ANALYSIS OF PLASMID DNA FROM *PASTEURELLA HAEMOLYTICA* AND CONSTRUCTION OF CLONING VECTORS

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"Even though we have seen some dimming of unblinking support for scientific research and molecular biology is of course much more crowded, any of my students still has opportunity of a crack at revolutionary discovery if they will but seize the day".

Joshua Lederberg

(Pioneer of phage & plasmid DNA, Nobel Laureate of 1958 and first Editor-in-Chief of Encyclopedia of Microbiology of 1992).

DECLARATION

The thesis is the original work of the author:

A. K. Azad

DEDICATION

This thesis is dedicated to my elder brother, Alhaj Md. A. Khair, who supported me tirelessly throughout my academic career before I came to Glasgow.

It is also dedicated to my wife, Anwari, and to my son, Rakin, who are always sources of mental support, happiness, love and inspiration for my future.

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ABBREVIATIONS USED IN THIS STUDY*

Ap^R	Ampicillin resistance
BHI	Brain heart infusion
bla	Beta-lactamase gene
bp	Base pair(s)
BRDC	Bovine respiratory disease complex
BSA	Bovine serum albumin
°C	Degree(s) Celsius
CCC	Covalently closed circular
CIP	Calf intestinal phosphatase
Col	Colicin
dATP	deoxy-Adenosine triphosphate
dCTP	deoxy-Cytidine triphosphate
dGTP	deoxy-Guanosine triphosphate
dTTP	deoxy-Thymidine triphosphate
dNTP	deoxy-Nucleoside triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetate
ETEC	Enterotoxigenic *E. coli*
F	Fertility
g	Gram(s) (but 'xg' indicates 'acceleration due to gravity')
h	Hour(s)
Inc	Incompatibilty
IPTG	Isopropylthio-β-D-galactoside
IRP	Iron-regulated protein
kb	Kilobase pair(s)
kDa	Kilo Dalton
lac	Lactose operon gene of *E. coli*
lacI	Regulatory gene for the *lac* operon
lacZ'	A part of the *lac* operon
LB	Luria-Bertani
LMP	Low melting point

Abbreviations contd.

LPS	Lipopolysaccharide
LT	Heat-labile toxin
M	Molar
mM	Millimolar
MCS	Multiple cloning site
mg	Milligram(s)
µg	Microgram(s)
MIC	Minimum inhibitory concentration
min	minute(s)
ml	Millilitre(s)
µl	Microlitre(s)
mob	Mobility (gene) region
MW	Molecular weight
ng	Nanogram(s)
nic	Nicking site
nm	Nanometre(s)
OC	Open circular
OD	Optical density
OMP	Outer-membrane protein
*ori*T	Origin of transfer replication
*ori*V	Origin of vegetative replication
^{32}P	Radioactive phosphorus
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pI	Isoelectric point
p.s.i.	Pounds per square inch
R	Resistance
RBC	Red blood cell
recA^{+}	Recombination-proficient
recA^{-}	Recombination-deficient
rep	Replication genes
RNA	Ribonucleic acid
RNase	Ribonuclease

Abbreviations contd.

rop	Repression of primer
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Second(s)
SEM	Standard error of mean
SPF	Specific pathogen free
ST	Heat-stable toxin
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
Tn	Transposon
tra	Transfer genes
UV	Ultra-violet
V	Volt(s)
v/v	Volume/volume ratio
W	Watt(s)
w/v	Weight/volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YNB	Yeast nitrogen base

* Abbreviations of antibiotics used in this study are described in the text (Materials and Methods: Section 2.3) and in the footnotes to Tables 2, 3, and 9.

SUMMARY

Pasteurella haemolytica, a Gram-negative bacterium, is the principal causative agent of pneumonic pasteurellosis in cattle and sheep. The presence of plasmids in various isolates of this species and their relationship to antibiotic resistance and to the health status of the source animal were investigated. Plasmids conferring resistance to ampicillin were analyzed and the plasmid-encoded β-lactamase enzymes characterized. A suitable plasmid was identified for the construction of shuttle cloning vectors for *P. haemolytica*.

Thirty five typable and untypable isolates of *P. 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. 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, 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, which was shown by transfer studies (transformation and conjugation) to be plasmid-mediated.

Plasmid DNA prepared from ampicillin-resistant *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 or by modifying the growth conditions. Terrific broth or broth supplemented with 1% yeast nitrogen base was found to be the best

growth medium for plasmid amplification. All Ap^R plasmids had a similar size (~4.3 kb), and one of them (pPH843) was highly amplifiable and more stable in *E. coli* compared to the other plasmids (pPH2, pPH33 and pPH821).

An improved method was developed for the large-scale purification of covalently closed circular plasmid DNA molecules over a wide size range. The protocol used an alkaline-lysis procedure followed by acid-phenol extraction and included several modifications to previously reported methods. Two principal modifications were the replacement of NaCl by $MgCl_2$ in the extraction buffer and vortexing instead of shaking the crude DNA suspensions to more efficiently remove chromosomal and other non-CCC plasmid DNA and to improve yield of purified CCC DNA forms.

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*, but a part of the DNA sequence (~0.7 kb) from the *Haemophilus* plasmid was not present in the *Pasteurella* plasmid. This suggested that one plasmid had been derived from the other during the course of evolution. From substrate profiles and isoelectric focusing data, the β-lactamases encoded by the *P. haemolytica* plasmids were found to be indistinguishable from the ROB-1 β-lactamase.

A restriction map of plasmid pPH843 was constructed and the putative Ap^R, *oriV*, and *mob* regions of the plasmid were located by deletion and fusion experiments. The plasmid was converted into a series of possible cloning vectors (pAKA15, pAKA15-1, pAKA16, pAKA16-1 and pAKA17) by insertion at different points in the plasmid of the *lacZ* α-peptide-coding gene and a multiple cloning site (for insertional inactivation of β-

galactosidase activity) from the *E. coli* vector pIC20H and an *Inc*P mobilization function from plasmid RK2. Transconjugants obtained by transfer of the constructs from *E. coli* to *P. haemolytica* were found to be unstable on subculture, although they were stable upon transfer to another *E. coli* strain. This indicated that *P. haemolytica* might have a restriction/modification system which affected plasmid DNA previously propagated in *E. coli*.

1. INTRODUCTION

1.1. Pasteurellosis - The Disease.

Pasteurellosis is a broad term relating to the bacterial diseases caused by members of the genus *Pasteurella*. Diseases caused by *Pasteurella haemolytica* are predominant in domestic ruminant animals. The most important is an acute respiratory-tract disease (pneumonic pasteurellosis) in cattle, associated with a condition known as "shipping fever" in North America or "transit fever" in Europe (Collier, 1968a; 1968b; Lillie, 1974; Frank, 1979; Thomson, 1980; Rehmtulla & Thomson, 1981; Yates, 1982; Allan *et al.*, 1983; Gibbs *et al.*, 1983; Frank, 1989). Young calves, especially when gathered under crowded conditions in closed buildings with insufficient ventilation, can develop a respiratory syndrome known as 'enzootic pneumonia' which may eventually lead to shipping fever or pneumonic pasteurellosis (Frank, 1989). *P. haemolytica* also causes septicaemia and pneumonia in lambs and sheep (Gilmour, 1980; Gilmour & Gilmour, 1989). The other animal hosts in which this organism may occasionally cause disease are: goats, swine, chickens and turkeys (Carter, 1981). Apart from respiratory and septicaemic diseases, other *P. haemolytica* infections are: mastitis in ewes, arthritis and meningitis in sheep and lambs (Gilmour, 1980), and salpingitis in poultry (Carter, 1981). *P. haemolytica* infections are rare in human hosts and only a few cases of such infections are evident from the annual reports of the Public Health Laboratory Service in London (Frederiksen, 1989).

Pasteurelloses caused by *Pasteurella multocida* are widespread among different avian and animal species, and among human hosts. These diseases include: fowl cholera in birds, haemorrhagic septicaemia in cattle and buffaloes (Carter, 1984), atrophic rhinitis and pneumonia in pigs (Chanter

& Rutter, 1989) and a variety of sporadic infections such as bite-wound infections, pneumonia, pleural empyemia, meningitis and encephalitis in man (Frederiksen, 1989).

1.1.1. Historical background of *Pasteurella* and Pasteurellosis.

In 1887, Trevisan first established the genus *Pasteurella*, in recognition of the work of Louis Pasteur on fowl cholera caused by a bacterium (later identified as *P. multocida*) of this group (cited by Zinnemann, 1981). The name '*Pasteurella haemolytica*' was first given by Newsom & Cross (1932) to the species of this genus for its haemolytic nature on blood agar. They isolated this organism from sheep with pneumonia. *Bacillus bovisepticus* Group 1 found in bovine diseases (Jones, 1921) and *Pasteurella mastidis* isolated from ewes with mastitis (Marsh, 1932) were eventually identified as the same organism, *P. haemolytica*.

P. multocida was first isolated in France in 1879 by Toussaint and then in 1880 by Louis Pasteur, while working on fowl cholera in chickens. The organism has had many names including *Pasteurella cholerae-gallinarum* and *Pasteurella septica*. The name *Pasteurella multocida* was finally introduced by Rosenbusch & Merchant (1939).

Pasteurellosis in cattle was first described as haemorrhagic septicaemia (Marshall, 1922), a syndrome which was characterized by fibrinous pleuropneumonia. The pneumonic form of this disease was subsequently referred to as bovine pasteurellosis rather than haemorrhagic septicaemia (Shirlaw, 1938).

Early reports of pneumonic pasteurellosis in housed sheep were obtained from Iceland (Dungal, 1931), Australia (Beveridge, 1937) and England

(Montgomerie *et al.*, 1938). A septicaemic form of the fatal disease in lambs, caused by *P. haemolytica*, was described by Stamp *et al.* (1955) in Scotland and this was probably the first report on the so-called septicaemic pasteurellosis. Septicaemia in lambs was also reported from North America at about this time (Biberstein & Kennedy, 1959).

1.1.2. Definitions and clinical symptoms of pneumonic pasteurellosis.

Though pneumonic pasteurellosis refers to both cattle and sheep as the hosts, the disease in cattle has been investigated most extensively. Shipping fever is the most important component of 'bovine respiratory disease complex' (BRDC) and defined as a fibrinous pneumonia resulting from an interaction between *Pasteurella* spp., various stresses (e.g. transport) and predisposing factors (e.g. viral infection) (Collier, 1968a; Yates, 1982). Different authors presented definitions of this disease on the basis of clinical symptoms and pathological parameters observed in infected animals. Several investigators defined shipping fever as an acute infectious disease of cattle characterized by fever, dyspnoea, and fibrinous pneumonia of unknown cause (Adams *et al.*, 1959), which is usually seen several days after shipment and in adverse weather conditions (Sinha & Abinanti, 1962). Another definition is "an acute infection of the respiratory tract in young cattle characterized by fever, depression, nasal discharge, and referred bronchial and pleuritic sounds in ventral parts of the lungs" (Jensen & Mackey, 1979). Yates (1982), however, accumulated opinions from different authors and set down a definition as follows: "Shipping fever is an entity within the BRDC. It is a pneumonic condition of cattle which is of undetermined though multifactorial aetiology, and is

usually associated with *P. haemolytica* or, less commonly, *P. multocida*, in which instances the term 'pneumonic pasteurellosis' can be used synonymously. Shipping fever is characterized pathologically by lobar, anteroventrally distributed, exudative pneumonia in which fibrin is usually a prominent part of the exudate and fibrinous pleuritis is common".

Throughout his review, Yates (1982) mentioned this bovine disease as 'shipping fever pneumonia'. Pneumonic pasteurellosis appears to be the appropriate term, now being widely used, to indicate pneumonia caused by *Pasteurella* spp. That pneumonic pasteurellosis is the synonym for BRDC (Maheswaran *et al.*, 1980) is not true, because the latter also includes diseases caused by microorganisms other than pasteurellae. Rehmtulla & Thomson (1981) agreed that the term pneumonic pasteurellosis has attained recognition as a distinct disease entity and they used the terms pneumonic pasteurellosis and shipping fever interchangeably, the latter being often put within quotation marks.

Bovine pneumonic pasteurellosis in Britain is primarily a disease of recently weaned, newly housed, single-suckled beef calves (Allan *et al.*, 1983) and is associated with the clinical symptoms of sudden onset of dullness, anorexia, pyrexia (106-107°F), tachypnoea, rare adventitious respiratory sounds and/or serous nasal discharge (Dalgleish, 1990).

1.1.3. Aetiology of pneumonic pasteurellosis.

There can be no reasonable doubt that *P. haemolytica* is an essential component in the aetiology of shipping fever and that pasteurellosis is responsible for the clinical signs and death of cattle (Collier, 1968b). Pneumonic pasteurellosis is often promoted by stress and preinfections with

respiratory viruses. The putative stressing factors may include: weaning, prolonged transport with consequent anxiety and fatigue, temperature extremes, inadequate and irregular feeding and watering, changes in diet, increased humidity, crowding, inhalation of dust, draughts, dehorning, castration or vaccinations (Yates, 1982).

The probable aetiological agents involved in the disease have been extensively investigated. In one experiment, Lopez *et al.* (1976) used parainfluenzae-type 3 (PI-3) virus which depressed bacterial clearance from lungs. In other experiments, the use of aerosols of bovine herpes virus 1 (BHV 1) prior to *P. haemolytica* infection caused fibrinous pneumonia similar to field conditions (Jericho *et al.*, 1976; Jericho & Langford, 1978). On the other hand, Thomson (1980) believed that *P. haemolytica* could cause shipping fever by itself, although PI-3 and infectious bovine rhinotracheitis (IBR) viruses may facilitate the process (Rehmtulla & Thomson, 1981). It is now known that preinfecting bovine lungs with an agent like IBR virus, PI-3 virus, bovine respiratory syncytial virus (BRSV) or *Mycoplasma bovis* increases susceptibility to challenge with *P. haemolytica* A1. But such preinfection is not necessarily required in all cases to develop pneumonia, and the severity of this disease is a function of the dose and virulence of *P. haemolytica* A1 and not of the other infectious agents (Yates *et al.*, 1983a; Gourlay & Houghton, 1985). Several other workers produced similar results whereby *P. haemolytica* A1 caused pneumonic pasteurellosis independently of viral or other microbial agents when virulent, rapidly growing bacteria were injected intrabronchially (Friend *et al.*, 1977) or transthoracically (Newman *et al.*, 1982). Gibbs *et al.* (1984) reproduced typical pneumonic pasteurellosis in carefully selected, unstressed, conventionally-reared calves by intranasal and

intratracheal inoculation of a virulent strain of *P. haemolytica* A1 alone.

