basic Microbiology* **28**, 553–555.
- SCHWARZ, ST., SPIES, U., REITZ, B., SEYFERT, H.-M., LAMMLER, CH. & BLOBEL, H. (1989). Detection and interspecies-transformation of a β -lactamase-encoding plasmid from *Pasteurella haemolytica*. *Zentralblatt für Bakteriologie und Hygiene A270*, 462–469.
- SHOO, M. K. (1989). Comparing different isolates of *Pasteurella haemolytica* from beef calves using their *in vitro* antimicrobial sensitivity patterns. *Veterinary Microbiology* **20**, 73–78.
- TARTOF, K. D. & HOBBS, C. A. (1987). Improved media for growing plasmid and cosmid clones. *Bethesda Research Laboratory Focus* **9**, 12.
- VIEIRA, J. & MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- WASHINGTON, J. A. & SUTTER, V. L. (1980). Dilution susceptibility test: agar and macro-broth dilution procedures. In *Manual of Clinical Microbiology*, pp. 453–456. Edited by E. H. Lennette, A. Balows, W. J. Hausler & J. P. Truant. Washington, DC: American Society for Microbiology.
- WRAY, C. & MORRISON, J. R. A. (1983). Antibiotic resistant *Pasteurella haemolytica*. *Veterinary Record* **113**, 143.
- YATES, W. D. G. (1982). A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Canadian Journal of Comparative Medicine* **46**, 225–263.
- ZASLOFF, M., GINDER, G. D. & FELSENFELD, G. (1978). A new method for the purification and identification of covalently closed circular DNA molecules. *Nucleic Acids Research* **5**, 1139–1151.
- ZIMMERMAN, M. L. & HIRSH, D. C. (1980). Demonstration of an R plasmid in a strain of *Pasteurella haemolytica* isolated from feedlot cattle. *American Journal of Veterinary Research* **41**, 166–169.