Frank (1988), however, suggested that *P. haemolytica* remains as part of the normal flora in low numbers in the nasopharynx of healthy calves until transport stress, viral respiratory disease or a climatic change triggers a population explosion of *P. haemolytica* which heavily colonizes the nasopharynx. In one such study, calves exposed to an aerosol infection and then subjected to an abrupt change from a cold and dry to a hot and humid climate were found to be rapidly colonized by *P. haemolytica* (Jones, 1987).

1.1.4. Pathogenesis of pneumonic pasteurellosis.

Although the involvement of *P. haemolytica* in pneumonic pasteurellosis has been studied for many years, the pathogenesis of this disease is still not well understood. The mechanism has remained obscure because of the difficulty in producing the disease under laboratory conditions (Frank, 1979). However, recent research on experimental infections in cattle and sheep has provided some understanding of the pathogenesis of pneumonic pasteurellosis.

It is now established that *P. haemolytica* A1 plays a key role in the pathogenesis of pneumonic pasteurellosis in cattle. In the course of assembling, shipping and processing cattle for entry into the feedlot, the animals are stressed, resulting in the overgrowth of *P. haemolytica* A1 in the nasal passages and tracheal airways (Frank & Smith, 1983; Wilkie & Shewen, 1988). The organisms travel from the upper respiratory tract to the lungs via aerosolized droplets (Grey & Thomson, 1971; Jericho *et al.*, 1986). Once in the lung, several pathophysiological events occur, that combine to cause severe pneumonia (Wilkie & Shewen, 1988).

It was assumed that inhaled droplets were important in the pathogenesis and that the lesions of fibrinous pneumonia start at the level of the respiratory bronchiole and spread within the lung primarily through the connective tissue around blood and lymph vessels and respiratory airways (Jubb & Kennedy, 1970). These authors, in the final analysis of their study, stated that "it is the breakdown of the phagocytic barrier which allows the infection to be established". Alveolar macrophages in the lungs are considered to be the primary phagocytic cells which can phagocytose *P. haemolytica*, especially when the bacteria are present in small numbers. However, if a large number of bacteria are present, many escape phagocytosis and produce a cytotoxin (leukotoxin) which can destroy the macrophages (Frank, 1989). Moreover, *P. haemolytica* cells are readily phagocytosed during the stationary phase, but not during active growth in the logarithmic phase (Walker *et al.*, 1984) when the bacteria produce the potent leukotoxin (Shewen & Wilkie, 1985).

During *P. haemolytica* infections, neutrophils have been demonstrated to be responsible for acute lung injury in cattle and thereby play an important role in the pathogenesis of pneumonic pasteurellosis (Solocombe *et al.*, 1985). These workers showed that calves depleted of neutrophils by prior administration of hydroxyurea were resistant to intratracheal challenge with live *P. haemolytica* A1, while neutrophil-sufficient normal calves developed severe exudative and necrotizing bronchopneumonia.

Alveolar macrophages and neutrophils can engulf and kill *P. haemolytica* organisms, but eventually both are killed by the leukotoxin produced by actively-growing bacteria (Czuprynski *et al.*, 1987). Neutrophils are more sensitive than alveolar macrophages to destruction by the leukotoxin (Czuprinski *et al.*, 1987; O'Brien & Duffas, 1987). Walker *et al.* (1985)

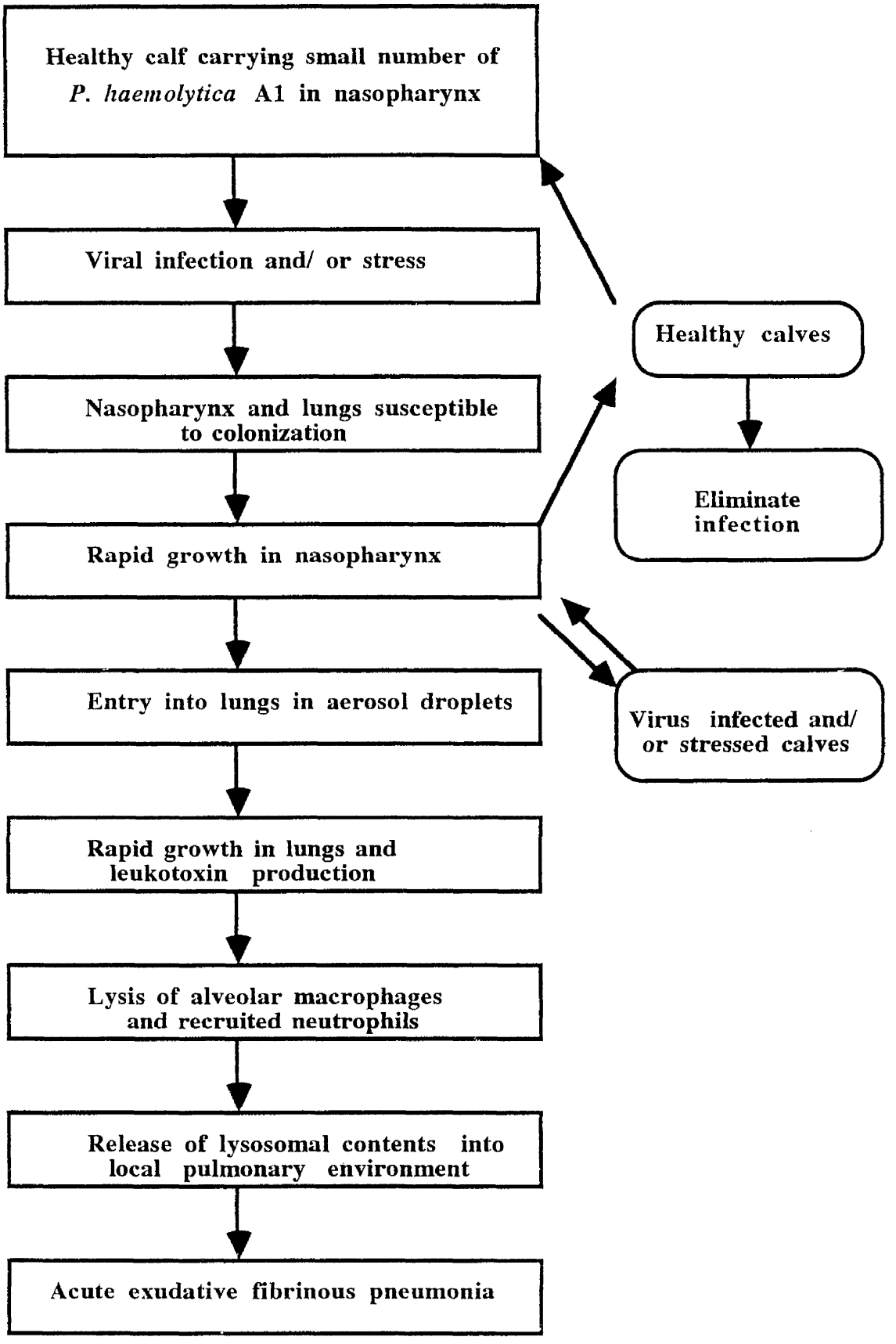
argued that the leukotoxin is indirectly responsible for pneumonia, because it kills neutrophils, the principal defensive blood cells, which enter the lung in response to *P. haemolytica* infections and become the predominant cell type in the alveolar spaces within four hours of infection. The cytolytic effect of leukotoxin is initiated by the formation of pores in the cell membrane which allows the influx of calcium and results in a sequence of cell-damaging events (Clinkenbeard *et al.*, 1989). Leukotoxin thus contributes to the pathogenesis of pneumonia by inducing lysis of neutrophils and macrophages and liberating, from these leukocytes, intracellular contents such as lysosomal enzymes and oxygen-derived toxic free radicals which are responsible for the acute lesions of fibrinous pneumonia (Sibille & Reynolds, 1990; Czuprynski *et al.*, 1991; Maheswaran *et al.*, 1992).

A flow diagram of the pathogenesis of bovine pneumonic pasteurellosis involving the leukotoxin is shown in Figure 1.

In addition to leukotoxin, endotoxin (lipopolysaccharide), derived from *P. haemolytica*, was also implicated in the pathogenesis of bovine pneumonic pasteurellosis (Walker *et al.*, 1985; Confer & Simons, 1986). Deposition of lipopolysaccharide (LPS) into sheep's lungs caused an influx of a huge number of neutrophils and contributed to the lesions of pneumonic pasteurellosis (Brogden *et al.*, 1984). An intratracheal inoculation of LPS was found to cause significant pulmonary lesions in cattle (Solocombe *et al.*, 1990). Recent *in vitro* studies by Breider *et al.* (1990, 1991) have shown that LPS was a major toxic factor which caused damage to a bovine pulmonary endothelial cell line and that the addition of bovine neutrophils could prevent endothelial cell damage. These *in vitro* findings contrast

FIGURE 1. FLOW DIAGRAM SHOWING THE PROPOSED MECHANISM OF THE PATHOGENESIS OF BOVINE PNEUMONIC PASTEURELLOSIS CAUSED BY *P. HAEMOLYTICA* A1.

The diagram was adapted from Frank (1989) and Dalgleish (1990).


Healthy calf carrying small number of P. haemolytica A1 in nasopharynx
Viral infection and/ or stress
Nasopharynx and lungs susceptible to colonization
Rapid growth in nasopharynx
Entry into lungs in aerosol droplets
Rapid growth in lungs and leukotoxin production
Lysis of alveolar macrophages and recruited neutrophils
Release of lysosomal contents into local pulmonary environment
Acute exudative fibrinous pneumonia
Healthy calves
Eliminate infection
Virus infected and/ or stressed calves

with the primary role attributed to the leukotoxin in the pathogenesis of bovine pneumonic pasteurellosis.

1.1.5. Prevalence and significance of pneumonic pasteurellosis.

Pneumonic pasteurellosis, caused by *P. haemolytica*, is an economically important disease of food-producing animals because it causes substantial economic loss to the farming (especially, feedlot cattle and sheep) industries (McKercher, 1978; Blood *et al.*, 1979; Thomson, 1980; Gilmour, 1980; Yates, 1982; Wilkie & Shewen, 1988; Donachie, 1988). The disease is most prevalent in North America and severe morbidity and mortality due to respiratory illness in North American feedlot cattle result mainly from *P. haemolytica* infections (Roth, 1988). The economic losses to the beef and dairy industries have been estimated to be about 800 million dollars annually in the United States (Frank, 1989). Two-thirds of sickness and deaths in Alberta feedlots were due to bovine respiratory diseases, including IBR and shipping fever, which caused an annual loss of 9.6 million dollars in Alberta (Church & Radostits, 1981). In specific survey results, Jensen *et al.* (1976) found that 64% of 1988 necropsy diagnoses were respiratory disease and, of these, 75% were shipping fever pneumonia. The results of Rothwell *et al.* (1979) on 168 deaths from a large Saskatchewan feedlot were remarkably similar, with respiratory disease causing 71% of deaths, and 73% of these being pneumonic pasteurellosis or shipping fever. In an Ontario feedlot study, fibrinous pneumonia was found to be the most frequent diagnosis at post-mortem, accounting for 41% of 168 deaths in one year (Martin *et al.*, 1980) and 45% of 167 deaths the next (Martin *et al.*, 1981).

Relatively few references are available on *P. haemolytica* infections in Europe, though Blood *et al.* (1979) stated that pneumonic pasteurellosis is common in Europe including the United Kingdom. Allan *et al.* (1983) and Gibbs *et al.* (1983) described a series of field outbreaks of pneumonic pasteurellosis in Scotland which seriously affected recently weaned, single-suckled calves. Gilmour (1978a, 1980) considered pasteurellosis as the most significant and fatal disease of sheep in Britain. The author highlighted the importance of this disease in Britain by presenting the results of two surveys reported from the Ministry of Agriculture, Fisheries and Food. In one survey in 1964, pneumonia was found in 82% of 9550 sheep submitted to the Veterinary Investigation Centres in England and Wales, and in another survey during 1976-78, *Pasteurella* infections were diagnosed in sheep much more than any other bacterial diseases at the UK Veterinary Investigation Centres.

1.1.6. Vaccines for *P. haemolytica* infections.

P. haemolytica infections in cattle and sheep have long been considered to be multifactorial with regard to the different serotypes and the virulence factors involved. The most efficient and practical means of control of infection depends upon the development of an effective vaccine against the various antigens involved.

Since the late 1950s, bacterins (killed bacteria) from *P. haemolytica* (or *P. multocida*) have been used as the primary type of vaccine (Rice *et al.*, 1955; Palotay *et al.*, 1963), but these vaccines have been shown to be of questionable protective value. When viruses were found to be associated with the pathogenesis of pneumonic pasteurellosis, attempts were made to

prevent the disease with viral vaccines. Use of vaccines containing PI-3 virus (Mohanty & Lillie, 1964), IBR virus (Jericho *et al.*, 1976) or a combination of PI-3 and IBR viruses (Hamdy *et al.*, 1965) protected calves against experimental challenge with the respective virus and *P. haemolytica*. However, protection was variable with viral vaccines alone. Combinations of bacterins and viral vaccines were also developed and evaluated. Vaccination of calves with a combination of inactivated PI-3 virus and *P. haemolytica* bacterins provided protection against a combined experimental challenge infection (Matsuoka *et al.*, 1966). In field trials, however, this combined vaccine had no effect on reducing the incidence of respiratory disease (Hamdy *et al.*, 1965).

The experimental production of pneumonic pasteurellosis in calves with *P. haemolytica* in the absence of other infectious agents (Friend *et al.*, 1977; Gibbs *et al.*, 1984; Wiseman *et al.*, 1984) stimulated interest in evaluating the effects of bacterins or live bacteria, when used alone. Use of formalin-killed *P. haemolytica* bacterin produced adverse effects in an experimental study (Wilkie *et al.*, 1980), while in a field trial, use of a commercial bacterin did not produce any significant effects (Martin *et al.*, 1984). *P. haemolytica* bacterins with different adjuvants have been shown to stimulate high antibody responses to somatic antigens but low titres to leukotoxin (Confer *et al.*, 1987). Since both capsule and leukotoxin production are associated with rapidly growing bacteria, the use of live vaccines has yielded some promising results in reducing the incidence of pneumonic pasteurellosis (Newman *et al.*, 1982). Henry (1984) used live, lyophilized preparations of *P. haemolytica* which protected feeder calves from disease under field conditions. Modified live vaccines containing chemically-altered strains (Kucera *et al.*, 1983) or streptomycin-dependent

strains (Catt *et al.*, 1985) of *P. haemolytica* have also been used to obtain an enhanced protection. Dosage of organism, culture age and route of administration were, however, important for live vaccines (Confer *et al.*, 1987). Vaccines prepared from various surface antigen extracts have been evaluated for their protective role. Potassium thiocyanate or sodium salicylate extracts of *P. haemolytica* have been used as experimental vaccines but gave inconsistent results (Yates *et al.*, 1983b; Gilmour *et al.*, 1987). It appears to be necessary to immunize calves and sheep with both leukotoxin and serotype-specific antigens for consistent protection against pneumonic pasteurellosis (Shewen & Wilkie, 1988; Sutherland *et al.*, 1989; Mosier *et al.*, 1989). The failure of *P. haemolytica* bacterins to protect cattle against this disease is due to the fact that they induce bacterial surface antigen-specific antibody but not toxin neutralizing (anti-leukotoxin) antibody (Wilkie & Shewen, 1988). In one study, a vaccine containing a crude leukotoxin preparation, which is believed to contain some other soluble antigens, from *P. haemolytica* A2 was found to protect sheep (86%) against a homologous challenge infection (Sutherland *et al.*, 1989). A similar preparation from serotype A1 protected two of three calves in another experimental study (Shewen & Wilkie, 1988).

Current research on vaccine development is, therefore, aimed at identifying the bacterial surface components responsible for adhesion and colonization of *P. haemolytica* and at inducing neutralizing antibodies against the leukotoxin. A crude vaccine called "Presponse" (Langford Laboratories, Guelph, Ontario), derived from the supernate of a log-phase culture of *P. haemolytica* and containing both leukotoxin and certain soluble antigens, is now commercially available in Canada (Dalgleish, 1990; Conlon *et al.*, 1991). This type of vaccine has already shown promise in

reducing the incidence and severity of pneumonia following experimental challenge (Shewen *et al.*, 1988) and field trials are currently underway to assess its general efficacy.

In a recent experimental study, the recombinant leukotoxin has been used as a component of a vaccine against *P. haemolytica* infections in cattle (Conlon *et al.*, 1991). Use of the recombinant leukotoxin alone in the vaccine did not give any protection; however, it enhanced the efficacy of the culture supernate vaccine, Presponse, when used in combination.

A vaccine consisting of sodium salicylate extracts of *P. haemolytica* A2, which harboured inducible iron-regulated proteins (IRPs) (see Section 1.2.5.8) has been recently shown to confer protection against experimental infection with serotype A2 in specific pathogen-free (SPF) lambs (Gilmour *et al.*, 1991).

Thus, most studies indicate that several cellular and extracellular components of *P. haemolytica* are likely to be required in an efficient and successful acellular vaccine.

No molecular genetic approach is yet available towards the development of attenuated *Pasteurella* strains, defective in individual antigens, which could be used as whole-cell or live vaccines.

1.2. *Pasteurella haemolytica* - The Organism.

1.2.1. Taxonomic status.

P. haemolytica is one of six species of the genus *Pasteurella* belonging to the family *Pasteurellaceae*, which has been mentioned as family III under the section 'facultatively anaerobic Gram-negative rods' in Bergey's Manual of Systemic Bacteriology [Holt & Kreig (eds.), 1984]. Other recognized genera under this family include *Haemophilus* and *Actinobacillus*. The other five member-species of the genus *Pasteurella* are: *Pasteurella multocida*, *Pasteurella pneumotropica*, *Pasteurella ureae*, *Pasteurella aerogenes* and *Pasteurella gallinarum*. The mole percent G+C of the DNA of *P. haemolytica* is 42.3-43.6. It is interesting to note that *P. haemolytica* (biotype A) and *P. ureae* appear to be closer to the genus *Actinobacillus* than to *Pasteurella* on the basis of the results of DNA-DNA hybridization studies (Pohl, 1981).

Among the members of the genus *Pasteurella*, *P. haemolytica* and *P. multocida* are the most widely studied species from the clincal point of view, because the pathological effects of these organisms are evident within a wide variety of hosts including animals, birds and humans.

1.2.2. Definition and characteristics.

The definition and relevant characteristics of *P. haemolytica* were described in detail by Carter (1981, 1984). The bacterium is a Gram-negative, non-motile, facultatively anaerobic small rod whose colonies show characteristically weak haemolysis on sheep blood agar. This is the only species of *Pasteurella* to produce a distinct zone of β-haemolysis. Wider

zones of haemolysis were obtained with avian strains than with strains from cattle and sheep (Heddleston, 1975). *P. haemolytica* is catalase and oxidase positive, and fermentative in nature by producing acid but not gas from a number of carbohydrates. The growth of this organism occurs between 25° and 40°C; 37°C being the optimum growth temperature. Blood agar, brain heart infusion agar and dextrose agar are used for isolation of *P. haemolytica*. Growth also occurs on MacConkey's agar. Complete mature colonies develop after 18-24 h of incubation and are round and greyish. Lactose-fermenting strains of *P. haemolytica* (predominantly A1 serotype) yield small, round, pink to red colonies on MacConkey's agar (Biberstein *et al.*, 1960). Capsules are commonly seen in cultures from diseased tissue.

Microscopically, cells of *P. haemolytica* are slightly larger than those of *P. multocida*. Carter (1967) stated that these two organisms are not different in morphology. *P. haemolytica* strains can be differentiated from *P. multocida* strains by their ability to grow on MacConkey's agar and to cause haemolysis on blood agar media, and by their inability to produce indole from tryptophan.

1.2.3. Biotype and serotype.

Strains of *P. haemolytica* can be divided into two biotypes: A and T, on the basis of several *in vitro* criteria including carbohydrate fermentation reactions (Smith, 1961). Biotype A includes strains which usually ferment arabinose but not trehalose, whereas, all biotype T strains ferment trehalose. Both biotypes were further differentially characterized by nucleic acid homology (Biberstein & Francis, 1968) and antibiotic sensitivity (Biberstein & Kirkham, 1979). The differential characteristics

of A and T biotypes of *P. haemolytica* are presented in Table 1.

P. haemolytica biotype A is predominantly associated with the pneumonic form and biotype T with the septicaemic form of pasteurellosis in sheep (Gilmour, 1978a). Biotype A also causes septicaemia in young lambs (Smith, 1961; Gilmour, 1980). In cattle, biotype A is associated with pneumonia and biotype T has not been reported to cause disease (Yates, 1982), but Allan *et al.* (1985a) reported the isolation of biotype T strains from cases of pneumonic pasteurellosis in calves.

Currently 16 serotypes of *P. haemolytica* are known, which have been differentiated on the basis of their specific capsular antigens, as determined by the indirect haemagglutination test. Twelve serotypes were described first (Biberstein *et al.*, 1960). Two other serotypes were identified from sheep isolates in Ethiopia (Pegram *et al.*, 1979), another serotype (of biotype T) from sheep in Scotland (Fraser *et al.*, 1982a) and also one from Hungary (Fodor *et al.*, 1987). Serologically untypable strains also occur in both sheep (Fraser *et al.*, 1982b) and cattle (Quirie *et al.*, 1986). Such strains are presumably uncapsulated variants of *P. haemolytica* (Biberstein, 1978).

On the basis of a relationship between serotypes and biotypes established by Biberstein & Gills (1962), serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, and 16 have been placed under biotype A and serotypes 3, 4, 10, and 15 under biotype T.

P. haemolytica biotype A serotype 1 (A1) has been found predominantly to be associated with pasteurellosis of cattle and biotype A serotype 2 (A2) with that of sheep (Wray & Thomson, 1971; Thomson *et al.*, 1977; Fraser *et al.*, 1982b; Allan *et al.*, 1983, 1985a; Quirie *et al.*, 1986; Donachie,

TABLE 1. DIFFERENTIAL CHARACTERISTICS OF THE TWO BIOTYPES (A & T) OF *PASTEURELLA HAEMOLYTICA**

Characteristics	Biotype A	Biotype T
Acid production from arabinose and xylose	+	-
Acid production from trehalose and salicin	-	+
Susceptibility to penicillin (*in vitro*)	High	Low
Survival period of culture	Short	Long
Colonies	Small, grey	Large with brownish centre
Capsular serotypes	1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16	3, 4, 10, 15
Principal location in natural host (cattle and sheep)	Nasopharynx	Tonsils
Principal disease association	Pneumonia of cattle and sheep; septicaemia of new-born lambs	Septicaemia of feeder lambs (3 months of age or more)

*Adapted from Carter (1981, 1984).

1988). Biotype T serotype 10 (T10) has been identified as the most common pathogen in sheep (Biberstein & Thomson, 1966; Fraser *et al.*, 1982b).

1.2.4. Source and frequency of isolation.

Numerous investigators have reported the presence of *Pasteurella* spp. in the upper respiratory tract of apparently healthy cattle (Collier, 1968a; Wessman & Hilker, 1968; Gilmour, 1978b; Wilkie & Shewen, 1988). Several other workers stated that *Pasteurella* spp. were not normal microflora of the lower respiratory tract including the lungs (Collier & Rossow, 1964; Grey & Thomson, 1971), although both *P. haemolytica* and *P. multocida* have been found in normal bovine lungs in one study (Allan, 1978). In fact, these organisms can be present in both pneumonic and non-pneumonic calves (Allan, 1978; Allan *et al.*, 1985a), but the numbers are large in the former and small in the latter (Pirie, 1978). In general, *P. haemolytica* occurred more frequently than *P. multocida* in pneumonic calves, and equal percentages of the two bacteria were found in non-pneumonic animals (Allan, 1978). It was unusual to isolate both *P. haemolytica* A1 and *P. multocida* from the same infected animal (Allan *et al.*, 1985a).

Gilmour (1978a) considered *P. haemolytica* to be an opportunistic pathogen as he recovered the organism from 95% of tonsils and 64% of nasopharyngeal swabs from apparently healthy sheep. The organism is commonly recovered from the nasal passages of cattle with respiratory tract disease and from pneumonic lungs (Wray & Thomson, 1971; 1973; Reggiardo, 1979; Frank & Smith, 1983). The occurrence and overgrowth of the bacterium in the respiratory tract often depend on the stressful situations experienced by the animals. In a study of 356 transported calves, samples

were collected from the nasal passages of calves at three different and distant places (e.g. farm, auction barn, and feedyard) at different intervals of time (Frank & Smith, 1983). These workers isolated *P. haemolytica* at a low frequency from calves when they were at the farm of origin, at a greater frequency at the auction barn, and at a markedly higher frequency at the feedyard.

P. haemolytica A1 is the predominant serotype consistently isolated from cattle with pneumonic pasteurellosis, both in Britain (Wray & Thomson, 1971; Gibbs *et al.*, 1983; Allan *et al.*, 1983; 1985a; Quirie *et al.*, 1986) and in North America (Jubb & Kennedy, 1970; Biberstein, 1978; Reggiardo, 1979). Of 16 bovine *P. haemolytica* strains tested for typing, 14 (88%) were identified as serotype A1 (Biberstein *et al.*, 1960). In a case study, 94% of *P. haemolytica* isolates from cattle dying of shipping fever in Texas were found to be type 1 (Reggiardo, 1979). Quirie *et al.* (1986) observed that 63% of lung isolates and 48% of nasal swab isolates were serotype A1 and that untypable strains predominantly occurred in the female genital tract (including the foetus and placenta), udder, milk, and intestinal tract of cattle. A1 strains were isolated from each of the cases of acute exudative fibrinous pneumonia in recently housed, weaned, single-suckled calves (Allan *et al.*, 1983; Gibbs *et al.*, 1983) and also from apparently healthy calves in houses adjacent to the infected groups (Wray & Thomson, 1973).

1.2.5. Virulence factors.

Several potential virulence factors in *P. haemolytica* have been identified, some of which have been examined intensively, while some others demand

further investigation. These factors are described as follows:

1.2.5.1. Cytotoxin (Leukotoxin).

The cytotoxin has been considered as the most important virulence determinant of *P. haemolytica* and has been studied extensively. Benson *et al.* (1978) first observed cytotoxic changes in bovine alveolar macrophages, which were ultimately killed in the presence of live *P. haemolytica* A1. The toxic factor was present in bacteria-free culture filtrates and sublethal concentrations of crude toxin were found to induce impaired phagocytosis (Markham & Wilkie, 1980), to reduce chemotaxin production in pulmonary macrophages (Markham *et al.*, 1982), and to inhibit the luminol-dependent chemiluminescent response of bovine neutrophils (Chang *et al.*, 1985). The cytotoxin from *P. haemolytica* has been shown to be a true exotoxin (Balyut *et al.*, 1981; Sutherland *et al.*, 1983). Shewen & Wilkie (1985) confirmed that the toxin is an extracellular product of metabolically-active bacteria, since it is produced *in vitro* during the logarithmic growth phase and is not found in bacterial lysates or sonic extracts. The presence of iron on a suitable carrier molecule, such as transferrin, encourages the optimal production of cytotoxin (Gentry *et al.*, 1986). The cytotoxin is a protein and the immuno-chemical properties of this toxin appeared to be common to all serotypes of *P. haemolytica* (Himmel *et al.*, 1982). All serotypable and non-serotypable strains of this species are toxigenic (Shewen & Wilkie, 1985; Sutherland & Donachie, 1986; Chang *et al.*, 1987), although not all are pathogenic. The activity of the cytotoxin is specific for ruminant leukocytes including alveolar macrophages, cultured blood monocytes, lymphocytes, and neutrophils (Kaehler *et al.*, 1980; Shewen & Wilkie, 1982; Sutherland *et al.*, 1983); hence the more current reference to

this virulence factor as a leukotoxin.

Currently, *P. haemolytica* leukotoxin is considered to be an important member of the RTX (repeats in toxin) toxin family from Gram-negative bacteria. The structural and functional relationships of these toxins have been reviewed recently (Coote, 1992).

1.2.5.2. Haemolysin.

Production of haemolysin is a distinguishing feature of *P. haemolytica*, and although haemolytic activity on blood agar is not prominent, it may be enhanced *in vivo*. One possible role of haemolysin *in vivo* is that it may liberate iron from the host red blood cells (RBCs) for use by the bacterium (Chengappa *et al.*, 1983). However, except in cases of septicaemia, it is difficult to visualize such a role in respiratory infection. The importance of iron for the production of leukotoxin (Gentry *et al.*, 1986) and in the pathogenesis of *P. haemolytica* infections in mice (Al-Sultan & Aitken, 1984) has been described.

Forestier & Welch (1990) believed that *P. haemolytica* haemolysin could be the leukotoxin, because they detected a weak haemolytic activity for the active form of leukotoxin against sheep RBCs, but this activity was not present if an inactive form of the toxin was examined. Haemolysin, however, is not generally considered as a potential virulence factor for *P. haemolytica*.

1.2.5.3. Capsule.

Production of a surface capsule in *P. haemolytica* is dependent on the age of the culture. Corstvet *et al.* (1982) demonstrated that young, rapidly growing cultures of *P. haemolytica* A1 produced large amounts of capsular

material compared to older cultures. Capsular size is likely to be important in resistance to phagocytosis.

The capsule carries the serotype-specific antigens (Adlam *et al.*, 1984), which probably mediate surface adherence between the bacterium and the epithelium of the host's respiratory tract. Once adhered, *P. haemolytica* can colonize, grow, and liberate exotoxin. Capsular polysaccharide, which is the basis of the serotyping scheme, has long been known as a virulence determinant (Biberstein *et al.*, 1960). Capsule-free, non-typable strains of *P. haemolytica* were found to be avirulent for lambs in an aerosol challenge experiment (Donachie *et al.*, 1984). Purified capsular polysaccharide from A1 serotypes reacts with sheep lung surfactant *in vitro* to form a precipitate, which is thought to facilitate bacterial attachment to the lining of the lung alveoli (Adlam, 1989). The composition, structure and immunogenicity of purified capsules from *P. haemolytica* serotypes A1 and A2 have been described by Adlam *et al.* (1984, 1987).

1.2.5.4. Lipopolysaccharide (LPS).

LPS is a major outer-membrane component of *P. haemolytica* and appears to possess the same endotoxic activities as the LPS from other Gram-negative bacteria (Rimsay *et al.*, 1981). It may be responsible for the high rate of morbidity and mortality in infected cattle and sheep (Adlam, 1989). Endotoxin has long been blamed for increased capillary permeability, thrombosis and coagulation necrosis in pneumonic pasteurellosis (Jensen & Mackey, 1979).

The effects of serotype A1 LPS on bovine peripheral blood leukocyte function were strictly dose-dependent (Confer & Simons, 1986). Low

concentrations of LPS decreased and high concentrations increased the phagocytic activity of polymorphonuclear leukocytes, and moderate concentrations were mitogenic for mononuclear cells. Solocombe *et al.* (1990) found significant lung lesions in cattle following intratracheal inoculation of LPS. Recent studies have demonstrated that *P. haemolytica* LPS was a predominant toxic factor which mediated bovine pulmonary endothelial cell damage *in vitro* (Breider *et al.*, 1990; 1991). In studies by Brogden *et al.* (1984, 1986), direct introduction of purified A1 LPS into sheep lungs caused a variety of clinical and pathological changes. The LPS bound tightly to and formed a complex with ovine lung surfactant, a natural lung lubricant, and produced lesions very similar to those found in disease under field conditions. Moreover, the bactericidal activity of immune sheep sera to *P. haemolytica* A2 could be eliminated by absorption with purified LPS, suggesting that LPS may be important in immunity as well as in pathogenesis (Sutherland, 1988).

The structure of *P. haemolytica* LPS is not known in detail. The LPS profiles of *P. haemolytica* serotype A1 indicate that they possess the typical LPS composition of O-side chain, core-oligosaccharide and lipid A component, whereas serotype A2 lacks O-side chain (Davies *et al.*, 1991). Heterogeneity of LPS between serotypes and among strains of the same serotype (A1 or A2) has also been described recently (Ali *et al.*, 1992). A study by crossed immunoelectrophoresis has shown distinct immunological differences between LPS molecules derived from different serotypes of *P. haemolytica* (Tsai *et al.*, 1988).

Expression of LPS and outer-membrane proteins (OMPs) in *P. haemolytica* A1 has been shown to vary with different growth conditions (Davies *et al.*,

1992).

1.2.5.5. Fimbriae.

By electron microscopy, fimbriae (pili), in association with glycocalyx (capsule), were found on the surface of a serotype A1 strain of *P. haemolytica* isolated from a bovine lung lesion (Morck *et al.*, 1987). Fimbriae are surface structures that help the bacterium attach to epithelial cells and thereby aid colonization. Two types of fimbriae were identified from A1 strains grown on agar (Potter *et al.*, 1988). These workers isolated the larger of the two types, which was 12 nm in diameter and up to 560 nm in length. The pilin subunit had a molecular mass of 35 kilodalton (kDa) and an isoelectric point of about 4.7.

1.2.5.6. Neuraminidase.

A majority of the *P. haemolytica* A isolates have been reported to produce large amounts of neuraminidase, when grown overnight on blood agar, and the enzyme appeared to be cell-associated in most cases (Frank & Tabatabai, 1981). Serotype A2 was found to be the highest producer of neuraminidase which was present in crude cytotoxin preparations or culture supernates of this organism (Otulakowski *et al.*, 1983).

Neuraminidase acts by hydrolyzing the host cell glycoproteins and glycolipids and aids in the spread of some infectious agents. Although neuraminidase plays an important role in the pathogenesis of several viral and bacterial diseases (Murray *et al.*, 1990), the significance of this enzyme in *P. haemolytica* infections is not clear. However, a possible role of neuraminidase in virulence of *P. haemolytica* may still exist.

1.2.5.7. Sialoglycoprotease.

Sialoglycoprotease is a proteolytic enzyme (protease) from *P. haemolytica*, and is so named because of its ability to release sialoglycopeptides from human RBCs (Otulakowski *et al.*, 1983). Its enzymic activity was tested by using culture supernates that were cytotoxic for bovine alveolar macrophages. Sialoglycoprotease activity can be demonstrated in strains of most of the serotypes of *P. haemolytica* (Adlam, 1989). The sialoglycoprotease gene from *P. haemolytica* A1 has been recently cloned and sequenced (Abdullah *et al.*, 1991). However, the role of this enzyme in disease is still unknown.

1.2.5.8. Iron-regulated proteins (IRPs).

Iron acquisition in pathogenic bacteria is an important virulence attribute (Weinberg, 1978). IRPs are bacterial outer-membrane proteins which serve as receptors for iron-containing compounds such as transferrins or iron-siderophore(iron-chelator) complexes.

IRPs with apparent molecular weights of 70 and 100 kDa have been identified in serotype A2 (Donachie & Gilmour, 1988) and those of 71, 77, and 100 kDa in serotype A1 (Deneer & Potter, 1989) of *P. haemolytica*, when grown under iron-limited conditions. Subsequent study has demonstrated that the 100-kDa IRP from an A1 isolate served as the specific receptor for bovine transferrin (Ogunnariwo & Schryvers, 1990). Very recently, *P. haemolytica* IRPs have been described for binding transferrins from ruminants including sheep, goat and cattle (Yu *et al.*, 1992). These IRPs, by direct binding of host transferrins, aid bacteria in iron uptake in order to multiply quickly enough to establish infection. The availability

of iron to *P. haemolytica* also influences the expression of leukotoxin (Gentry *et al.*, 1986).

1.2.6. Experiments in animal models.

P. haemolytica shows a low pathogenicity for laboratory animals including mice and rabbits (Carter, 1984). The organism was found to be pathogenic for mice only when inoculated intraperitoneally with gastric mucin or intracerebrally (Smith, 1958). Intraperitoneal inoculation of *P. haemolytica* also produced infections in mice after they had received haemoglobin (Chengappa *et al.*, 1983) or an aqueous iron solution (Al-Sultan & Aitken, 1984). It has not been possible to develop a suitable small animal model for pneumonic pasteurellosis because of the fact that the natural host range of *P. haemolytica* as a respiratory pathogen is confined to ruminants (Donachie, 1988).

Pneumonic pasteurellosis has been reproduced by various investigators, using different ruminant models and combinations of aetiological agents. Experimental infections have been established in calves and lambs by stress, infection with viruses and by intratracheal inoculation of *P. haemolytica*. In one study, a combination of stress, infection with IBR virus, and *P. haemolytica* was used in young calves to produce a typical pneumonic pasteurellosis (Jericho *et al.*, 1976). In a subsequent study, infection with PI-3 virus followed by exposure to *P. haemolytica* produced severe purulent pneumonia in calves (Jericho *et al.*, 1982). Smith (1986) produced respiratory infections in calves stressed by fasting, acetic acid instillation and cold-water spray prior to intratracheal injection of live *P. haemolytica*. However, several other workers reproduced in young,

unstressed calves a respiratory condition identical to the field disease, using strains of *P. haemolytica* alone (Friend *et al.*, 1977; Gibbs *et al.*, 1984; Wiseman *et al.*, 1984; Allan *et al.*, 1985b; Vestweber *et al.*, 1990). These authors confirmed the disease clinically, pathologically and microbiologically, and their success in experimental production of pneumonic pasteurellosis was likely to have been the result of a combination of the selection criteria applied in choosing the experimental calves (i.e. those lacking antibodies to *P. haemolytica*), the virulent strain of organism used, the use of log-phase cultures, and the size and route of the challenge doses. These studies strongly support the fact that *P. haemolytica* acts as a primary pathogen in the bovine respiratory tract and that pneumonic pasteurellosis may be caused by *P. haemolytica* alone. This concept is important from the view point of vaccine development.

SPF lambs were used in experimental reproduction of sheep pasteurellosis. The intratracheal injection of PI-3 virus, one week before the use of a bacterial aerosol, produced pasteurellosis in over 90% of lambs as opposed to 40% of those exposed to *P. haemolytica* alone (Gilmour, 1978a; Sharp *et al.*, 1978).

1.3. Resistance to Antimicrobials.

Watanabe (1963) has described the early history of drug (antimicrobial) resistance in bacteria. At the end of the World War II and until about 1950, sulphonamide was the drug of choice for the *Shigella* spp. that caused outbreaks of bacillary dysentery in Japan. This drug rapidly became less effective and, by 1952, more than 80% of *Shigella* isolates were highly resistant to sulphonamide. The use of tetracycline, streptomycin and

chloramphenicol for treating dysentery started in 1950. Some resistance to these agents also gradually began to develop. Initially strains were resistant to either tetracycline or streptomycin alone but Kitamoto, in 1956, reported the isolation of *Shigella flexneri* 4a strain resistant to streptomycin, tetracycline, chloramphenicol and sulphonamide. By 1957, a significant number of multiple-resistant strains of *Shigella* were isolated.

Multiple drug resistance is also prevalent in *Staphylococcus aureus*, a Gram-positive bacterium. Penicillin-resistant strains of *S. aureus* were rapidly selected after penicillin came into use in the 1940s. The proportion of resistant strains gradually rose to 14% in 1946, 38% in 1947 and 59% in 1949 in the hospital environment where penicillin was extensively used (Barber, 1957). Resistance to other antimicrobial agents such as methicillin, erythromycin, aminoglycosides and toxic metal ions is now commonly found in *S. aureus* (Lacey, 1984).

Due to the increasing incidence of infections caused by bacteria, multiple antimicrobial resistance has now become a problem of prime importance in the clinical and pharmaceutical sciences (Falkow, 1975).

1.3.1. Antimicrobial resistance in *P. haemolytica*.

P. haemolytica infections of cattle are often treated with antibiotics to reduce death and economic losses; this has undoubtedly led to an increased antimicrobial resistance in this bacterium. It is now apparent that multiple drug resistance in *P. haemolytica* is becoming an increasingly common phenomenon in feedlot cattle with respiratory disease in North America (Amstutz *et al.*, 1982; Fales *et al.*, 1982; Morter, 1983) as well as in Britain (Wray & Morrison, 1983; Allan *et al.*, 1985a; Shoo, 1989). The

prevalence of antimicrobial resistance has made it difficult to decide promptly as to which drug or dosage will be effective in treating cattle with pasteurellosis (Fales *et al.*, 1982). Multiple drug resistance in *P. haemolytica* reported from different countries is summarized in Table 2.

Chang & Carter (1976) first described multiple drug resistance in *Pasteurella* isolates from cattle and swine. They showed that about 92% of *P. haemolytica* isolates were resistant to a number of clinically important antimicrobial drugs including streptomycin, penicillin, tetracycline and chloramphenicol. Streptomycin resistance was most frequent, followed by penicillin and tetracycline resistance. An increasingly high resistance to antimicrobials in bovine isolates of *P. haemolytica* was reported in North America from two studies in the same year (Amstutz *et al.*, 1982; Fales *et al.*, 1982). In the former report, the resistance was shown in order of decreasing prevalence as: streptomycin, tetracycline (90%) > ampicillin (41%) > sulphonamides (23.5%) and, in the latter report, the order was: sulphonamides (84%) > streptomycin (70%) > ampicillin (58%) > penicillin (57%) > tetracycline (21%). The obvious differences in the incidence rates of tetracycline and sulphonamide resistance between these two studies may be due to antibiotic therapy of animals (untreated vs. treated), sample size (65 vs. 386), source of samples (nasal vs. lungs) or concentrations of drugs in the disks used in the drug-sensitivity tests. A high level of resistance to sulphonamides, tetracycline and ampicillin in cattle isolates of *P. haemolytica* was also reported by Morter (1983). In an investigation of plasmid profiles of *P. haemolytica* serotypes, plasmid-containing A1 isolates were shown to be multiply-resistant to ampicillin, penicillin, tetracycline, lincomycin, streptomycin and/or sulphonamides (Boyce & Morter, 1986). Chang *et al.* (1987) found that typable and untypable

TABLE 2. MULTIPLE DRUG RESISTANCE AND PLASMID INVESTIGATION IN *P. HAEMOLYTICA* ISOLATES FROM DIFFERENT COUNTRIES

Reference	Country	Drug-resistance phenotypes*	Plasmid investigated
Chang & Carter (1976)	USA	Pe, Tc, Cm, Sm	No
Zimmerman & Hirsh (1980)	USA	Ap, Pe, Tc, Sm	Yes
Amstutz *et al.* (1982)	USA	Ap, Tc, Sm, Su	No
Fales *et al.* (1982)	USA	Ap, Pe, Tc, Sm, Su	No
Morter (1983)	USA	Ap, Tc, Su	No
Boyce & Morter (1986)	USA	Ap, Pe, Tc, Lm, Sm, Su	Yes
Chang *et al.* (1987)	USA	Ap, Pe, Tc, Bac, Lm, Sm	Yes
Rossmanith *et al.* (1991)	USA	Ap, Pe, Tc, Ak, Em, Sm, Su	Yes
Haghour *et al.* (1987)	Germany	Ap, Pe, Tc, Oxa, Em, Sm, SxT	Yes
Schwarz *et al.* (1989b)	Germany	Ap, Pe, Carb, Tic, Tc, Sm, SxT	Yes
Livrelli *et al.* (1988a)	France	Amo, Tic, Tc, Sm, Su	Yes
Wray & Morrison (1983)	UK	Ap, Pe, Amo, Carb, Sm, Su	No
Allan *et al.* (1985a)	UK	Ap, Pe, Tc, Lm, Sm	No
Shoo (1989)	UK	Ap, Pe, Lm, Sm	No
Craig *et al.* (1989)	UK	Ap, Pe, Sm, Su	Yes

* Abbreviations: Ap = ampicillin, Pe = penicillin, Tc = tetracycline, Cm = chloramphenicol, Sm = streptomycin, Su = sulphonamides, Lm = lincomycin, Bac = bacitracin, Ak = amikacin, Em = erythromycin, Oxa = oxacillin, SxT = sulphamethoxazole-trimethoprim, Carb = carbenicillin, Tic = ticarcillin, Amo = amoxycillin.

isolates of *P. haemolytica*, regardless of plasmid content, were resistant to bacitracin, lincomycin, streptomycin, ampicillin, penicillin or tetracycline. More recently, multiple antibiotic resistance was observed in 76% of *P. haemolytica* strains examined in a study of twenty strains which included representatives of most serotypes together with field isolates, where resistance to streptomycin was most common followed by amikacin, erythromycin, sulphonamides, ampicillin, penicillin or tetracycline resistance; but no isolate was resistant to chloramphenicol (Rossmanith *et al.*, 1991).

In a report from Germany, all *P. haemolytica* isolates from cattle and sheep of different countries of origin were shown to be resistant to one or more of nine drugs including ampicillin, penicillin, oxacillin, cephalothin, erythromycin, tetracycline, chloramphenicol, streptomycin, and sulphamethoxazole-trimethoprim (Haghour *et al.*, 1987). A French isolate of *P. haemolytica*, obtained from a bovine source, showed production of ROB-1 β-lactamase (see following section) and multiple resistance to amoxycillin, ticarcillin, tetracycline, streptomycin, and sulphonamide (Livrelli *et al.*, 1988a). In Britain, one strain of *P. haemolytica* A1, isolated from a calf with pneumonia, was found to be resistant to penicillin, ampicillin, amoxycillin, carbenicillin, streptomycin and sulphonamide, and produced a β-lactamase enzyme (Wray & Morrison, 1983). Allan *et al.* (1985a) found that almost all bovine isolates (>97%) of *P. haemolytica* A1 they examined were resistant to streptomycin and lincomycin. Oxytetracycline, penicillin, and ampicillin were associated with resistance in 20%, 18%, and 15% of the isolates respectively. Notable differences were found between the antibiotic resistance patterns of isolates from the upper and lower respiratory tracts of the same animal, with the nasopharyngeal isolates

having greater resistance to penicillin, ampicillin and tetracycline. The reason for such differences was not explained but might be due to prolonged exposure of the upper respiratory tract of cattle to subclinical doses of antibiotics, used as growth-promoters in feeds. However, no consistent difference was observed in the resistance patterns between the isolates from antibiotic-treated and untreated animals. Shoo (1989) reported an almost similar pattern of drug resistance to that of Allan *et al.* (1985a) in *P. haemolytica* isolates from both healthy and pneumonic calves, and no significant difference in resistance patterns was detected in isolates of A1 and A2 serotypes or in isolates from healthy and diseased animals.

Different authors (Chang & Carter, 1976; Amstutz *et al.*, 1982) proposed that the emergence of drug resistance in pasteurellae may have been due to: i) frequent use of antibiotics in the treatment of animal disease, ii) inadequate use of antibiotics in terms of dosage, duration of therapy, and route of administration, iii) addition of antibiotics as growth-promoters to animal feeds, and iv) the presence, in *Pasteurella* spp., of resistance plasmids which might have been acquired from other bacteria and which conferred the property of antibiotic resistance upon the host bacteria.

1.3.2. Beta-lactamases.

The most important biochemical mechanism for β-lactam antibiotic resistance in Gram-negative bacteria is the production of β-lactamases. These enzymes cleave the β-lactam ring of penicillins and cephalosporins to give products devoid of antibacterial activity (Sykes & Matthew, 1976).

Beta-lactamases are encoded by genes present on either the bacterial chromosome or on a plasmid. Matthew (1979) described and differentiated 11

types of plasmid-determined β-lactamases on the basis of substrate profile (i.e. hydrolysis rate of penicillins vs. cephalosporins), reaction with inhibitors, isoelectric point (pI), and molecular weight (MW). Since then additional plasmid-mediated enzymes have been discovered, bringing their number to more than 20 in 1986 (Medeiros *et al.*, 1985; Livermore & Jones, 1986), and new enzymes are still being added to the existing list. Beta-lactamases described by Matthew (1979) fall into three broad classes: i) the 'broad spectrum' penicillinases which hydrolyze benzyl- penicillin and cephaloridine at similar rates, ii) the oxacillinases which hydrolyze oxacillin rapidly, and iii) the carbenicillinases which destroy carbenicillin. Based on amino acid composition and sequence homologies, Ambler (1980) proposed a classification scheme for β-lactamases, which included classes A and B, and which has now been extended to C and D.

The nomenclature of different plasmid-mediated β-lactamases is mainly attributed to their source or properties. TEM ('Temoniera', a patient's name from whom the β-lactamase-producing *E. coli* strain was isolated), ROB (according to the source strain *H. influenzae* ROB), SHV (Sulphydryl variable), OXA (Oxacillin-hydrolyzing), PSE (*Pseudomonas*-specific), and CARB (Carbenicillin-hydrolyzing) are several of the common β-lactamase enzymes. TEM (TEM-1 or TEM-2) is the most common plasmid-mediated enzyme and is found on a wide variety of naturally-occurring plasmids of Gram-negative bacteria (Kontomichalou *et al.*, 1970; Datta *et al.*, 1971), while ROB-1 is a novel enzyme first detected in *H. influenzae* type b strain isolated from a child with meningitis (Rubin *et al.*, 1981).

Comparison of some properties of representative plasmid-mediated β-lactamases is shown in Table 3. Apart from the MW and pI values as bases of distinction, OXA-1 can be distinguished by ready hydrolysis of oxacillin

TABLE 3. COMPARISON OF PROPERTIES OF PLASMID-MEDIATED BETA-LACTAMASES*

	Relative rate of hydrolysis§						Inhibited by			
β-lactamase	Ap	Carb	Oxa	Clox	Cer	Cet	Clox	NaCl	Mol. wt. (kDa)	pI
TEM-1	106	10	5	0	76	20	+	-	22	5.4
TEM-2	107	10	5	0	74	20	+	-	23.5	5.6
ROB-1	186	25	6	<2	24	2	+	-	...	8.1
SHV-1	212	8	0	<2	56	8	+	-	17	7.6
OXA-1	382	30	197	190	30	15	-	+	23.3	7.4
PSE-1	90	97	<2	<2	18	<2	-	...	26.5	5.7
CARB-1	88	150	8	<2	40	4	-	...	32	5.3

* Adapted from Rubin *et al.* (1981) and Medeiros (1984).

§ Ap = ampicillin, Carb = carbenicillin, Oxa = oxacillin, Clox = cloxacillin, Cer = cephaloridine, Cet = cephalothin.

and cloxacillin, and sensitivity to inhibition by NaCl but not by cloxacillin. In contrast, TEM-1, TEM-2, ROB-1, and SHV-1 have low or no activities against oxacillin and cloxacillin and are sensitive to inhibition by cloxacillin but not by NaCl. PSE-1 and CARB-1 readily hydrolyze carbenicillin. Furthermore, TEM- and ROB-type enzymes differ mainly in the hydrolysis rates of ampicillin (or carbenicillin) and cephaloridine (or cephalothin). ROB-1 has shown higher pI than all other enzymes.

1.4. Plasmids.

A plasmid is an autonomous, self-replicating extrachromosomal DNA element (Lewin, 1987). Following the discovery of conjugation in *Escherichia coli*, Lederberg (1952) first coined the term "plasmid" to refer to all extrachromosomal genetic elements present in a bacterium. He used to call these elements 'symbiotic particles' before the new name was given. Novick (1980) considered plasmids as 'subcellular organisms', found in a wide variety of bacteria, and not essential to the growth or metabolism of the host cell. Plasmids are normally circular molecules (Hardy, 1986), but linear forms of plasmids have also been reported in *Streptomyces rochei* (Hirochika *et al.*, 1984), *Borrelia burgdorferi* (Barbour & Garon, 1987), and *Thiobacillus versutus* (Wlodarczyk & Nowicka, 1988). They constitute between <1 to 3 percent of the host cell's genome and the size of a single plasmid may range from 1 to about 300 kilobase pairs (kb).

1.4.1. Topological forms, functions and types of plasmids.

Plasmids may exist in more than one form in cellular lysates of bacteria, but the covalently closed circular (CCC) double-stranded DNA form (component I) predominates. The CCC form has no breaks in either strand of DNA and is characteristically supercoiled or twisted upon itself (Bauer *et al.*, 1980). Open circular (OC) plasmid, resulting from nicks or breaks in either one or both DNA strands, is a second form (component II) of plasmid DNA that may be present in bacterial cell lysates. When both DNA strands are broken or nicked at points either exactly opposite or very close to each other, a linear form (component III) of plasmid DNA is generated. This may occur due to strong shearing stress during plasmid preparation or cleavage by endonuclease enzymes.

Various functions have been attributed to plasmids (Broda, 1979; Hardy, 1986): i) resistance to antibiotics and heavy metals, ii) production of antibiotics, iii) utilization of complex organic compounds (carbohydrates, hydrocarbons) as energy sources, iv) production of bacteriocins and haemolysins, v) production of restriction and modification enzymes, vi) production of toxins, vii) synthesis of virulence and colonization factors, viii) tumorigenicity and nitrogen-fixation in plants and ix) capability to mate with a suitable recipient cell (i.e. genetic transfer).

Three main types of plasmids have been characterized most extensively: i) F plasmids or fertility factors, ii) drug-resistance (R) plasmids or R factors and iii) colicinogenic (Col) plasmids. Plasmids associated with the expression of virulence factors in bacteria are often termed 'virulence plasmids'. Plasmids with unknown functions are termed 'cryptic plasmids'.

On the basis of replication control systems, plasmids are classified

into incompatibility (*Inc*) groups, a group comprising plasmids which cannot co-exist together in the same host cell. Incompatibility is a manifestation of relatedness: the sharing of common elements involved in plasmid replication control or equipartitioning (Novick, 1987). About 30 *Inc* groups have been established for the plasmids found in enterobacteria and 7 for the staphylococcal plasmids (Couturier *et al.*, 1988).

1.4.2. Antibiotic-resistance plasmids (R plasmids).

R plasmids were first discovered in multiple drug-resistant *Shigella* species in Japan during the late 1950s (Ochiai *et al.*, 1959) and they are important from the clinical and therapeutic points of view. In Europe, plasmids of this type were first reported in *Salmonella* species (Datta, 1962).

R plasmids contribute to multiple drug resistance in both Gram-positive and Gram-negative bacteria and make their bacterial hosts resistant to antibiotics by one of four mechanisms (Foster, 1983): i) by altering the target site of the antibiotic, ii) by modifying the antibiotic so that it is no longer active, iii) by preventing the antibiotic from entering the cell, and iv) by specifying an enzyme which provides a substitute for a host-specified enzyme which is the target of the antibiotic.

Typical R plasmids are large, broad host range, low copy number, self-transmissible or conjugative plasmids. They possess two functionally distinct parts: the resistance transfer factor (RTF), which contains the genes for replication (*rep*) and conjugation (*tra*), and the resistance determinant (R-det). R-dets are covalently linked to the *tra* genes and many of them are located on a transposable element (Mitsuhashi, 1977). In some

instances, the *tra* genes are clustered in a single region on the plasmid, whereas in others, they are scattered in several different regions (Willetts & Skurray, 1980). The *tra* genes determine the production and assembly of sex pili (tube-like surface appendages made of protein), necessary to mediate cell-to-cell contact and DNA transfer (Willetts & Wilkins, 1984). Non-transmissible or non-conjugative R plasmids are usually small, lack the *tra* genes, but carry the *mob* (mobility) gene in which the origin of transfer replication (*oriT*) is presumed to be present. A typical genetic structure of conjugative and non-conjugative R plasmids is shown in Figure 2.

Non-conjugative plasmids can be mobilized into recipients when an appropriate conjugation system (i.e. *tra*) is provided by a co-existing conjugative plasmid. Mobilization is characterized by lack of covalent union between the participating conjugative and non-conjugative plasmids. Hence the two plasmids are not necessarily co-transferred to recipient cells. The non-conjugative plasmid participates actively in the mobilization process. It has been shown that the *mob* genes of ColE1 and RSF1010 plasmids encode mobilization proteins (Warren & Sherratt, 1977; Derbyshire *et al.*, 1987) that recognize, and bind to, a specific site on the plasmid molecule called *nic* which is probably the *oriT* of the plasmid. In the donor cell, the plasmid DNA to be mobilized is cleaved on one strand at the *nic* or *oriT* site by the mobilization proteins in order to trigger or initiate DNA transfer (Willetts & Wilkins, 1984; Derbyshire & Willetts, 1987). *oriT* also serves as the site at which recircularization of the transferred DNA takes place in the recipient cell. Furthermore, the *oriT* sequence may be required for the formation of primers that initiate complementary strand synthesis in the donor and recipient cells (Derbyshire

FIGURE 2. **TYPICAL GENETIC ORGANIZATION OF CONJUGATIVE AND NON-CONJUGATIVE R PLASMIDS.**

The figure was adapted from Saunders (1984).

Key:

oriV = Origin of vegetative replication.

oriT = Origin of transfer replication.

tra = Transfer genes.

mob = Mobility region.

nic = Nicking site for mobility proteins.

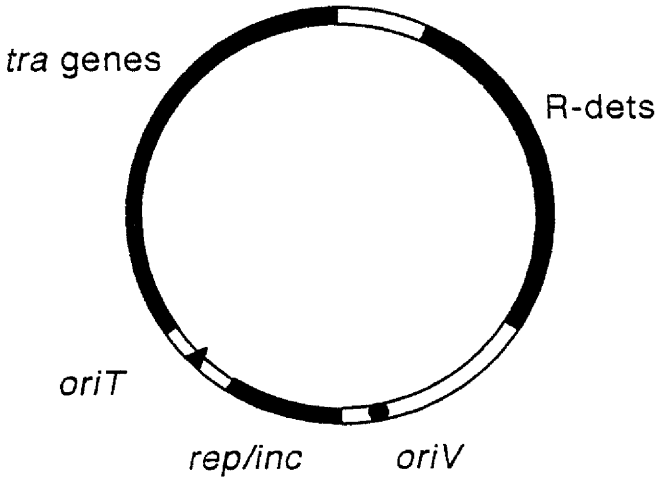
R-det = Resistance determinant.

rep = Replication genes.

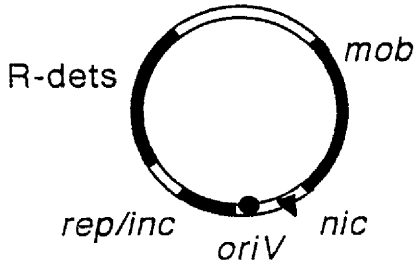
inc = Incompatibility determinant.

oriT represents a DNA sequence presumably located at the *nic* site within, or adjacent to, the *mob* region of both types of plasmid.

Conjugative



Non-conjugative



& Willetts, 1987).

The important features identified for the rapid evolution and dispersal of R plasmids are their conjugative ability and the presence within their sequences of transposable genetic elements known as 'transposons' (Cohen, 1976). Those plasmids which belong to the *Inc*P group in Gram-negative bacteria may be especially important in disseminating drug resistance to a wide range of organisms. Multiple drug resistance is thought to arise from the accumulation and insertion on a plasmid of individual transposons or 'jumping genes', derived from another plasmid or from the chromosome of the same or another bacterium. Transposon-encoded antibiotic resistance was first observed as a transfer of penicillinase activity between plasmids, and between plasmids and chromosomes (Datta *et al.*, 1971; Richmond & Sykes, 1972). The ampicillin resistance-encoding transposon, Tn*3*, is one of the most extensively studied drug-resistance transposons with regard to the mechanisms of transposition (Sherratt, 1989).

It has long been assumed that R plasmid evolution in bacteria is promoted by the selection pressure provided by the antibiotics used or present in the growth environment (Falkow, 1975).

1.4.2.1. R plasmids and plasmid-mediated β-lactamases in Gram-negative bacteria related to *Pasteurella*.

Since their discovery in *Shigella* spp., R plasmids have been found in numerous bacterial species pathogenic for man and animals and are particularly common in members of the *Enterobacteriaceae* including *E. coli*, salmonellae and shigellae, and in pseudomonads (Hardy, 1986).

Plasmids coding for resistance to a number of antibiotics have been demonstrated in the clinically important species of the genus *Haemophilus*. Two types of R plasmids have been found to occur in *Haemophilus influenzae*: 45 to 57-kb plasmids and 3.75 to 6.6-kb plasmids (De Graaff *et al.*, 1976; Elwell *et al.*, 1977; van Klingeren *et al.*, 1977; Kaulfers *et al.*, 1978; Laufs *et al.*, 1979). The large *Haemophilus* R plasmids that have been described show 65-100% homology in their base sequences independently of geographic origin and their antibiotic resistance markers (Elwell *et al.*, 1977; Laufs & Kaulfers, 1977). A small plasmid has been shown to be closely related to plasmids coding for β-lactamase in gonococci (Laufs *et al.*, 1979). R plasmids encoding multiple resistance to ampicillin, chloramphenicol and tetracycline have also been reported in *H. influenzae* (Bryan, 1978). Moreover, plasmids coding for β-lactamase production and sulphonamide resistance have been found in *Haemophilus ducreyi* (Brunton *et al.*, 1979; Albritton *et al.*, 1982) and *Haemophilus(Actinobacillus) pleuropneumoniae* (Hirsh *et al.*, 1982); the latter additionally carried a streptomycin-resistance plasmid.

In some large surveys, 3-5% of *H. influenzae* isolates carrying plasmid-mediated β-lactamase activity have been detected (Ward *et al.*, 1978; Green *et al.*, 1979). Beta-lactamase-encoding R plasmids are common in *H. influenzae*, and the β-lactamase enzymes have been found to be of the TEM-type in most cases (Elwell *et al.*, 1975; Thorne & Farrar, 1975; Laufs & Kaulfers, 1977; Gutmann *et al.*, 1988) and of the more recently described ROB-1 type in some instances (Rubin *et al.*, 1981; Medeiros *et al.*, 1986; Livrelli *et al.*, 1988b; Gutmann *et al.*, 1988). In an epidemiological survey for the ROB-1 β-lactamase among 161 ampicillin-resistant *H. influenzae* isolates in the United States, 8% of strains produced ROB-1, whereas 92%

produced TEM-1, as detected by DNA hybridization and β-lactamase characterization studies (Daum *et al.*, 1988). The ROB-1 enzyme has a substrate profile similar to the TEM-type but with a quite different isoelectric point (Table 3), and is more likely to be found in animal pathogens (Medeiros *et al.*, 1986; Gutmann *et al.*, 1988). The ROB-1 β-lactamase gene is not structurally related to the genes for the other known β-lactamases, including TEM-1 (Medeiros *et al.*, 1986; Levesque *et al.*, 1987; Daum *et al.*, 1988). The nucleotide sequence of the original (prototype) ROB-1 β-lactamase gene has been determined by Juteau & Levesque (1990).

A ROB-1 β-lactamase-encoding plasmid in association with a TEM-1 β-lactamase plasmid has been recently detected in the same isolate of *H. ducreyi*, and expression of one β-lactamase (TEM-1) appeared to suppress expression of the other (ROB-1) (Maclean *et al.*, 1992).

1.4.2.2. R plasmids and plasmid-mediated β-lactamases in *P. multocida* and *P. haemolytica*.

R plasmid-containing *Pasteurella* strains have emerged in different countries, particularly in the USA. Several workers reported the presence of R plasmids in antibiotic-resistant strains of *P. multocida* isolated from septicaemic turkeys (Berman & Hirsh, 1978; Hirsh *et al.*, 1981; 1985; 1989) and from pneumonic cattle (Silver *et al.*, 1979). These plasmids are small (3.5 to 15 kb in size) and non-conjugative, encoding resistance commonly to streptomycin, sulphonamides and tetracycline. Hirsh *et al.* (1989), in addition, isolated a large (105 kb), conjugative R plasmid. Another F-type conjugative plasmid was described earlier in *P. multocida* and shown to be

responsible for the transfer of a small and non-conjugative R plasmid (11 kb) from the same strain to *E. coli* and other strains of *P. multocida* (Hirsh *et al.*, 1981). A small streptomycin-resistance plasmid (5 kb) has been detected in *P. multocida* cultures derived from bovine and swine sources (Schwarz *et al.*, 1989a). Haghour *et al.* (1987) found plasmids, ranging from ~2 to 43 kb, in 35 of 163 isolates of *P. multocida* from different animal species, but could not show correlation with antimicrobial resistance.

Plasmids associated with antimicrobial resistance phenotypes in bovine isolates of *P. haemolytica* have been reported from the United States (Zimmerman & Hirsh, 1980; Boyce & Morter, 1986; Chang *et al.*, 1987), Germany (Haghour *et al.*, 1987; Schwarz *et al.*, 1989b), France (Livrelli *et al.*, 1988a; 1991) and Britain (Craig *et al.*, 1989). These plasmids are small (2 to 8.6 kb), non-conjugative, and associated with resistance commonly to β-lactam antibiotics, particularly ampicillin and penicillin, and rarely to tetracycline, streptomycin and sulphonamide compounds. Some of these reports have correlated particular antibiotic resistances with the presence of plasmid (R plasmid) by transfer studies such as transformation (Zimmerman & Hirsh, 1980; Livrelli *et al.*, 1988a; 1991; Schwarz *et al.*, 1989b; Craig *et al.*, 1989) and conjugation (Craig *et al.*, 1989). R plasmids in *P. haemolytica* and their confirmed antibiotic-resistance phenotypes are given in Table 4.

Zimmerman & Hirsh (1980) recovered two 8.6- and 8.3-kb plasmids from one *P. haemolytica* strain (T biotype), which were shown to code for tetracycline and streptomycin resistance respectively. These authors, in addition, noted that ampicillin resistance in the strain was β-lactamase-mediated, but this resistance could not be conclusively shown to be

TABLE 4. R PLASMIDS IN *P. HAEMOLYTICA* AND THEIR ENCODED PHENOTYPES

Reference	Country	R plasmid (kb)	Encoded phenotype*
Zimmerman & Hirsh (1980)	USA	8.3	Sm^R
		8.6	Tc^R
Livrelli *et al.* (1988a)	France	4.4	Ap^R
Livrelli *et al.* (1991)	,,	4.1, 4.4	Ap^R
Schwarz *et al.* (1989b)	Germany	4.3	Ap^R
Craig *et al.* (1989)	UK	4.5	Ap^R

* For abbreviations of antibiotics, see footnote (*) of Table 2.

^{R} = resistance.

plasmid-encoded. The work of Boyce & Morter (1986) demonstrated that only a single plasmid (~3 kb) was present in isolates of the A2 serotype, but two distinct plasmid profiles (one containing 3 plasmids and the other containing 2 plasmids, ranging from 3.9 to 5 kb) were found in isolates of the A1 serotype of *P. haemolytica*. It is important to note that only strains isolated from sick calves were studied and all were drug resistant and contained plasmids. Sulphonamide resistance was presumed to be encoded by a 4.5-kb plasmid. Chang *et al.* (1987) showed plasmids (2 to 5.4 kb) to be present in both typable and untypable strains of *P. haemolytica* and a correlation was found between a 5.2-kb plasmid and resistance to ampicillin and penicillin in A1 isolates. Several other workers have suggested a causal relationship between *P. haemolytica* plasmids (3.8 to 5.1 kb) and resistance to ampicillin and penicillin (Richards *et al.*, 1984; Rossmanith *et al.*, 1991). Haghour *et al.* (1987) isolated small plasmids (3.9 to 4.7 kb) exclusively from cattle isolates of *P. haemolytica* and found their association with resistance to ampicillin, penicillin, tetracycline, and sulphamethoxazole-trimethoprim. R plasmids (4.1-4.5 kb) encoding only β-lactam resistance (e.g. Ap^R) in bovine isolates of *P. haemolytica* have been identified and analyzed in France (Livrelli *et al.*, 1988a; 1991), Germany (Schwarz *et al.*, 1989b) and the UK (Craig *et al.*, 1989).

Plasmid-mediated β-lactamases have been demonstrated in *P. multocida* (Livrelli *et al.*, 1988a) and *P. haemolytica* (Livrelli *et al.*, 1988a; 1991; Schwarz *et al.*, 1989b; Azad *et al.*, 1992) and were found to be of the ROB-1 type (Livrelli *et al.*, 1988a; 1991; Azad *et al.*, 1992). The genetic determinant of this ROB-1 β-lactamase did not show homology with the other β-lactamase (TEM-1, OXA-1, SHV-1 or CARB-1)-encoding plasmid genes by hybridization experiments (Levesque *et al.*, 1987; Livrelli *et al.*, 1991).

The nucleotide sequence of the ROB-1 β-lactamase gene from *P. haemolytica* has been recently published (Livrelli *et al.*, 1991) and shown to be almost identical to that of the ROB-1 β-lactamase gene from *H. influenzae* (Juteau & Levesque, 1990). The only contrasting difference in the sequences indicated that the ROB-1 β-lactamase genes from *Pasteurella* and *Haemophilus* species have different promoters. Both types of ROB-1 β-lactamases were found to belong to the class A enzyme (see Section 1.3.2) and share several features including amino acid sequence homology with Gram-positive bacterial β-lactamases (Juteau & Levesque, 1990; Livrelli *et al.*, 1991).

Several R plasmids from *P. multocida* have been tested for *Inc* grouping and it was found that the plasmids did not belong to any of the recognized *Inc* groups, including *Inc*Q (Berman & Hirsh, 1978; Hirsh *et al.*, 1985). This is an unexpected observation, because the moles percent G+C contents of these plasmids suggested that they were of enteric origin (Hirsh *et al.*, 1985). There has been no report of *Inc* grouping of R plasmids from *P. haemolytica*.

1.4.2.3. Homologies and relationships among R plasmids.

Relationships among R plasmids, derived from different bacterial hosts, can be established by restriction endonuclease analysis, DNA-DNA hybridization and electron microscopy of DNA heteroduplexes. DNA-DNA hybridization is an efficient and reliable method which is performed by using one plasmid as a hybridization probe to seek homology with restriction fragments of other plasmids on Southern blots (Southern, 1975).

Several streptomycin and sulphonamide-resistance plasmids of the *Inc*Q group, isolated from a wide range of locations and bacterial species

(*Salmonella, Proteus, Providencia, Pseudomonas* and *E. coli*), were found to be closely related (Barth & Grinter, 1974; Grinter & Barth, 1976). By employing restriction endonuclease mapping and Southern hybridization techniques, homologies were shown between the broad-host-range plasmid RSF1010 (originally obtained from *Salmonella panama*) and the sulphonamide (and/or streptomycin)-resistance plasmids from *Bordetella bronchiseptica* (Lax & Walker, 1986), *A. pleuropneumoniae* (Wilson *et al.*, 1989a) and *H. ducreyi* (Wilson *et al.*, 1989b). Laufs & Kaulfers (1977) demonstrated high DNA homology of an ampicillin-resistance plasmid from *H. influenzae* with various R factors derived from the same species of wide geographic origin.

By DNA-DNA hybridization experiments, the probable origins of R plasmids can also be deduced. The sulphonamide-resistance plasmids in *H. ducreyi* were 80% homologous to RSF1010, suggesting that they represented an extension of the enteric plasmid pool into *Haemophilus* (Albritton *et al.*, 1982). An ampicillin-resistance plasmid from *H. influenzae* has been shown to contain DNA sequences characteristic of the ampicillin-resistance transposon (TnA) found on some R factors of enteric origin (Elwell *et al.*, 1975; Laufs & Kaulfers, 1977). Several workers hypothesized that the β-lactamase-encoding plasmids of *Haemophilus* spp. and *Neisseria gonorrhoeae* originated by insertion of TnA, possibly derived from an enteric source, into phenotypically cryptic indigenous plasmids (Elwell *et al.*, 1977; Roberts *et al.*, 1977; Brunton *et al.*, 1986).

1.4.3. Virulence plasmids in Gram-negative bacteria.

Some plasmids carry genetic determinants which may code for different virulence properties characteristic of a number of bacterial pathogens both

in man and animals (Broda, 1979; Elwell & Shipley, 1980). In many Gram-negative bacteria, plasmids contribute to a variety of virulence phenotypes including production of enterotoxin, haemolysin, colicin, and colonization or adherence antigen in *E. coli*; the invasive ability of *E. coli*, shigellae and salmonellae; and congo-red binding as a virulence marker in *Yersinia* and *Shigella* species.

Two kinds of *E. coli* enterotoxin, heat-labile toxin (LT) and heat-stable toxin (ST), are plasmid-specified, and have been reviewed in a paper on virulence plasmids of animal pathogens (Elwell & Shipley, 1980). That haemolysin production, colicin biosynthesis, and elaboration of colonization antigen in *E. coli* are plasmid-mediated, has also been reviewed (Novick, 1969; Helinski, 1973). Plasmid-encoded colonization antigens are K88 from pig isolates, K99 from calf or lamb isolates, and CFAI or CFAII from human isolates of enterotoxigenic *E. coli* (ETEC). Studies with pig and calf isolates of ETEC strains showed that enterotoxin production (Smith & Halls, 1968) and expression of both K88 (Ørskov & Ørskov, 1966) and K99 (Smith & Linggood, 1972) adherence proteins were encoded by plasmids, which were transmissible. The transmissible nature of these virulence plasmids was exploited to assess their role in the pathogenesis of porcine diarrhoea by a series of experiments (Smith, 1976). This author transferred the plasmids in different combinations into a set of non-pathogenic *E. coli* strains which were then used to infect young pigs. The presence of both enterotoxin (Ent) plasmid and K88 plasmid made *E. coli* enteropathogenic, causing diarrhoea in pigs. Further molecular studies with the Ent plasmid revealed that there was no transposon for LT, but that the ST gene was part of a transposon, Tn*1681* (So *et al.*, 1979). Production of colicin V (ColV) by *E. coli* is also mediated by a plasmid and

may enhance virulence by conferring the property of serum resistance upon its host (Smith, 1974). Smith & Huggins (1976, 1978) cured human, bovine, ovine, and avian *E. coli* strains of ColV plasmids and showed that loss of this virulence plasmid was accompanied by a decrease in pathogenicity. Reintroduction of the same plasmid into the cured derivative of *E. coli* by conjugation restored pathogenicity to its original level.

Two large virulence plasmids, one (210 kb) associated with invasiveness of *Shigella flexneri* (Sansonetti *et al.*, 1982) and enteroinvasive *E. coli* (Hale *et al.*, 1983), and the other plasmid (180 kb) involved in the expression of virulence-related O-side chains of LPS in the cell wall of *Shigella sonnei* (Sansonetti *et al.*, 1981), have been described. Plasmids of different sizes were also found to be associated with virulence and O-antigen expression in *Shigella dysenteriae* (Watanabe & Timmis, 1984; Haider *et al.*, 1990). The genetic basis of virulence, including the plasmid-borne genes, in *Shigella* spp. has been recently reviewed (Hale, 1991).

Plasmid-mediated congo-red binding has been shown to be an indicator of virulence in *Shigella* spp. (Maurelli *et al.*, 1984; Qadri *et al.*, 1988) and *Yersinia enterocolitica* (Bhaduri *et al.*, 1987). This dye-binding ability was previously shown to be linked to virulence in *Pasteurella pestis* (now called, *Yersinia pestis*) (Surgalla & Beesley, 1969). Production of two virulence factors, coagulase and plasminogen activator, by *Y. pestis* was encoded by a 9.5-kb plasmid (Sodeinde & Goguen, 1988). A 70-kb virulence plasmid has been shown to encode a set of outer-membrane proteins (called Yops) and surface-associated fibrillar proteins (adhesins) in the pathogenic species of *Yersinia* (Cornelis *et al.*, 1989). The loss of these proteins correlates with loss of pathogenicity.

Large plasmids have been implicated in the virulence of many serotypes

of *Salmonella* (Gulig, 1990). The virulence plasmids are primarily responsible for spreading infection beyond the host intestines, but the exact mechanism is unknown. Initially, Jones *et al.* (1982) described that a 90-kb plasmid was required for the invasive phenotype of *Salmonella typhimurium*. In different serotypes of non-typhoid *Salmonella*, Helmuth *et al.* (1985) identified high molecular-weight plasmids which were found to be responsible for virulence in animal models. *Salmonella* plasmids have also been reported to be associated with increased serum resistance, a virulence phenotype of bacteria, encoded by a number of genes including *traT* involved in conjugative functions (Hackett *et al.*, 1987; Rhen & Sukupolvi, 1988; Sukupolvi *et al.*, 1992). The virulence genes on *Salmonella* plasmids have been identified and analyzed (Williamson *et al.*, 1988; Lax *et al.*, 1990) and found to be distributed among different species of *Salmonella* (Woodward *et al.*, 1989).

A large virulence plasmid (180 kb) has been isolated from *Klebsiella pneumoniae* (Nassif *et al.*, 1989). This plasmid was shown to code for the mucoid phenotype and aerobactin production, both of which contribute to virulence properties of this bacterium.

To date, there have been no reports on virulence-related extra-chromosomal genetic elements (plasmids or bacteriophages) which might play a role in the pathogenicity of *Pasteurella*. Haemolysin production in *P. haemolytica* does not appear to be plasmid-mediated (Chang *et al.*, 1987). Production of leukotoxin, the principal virulence factor of this species, is not related to the presence of plasmids and has been shown to be chromosomally-mediated (Richards, 1985; Lo *et al.*, 1985; Chang *et al.*, 1987). The chromosomal gene coding for the leukotoxin of *P. haemolytica* A1 has been cloned (Lo *et al.*, 1985), sequenced (Lo *et al.*, 1987) and found to

have a significant degree of DNA sequence homology with the α-haemolysin gene of *E. coli* (Strathdee & Lo, 1987).

1.4.3.1. The R factor as a virulence plasmid.

R factors (R plasmids) have been associated with virulence properties of bacteria on several occasions (Elwell & Shipley, 1980). The multiple drug-resistance plasmids have been shown to increase the virulence of *Salmonella typhi* and *S. dysenteriae* (Gangarosa *et al.*, 1972). A K99-encoding virulence plasmid (78 kb) isolated from a bovine strain of *E. coli* was found to carry the genes for resistance to tetracycline and streptomycin (So *et al.*, 1976). Several workers described plasmids which were simultaneously associated with the synthesis of enterotoxin of *E. coli* (LT and/or ST) and resistance to tetracycline and streptomycin (Gyles *et al.*, 1977; Echeverria & Murphy, 1978). These plasmids were thought to be the result of recombination between an Ent plasmid and an R factor. A conjugative R plasmid (R6-5) has been shown to increase the pathogenic potential in *E. coli* by endowing the host with the property of high level serum resistance, and the *traT* gene product (a major OMP) has been found to be responsible for this phenomenon (Moll *et al.*, 1980). A large, self-transmissible R plasmid (117 kb) in an enteropathogenic *E. coli* has been recently reported to play a role in colonization of the rabbit intestinal tract and thus contributed to the virulence of this strain (Reynaud *et al.*, 1991). Plasmid-encoded colonization factors were probably fimbrial-like structures found on the cell surface.

1.4.4. Plasmid cloning vectors for *E. coli* and other Gram-negative bacteria.

"Because of their reliability and ease of handling, plasmids have become the workhorses of molecular cloning" (Sambrook *et al.*, 1989). Plasmids are often used to construct shuttle vectors to transfer foreign genes between *E. coli* and other Gram-negative bacteria in the field of genetic engineering.

The desirable features of an ideal plasmid cloning vector are: i) small size, ii) broad-host-range origin of replication, iii) selectable marker(s), iv) wide range of unique restriction sites, v) high copy number, vi) capacity for amplification, vii) stability and viii) conjugative or mobilization ability.

Plasmid vectors originally designed for use with *E. coli* employed insertional inactivation of one of two antibiotic-resistance genes present in the vector for recognition of recombinant clones. Perhaps the best-known and widely-used cloning vector of this kind is pBR322 (Bolivar *et al.*, 1977), based on the pMB1 replicon. This has a size of 4.36 kb, encodes resistance to ampicillin and tetracycline, and contains unique sites for 20 different restriction enzymes. Another such cloning vector is pACYC184 (Chang & Cohen, 1978), constructed from the P15A replicon. This has a size of 4.2 kb, and contains tetracycline- and chloramphenicol-resistance genes and unique sites for various restriction enzymes. Replication of these vectors is restricted to *E. coli*.

Commonly used *E. coli* cloning vectors at the present time are those of the pUC series constructed by Messing & co-workers (Vieira & Messing, 1982; Norrander *et al.*, 1983; Yanisch-Perron *et al.*, 1985). The pUC vectors are

small derivatives of pBR322, 2.69 kb in size. They contain a gene encoding resistance to ampicillin. They also carry a segment of DNA derived from the *lac* operon of *E. coli* that encodes the amino-terminal fragment (α-peptide) of β-galactosidase. This fragment, whose synthesis can be induced by isopropylthio-β-D-galactoside (IPTG), is capable of intra-allelic (α) complementation with a defective form of β-galactosidase produced by the host. Appropriate *E. coli* strains, transformed with the pUC plasmids and then exposed to IPTG, synthesize both fragments of the enzyme and form blue colonies when plated on media containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). The α-peptide-coding region contains a closely arranged series of synthetic cloning sites called a polylinker, or polycloning or multiple cloning site. Insertion of foreign DNA into one of these sites inactivates the α-peptide resulting in the production of white colonies and thus enables immediate visual identification of recombinant clones (histochemical screening).

The pIC plasmid vectors (Marsh *et al.*, 1984) are derivatives and improved versions of the pUC plasmids. A greater range of restriction sites in the polylinker region have made these improved vectors more versatile. However, the vectors of either the pUC or the pIC series appear to lack the *mob* gene and are incapable of transfer by conjugation to another *E. coli* host (Sambrook *et al.*, 1989; Schweizer, 1991).

A set of broad-host-range cloning vehicles was constructed for Gram-negative bacteria based on the pSa origin of replication (Tait *et al.*, 1983). Concurrently, Simon *et al.* (1983) developed a mobilization system which involved the construction of mobilizing *E. coli* strains, containing RP4 inserted into the chromosome, and mobilizable vectors useful in transposon-mutagenesis of other species. Another group of workers described

several vector derivatives of RK2, including pRK290 and pRK404, which could be used as broad-host-range cloning vectors for a wide variety of Gram-negative bacteria (Ditta *et al.*, 1985). Similar vectors based on plasmid RSF1010 have been developed by Labes *et al.* (1990). These are expression and *lac* fusion vectors, used for the study of function and expression of foreign genes in Gram-negative bacteria.

Commonly used broad-host-range vectors (e.g. pRK290, pSa4) could not be propagated in *P. haemolytica* (Craig *et al.*, 1989). The same was found to be true for *Campylobacter jejuni* and a shuttle vector was therefore constructed based on a plasmid replicon of *C. jejuni* origin (Labigne-Roussel *et al.*, 1987). Development of shuttle cloning vectors has also been reported for other Gram-negative bacteria such as *Pseudomonas* spp. (Nieto *et al.*, 1990; Schweizer, 1991), *H. influenzae* (Trieu & McCarthy, 1990; Chandler, 1991), *A. pleuropneumoniae* (Lalonde & O'Hanley, 1989) and *Actinobacillus actinomycetemcomitans* (Sreenivasan *et al.*, 1991). Some of these vectors were mobilizable by *tra* functions supplied by another plasmid (RP4 or pRK2013), whereas some were not and could only be transferred to recipient bacteria by transformation or electroporation (for definition, see Section 1.5.1).

Very recently, construction of a broad-host-range shuttle expression vector has been described for *A. pleuropneumoniae* and *P. haemolytica* (Frey, 1992). This vector is based on the minimal autonomous replicon of plasmid RSF1010, and a type II chloramphenicol acetyl transferase gene from plasmid pSa for selection purposes.

A detailed review of plasmid cloning vectors for Gram-negative bacteria appeared in an article by Schmidhauser *et al.* (1988).

1.5. Genetic Transfer Mechanisms.

Three mechanisms exist which allow transfer of genetic information (DNA) between bacteria. These mechanisms are: transformation (Griffith, 1928), conjugation (Davis, 1950) and transduction (Zinder & Lederberg, 1952).

1.5.1. Transformation in *Pasteurella* and related species.

Genetic transformation is a process by which a bacterial cell takes up DNA from the surrounding medium. In the case of plasmid DNA this may replicate autonomously, whereas chromosomal DNA may be incorporated into the genome and bestow an altered genotype that is heritable. Transformation of *E. coli* K-12 by R factor DNA from another bacterium helps to define the antibiotic-resistance phenotype encoded by individual plasmids, especially when more than one is present.

Numerous reports are now available on the transformation of *E. coli* by R plasmid DNA obtained from *P. multocida* (Berman & Hirsh, 1978; Silver *et al.*, 1979; Hirsh *et al.*, 1981; 1989; Schwarz *et al.*, 1989a) and *P. haemolytica* (Zimmerman & Hirsh, 1980; Livrelli *et al.*, 1988a; Schwarz *et al.*, 1989b; Craig *et al.*, 1989). Transformation of the *P. haemolytica* strains used by Craig *et al.* (1989) was not possible by the conventional $CaCl_2$-mediated procedure used with *E. coli*, but was possible by electroporation, a method which is based on the pulsing of a suspension of recipient bacteria with high voltages that may cause the temporary appearance of small holes in the cell envelope allowing entry of DNA molecules (Dower *et al.*, 1988). Electroporation-mediated transformation of plasmid DNA has also been reported for *P. multocida* (Jablonski *et al.*, 1992) and *A. pleuropneumoniae* (Lalonde *et al.*, 1989).

Antibiotic-resistance genes located on the chromosome as well as on small plasmids have been transferred between *H. influenzae* strains by the transformation process (Stuy, 1979). Genetic transformation in *H. influenzae* is a natural process and occurs more efficiently than in enteric bacteria, where it has to be induced by $CaCl_2$ or other treatments, and has been well characterized (Goodgal, 1982). Both intra- and interspecies transformation has been shown in *Haemophilus* (Steinhardt & Herriott, 1968; Beattie & Setlow, 1970). An intraspecies DNA transformation has been reported in encapsulated isolates of *H. influenzae* type b by the static aerobic procedure (Rowji *et al.*, 1989), in which the presence of capsule did not hinder the transformation process.

1.5.2. Conjugation in *Pasteurella* and related species.

Conjugation is a process whereby DNA is transferred between bacterial cells (donor and recipient) by a mechanism requiring cell-to-cell contact.

The earliest report of R factor transfer from *E. coli* to *Pasteurella (Yersinia) pestis* by conjugation is that of Ginoza & Matney (1963). Although most of the R plasmids identified so far in *Pasteurella* species are small and non-conjugative, both inter- and intraspecies transfer of plasmid DNA has been reported for *P. multocida* and *P. haemolytica*. Hirsh *et al.* (1989) found that a large conjugal R plasmid (105 kb) from *P. multocida* could be easily transferred to *E. coli* and *P. multocida* by conjugation. In an earlier study, a 43-kb fertility plasmid in *P. multocida* was shown to transfer an R plasmid (11 kb) present in the same strain to both *E. coli* and other strains of *P. multocida* (Hirsh *et al.*, 1981). Transfer of several natural and suicide plasmids from *E. coli* to *P. multocida* has been

described by Nnalue & Stocker (1989). An attempt to transfer an ampicillin-resistance factor from *P. haemolytica* to *E. coli* by conjugation was unsuccessful (Wray & Morrison, 1983), but Craig *et al.* (1989) successfully mobilized the same R plasmid from *E. coli* transformants to *P. haemolytica* and other *E. coli* strains by conjugation, using the transfer functions of the *Inc*P plasmid pRK2013 (Figurski & Helinski, 1979).

Large plasmids coding for resistance to one or more antibiotics have been shown to be transferable between strains of *H. influenzae* and between *H. influenzae* and *E. coli* (Thorne & Farrar, 1975; van Klingeren *et al.*, 1977; Stuy, 1979). The genetic transfer is likely to be mediated by conjugation although sex pili have not been described in haemophili. The same transfer mechanism has been reported in *H. ducreyi* (Brunton *et al.*, 1979; Deneer *et al.*, 1982).

1.5.3. Transduction in *Pasteurella* and related species.

Transduction involves the transfer of bacterial DNA from one bacterium to another by the agency of a virus particle (temperate or defective virus).

Four different transducing bacteriophages of *H. influenzae* have been reported: HP1, HP3, S2, and N3 (Stuy, 1978). Differences in phage sensitivity and lysogeny have been demonstrated among *H. influenzae* serotypes and a number of *Haemophilus* species (Stuy, 1978).

Nothing has been reported on bacteriophage-mediated transduction in *Pasteurella* spp. A common group of bacteriophage (group 5 phage) has been reported to be induced from all isolates of *P. haemolytica* tested (Richards *et al.*, 1984), and the kinetics of induction of the bacteriophages and their morphology have been determined (Richards, 1985).

OBJECTIVES OF THE RESEARCH

Currently, very little is known about the genetics of *P. haemolytica*. Because of the use of antibiotics in the control of disease caused by *P. haemolytica*, a knowledge of the genetic basis of antibiotic resistance in this bacterium is important. The initial aim of the project was to correlate the presence of plasmids in *P. haemolytica* isolates with the source and health status of the host animal, and with resistance to antibiotics exhibited by the isolates. The project also had the objective of determining whether different serotypes and untypable isolates of *P. haemolytica* had their own distinct plasmid profiles and then to characterize any R plasmids by transfer studies between *E. coli* and *P. haemolytica*, by examining their stability in *E. coli*, by seeking homology to other known drug-resistance plasmid(s), and by analyzing the plasmid-encoded enzymes.

Previous reports had indicated that plasmid DNA of *P. haemolytica* origin was difficult to reisolate or recover from *E. coli* after transformation and, therefore, another aim of the investigation was to devise procedures to amplify plasmid DNA in *E. coli* to obtain a good yield useful for characterization and cloning purposes.

An understanding of the role of virulence factors of *P. haemolytica* in the disease process has been hampered by a lack of vectors for transfer of cloned genes between *E. coli* and *P. haemolytica* and vectors to mediate transposon mutagenesis. The ultimate aim of this project was to identify a suitable plasmid which would provide the basis for construction of a shuttle vector for manipulation of *Pasteurella* genes to facilitate virulence studies and vaccine development.

2. MATERIALS AND METHODS

2.1. Bacterial Strains.

2.1.1. *P. haemolytica* strains.

Thirty five isolates of *P. haemolytica* from cattle or sheep were used in this study. These strains and their sources are listed in Table 5. Eighteen were serotype A1, 7 were A2, 1 each was T4 and T10, and 8 were untypable strains. Twenty three were bovine and 12 were ovine isolates. The majority of the isolates (30) were from pneumonic cases, only 3 were from healthy animals and 2 were from animals in contact with pneumonic cases. Nineteen *P. haemolytica* isolates of both ovine and bovine origin were kindly provided by Dr. W. Donachie of the Moredun Research Institute, Edinburgh; 15 bovine isolates were kindly supplied by Dr. H. A. Gibbs of the Glasgow University Veterinary School; and 1 bovine isolate (CVL33) was a gift from Dr. C. Wray of the Central Veterinary Laboratory, Surrey, UK. Apart from the source and the health status of the host animal (Table 5), details such as the source case (experimental or field case), antibiotic therapy of the animal (treated or untreated) and the site of isolation (nasopharynx or lungs) for several isolates were also known. In addition, strains A2S and A2N were used in this study. These were spontaneously-derived streptomycin (Sm) and nalidixic acid (Nal) resistant mutants, respectively, of *P. haemolytica* strain FA2 (serotype A2), isolated by Dr. F. F. Craig in our laboratory. Similarly, strains A1S and A1N were isolated in the present study (see following section).

All isolates were further characterized by the API 20NE system and serotyped by the indirect haemagglutination test (Q. Ali, personal communication).

TABLE 5. *P. HAEMOLYTICA* STRAINS USED IN THIS STUDY

Strain	Source		
	Animal	Health status of animal	Organization*
Serotype A1			
FA1	Ovine	Pneumonic	MRI
B2070	Bovine	Pneumonic	,,
B2086	Bovine	Pneumonic	,,
V965B	Ovine	Pneumonic	,,
W629	Ovine	Pneumonic	,,
CVL33	Bovine	Pneumonic	CVL
S/B 82/1	Bovine	Pneumonic	GUVS
S/C 82/1	Bovine	Pneumonic	,,
S/L 82/1	Bovine	Pneumonic	,,
G/A 83/5	Bovine	Pneumonic	,,
H/L 82/1	Bovine	Pneumonic	,,
W/S 82/1	Bovine	Pneumonic	,,
H/D 84/1	Bovine	Pneumonic	,,
M/F 83/1	Bovine	Pneumonic	,,
D/C 83/6	Bovine	In-contact with pneumonic	,,
S/C 84/3	Bovine	In-contact with pneumonic	,,
G/A 83/1	Bovine	Healthy	,,
W/D 83/4	Bovine	Healthy	,,
Serotype A2			
FA2	Ovine	Pneumonic	MRI
B664	Ovine	Pneumonic	,,

Continued on next page

TABLE 5 (Continued)

Strain	Source		
	Animal	Health status of animal	Organization*
T884	Ovine	Pneumonic	MRI
Y510	Ovine	Pneumonic	,,
C/P 84/2	Bovine	Pneumonic	GUVS
D/I 85/1	Bovine	Pneumonic	,,
G/T 85/15	Bovine	Healthy	,,
Serotype T4			
FT4	Ovine	Pneumonic	MRI
Serotype T10			
FT10	Ovine	Pneumonic	,,
Untypable			
T1262A	Ovine	Pneumonic	,,
T1368	Ovine	Pneumonic	,,
P709C	Bovine	Pneumonic	,,
U133	Bovine	Pneumonic	,,
B1031	Bovine	Pneumonic	,,
U300	Bovine	Pneumonic	,,
U1284	Bovine	Pneumonic	,,
U1447	Ovine	Pneumonic	,,

* MRI: Moredun Research Institute, Edinburgh EH17 7JH, U.K.

CVL: Central Veterinary Laboratory, Surrey KT15 3NB, U.K.

GUVS: Glasgow University Veterinary School, Glasgow G61 1QH, U.K.

2.1.1.1. Isolation of spontaneous antibiotic-resistant mutant strains of *P. haemolytica* A1.

P. haemolytica strain FA1 (serotype A1) was grown overnight (~10^9 cells ml^{-1}) in brain heart infusion broth (see Section 2.2) with shaking at 37°C. A 100 µl portion of the culture was spread on brain heart infusion agar containing either Sm (200 µg ml^{-1}) or Nal (20 µg ml^{-1}). The seeded plate was allowed to dry and then incubated at 37°C for 2-3 days. Antibiotic-resistant colonies (one or two) grown on the plate were picked, and their phenotype was confirmed by subculture on agar medium containing the same concentrations of antibiotics. The Sm- and Nal-resistant mutants were designated A1S and A1N respectively.

2.1.2. *E. coli* strains.

E. coli strains used in this study are given in Table 6. All strains were available from the departmental culture collection, except J53 which was kindly provided by Dr. D. J. Platt of the University Department of Bacteriology, Glasgow Royal Infirmary, Glasgow, UK. Competent cells of *E. coli* XL-1 Blue were obtained from Dr. G. Westrop of our laboratory.

2.2. Media and Growth of Bacterial Strains.

For composition and preparation of bacterial growth media, see Appendix 1.

All strains were stored frozen in 50% (v/v) glycerol in brain heart infusion (BHI) broth (Oxoid, Basingstoke, UK) at-70°C. *P. haemolytica* strains from the frozen glycerol stocks were subcultured routinely on BHI agar medium with or without the supplementation of 5% (v/v) defibrinated

TABLE 6. *E. COLI* STRAINS USED IN THIS STUDY

Strain	Genotype/Phenotype	Reference
C600	*supE*44 *hsdR thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21	Appleyard (1954)
DH1	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan (1983)
DH5	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan (1983)
HB101	*supE*44 *hsdS*20($r_B^-m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1	Boyer & Roulland-Dussoix (1969)
JM83	*ara* Δ(*lac-proAB*) *rpsL* φ80d*lacZ*ΔM15	Vieira & Messing (1982)
JC3272	$His^-Lys^-Trp^-Sm^R$	Achtman *et al.* (1971)
J53	Pro^-Met^-	Meynell & Datta (1966)
XL1-Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]	Bullock *et al.* (1987)

sheep blood (Becton Dickinson, UK). Blood agar was occasionally used for enrichment purposes for rapid growth, and for periodical confirmation of *P. haemolytica* strains by virtue of their characteristic haemolytic activity. *E. coli* strains were subcultured on either BHI agar or nutrient agar (Oxoid) or Luria-Bertani (LB) agar medium. All plates were incubated at 37°C overnight. Liquid cultures of the strains were grown in the corresponding broth media in dimpled or plain conical flasks with shaking overnight on an orbital shaker at 37°C. Where necessary, antibiotic-resistant strains were grown on agar or in liquid media containing appropriate antibiotics (concentrations of antibiotics are given in the following section).

2.3. Antibiotics.

Beta-lactam antibiotics used in this study were: penicillin G or benzyl-penicillin (Pe), ampicillin (Ap), carbenicillin (Carb), ticarcillin (Tic), oxacillin (Oxa), cloxacillin (Clox), cephaloridine (Cer), cephalothin (Cet) and cephalexin (Cex). Other antibiotics used were: chloramphenicol (Cm), tetracycline (Tc), kanamycin (Km), trimethoprim (Tp), Sm and Nal. All antibiotics were obtained from Sigma, St. Louis, MO., except Carb which was from Beecham, Brentford, UK. Preparations of stock solutions of antibiotics are described in Appendix 2. The sterile antibiotic solution was added to broth or agar media, after they had been autoclaved and then cooled to 50°C. The concentrations of the antibiotics used in selective media were ($\mu g\ ml^{-1}$): Ap (50 or 100), carb (100), Tc (10), Cm (25), Km (50), Tp (100), Sm (200) and Nal (20).

2.4. Plasmids.

The following plasmids were used in this study:-

pRK2013: A broad-host-range helper plasmid (48.0 kb) of the *Inc*P group (Figurski & Helinski, 1979), used in mobilizing the non-conjugative plasmids. This plasmid carries the transfer gene functions (*tra*) from RK2, the Km^R gene and a ColE1 replication origin. This was obtained from Dr. S. Long, Stanford University, California, USA.

pRM3022: A prototype ROB-1 β-lactamase-encoding plasmid (4.4 kb), originally isolated from *H. influenzae* strain F990 and designated R_{ROB} (Rubin *et al.*, 1981). This plasmid in *E. coli* HB101 was a generous gift from Professor E. R. Moxon, Institute of Molecular Medicine, Oxford University, Oxford, UK.

RP4: A broad-host-range conjugative (tra^+) plasmid (54.0 kb), encoding resistance to Ap, Km and Tc, and producing the prototype TEM-2 β-lactamase (Datta *et al.*, 1971). This plasmid is a member of the *Inc*P group and indistinguishable morphologically and biochemically from RP1, RK2, and R68 (Burkardt *et al.*, 1979). It carries transposon Tn*1*. This plasmid in *E. coli* J53 was kindly provided by Dr. D. J. Platt, University Department of Bacteriology, Royal Infirmary, Glasgow, UK.

R6K: A prototype TEM-1 β-lactamase-encoding plasmid (40.0 kb), which is conjugative (tra^+) and also encodes Sm^R (Kontomichalou *et al.*, 1970). This plasmid belongs to the *Inc*X group and carries a Tn*3*-like transposon Tn*2660*. It was also kindly supplied in *E. coli* J53 by Dr. D. J. Platt of Glasgow Royal Infirmary.

pUW964: A chimeric plasmid (73.0 kb) constructed from pRKTV5, a derivative of pRK2013, in which the pre-existing kanamycin resistance determinant had been inactivated by a Tn*7* (Tp^R, Sm^R and spectinomycin^R) insertion, followed by a transposition of Tn*5* (Km^R) onto the plasmid (Weiss *et al.*, 1983). This plasmid was obtained from Dr. A. A. Weiss, University of Virginia Medical School, USA.

pBR322::Tn*5*Cm^R: A suicide transposon vector, obtained by insertion of Tn*5*Cm^R (Sasakawa & Yoshikawa, 1987) into the Tc^R gene of plasmid pBR322 (Bolivar *et al.*, 1977). This was constructed by Dr. F. F. Craig in the Microbiology Department, Glasgow University.

pRK404::Tn*5*Cm^R: A transposon-carrying plasmid vector, obtained by insertion of Tn*5*Cm^R onto the Tc^R broad-host-range plasmid pRK404 (Ditta *et al.*, 1985). This was constructed by Dr. J. G. Coote in the Microbiology Department, Glasgow University.

pIC20H: An improved version of the *E. coli* cloning vector pUC19 and one of the vectors of the pIC series (Marsh *et al.*, 1984). This vector contains a β-lactamase Ap^R gene, an origin of replication and a portion of the β-galactosidase *lac*Z gene. It has a multiple cloning site (MCS) or polylinker within the *lac*Z region and DNA fragment insertion into the MCS results in loss of β-galactosidase activity, producing white colonies on X-gal media (Appendix 25). For more information on this plasmid vector, see Results (Section 3.15). It was obtained from Dr. J. Marsh, University of California, USA.

pILL514: A shuttle cloning vector for *Campylobacter jejuni* (Labigne-Roussel *et al.*, 1987). It contains a Km^R gene and a 760-bp fragment

containing *oriT* (*mob* functions) of RK2. This vector in *E. coli* DH1 was obtained from Dr. A. Labigne-Roussel, Pasteur Institute, Paris, France.

2.5. Antimicrobial Sensitivity Tests.

An antimicrobial sensitivity test or antibiogram for *P. haemolytica* strains was carried out by the disk-diffusion method (Barry & Thornsberry, 1980) using cartridge-borne antibiotic disks and a multiple disk dispenser (Oxoid). Sixteen antimicrobial agents were tested at the following concentrations per disk (μg): Ap (10), Cm (30), colistin sulphate (10), erythromycin (15), gentamicin (10), Km (30), lincomycin (2), Nal (30), neomycin (30), nitrofurantoin (300), polymyxin B (300 I.U.), Sm (10), sulphonamide compound (3300), sulphamethoxazole-trimethoprim (25), Tc (30) and Tp (1.25).

Two or three isolated colonies of *P. haemolytica* from an overnight culture plate were inoculated into a 50-ml flask containing 5 ml BHI broth and grown for 3 to 4 h with shaking on an orbital incubator at 37°C. The turbidity of the broth culture was adjusted, if necessary, with sterile normal saline (0.85% NaCl) to match that of the McFarland 0.5 standard (API system, France). Using a sterile bent glass rod, 100 μl of the culture suspension was spread over the entire surface of a pre-dried Mueller-Hinton agar medium (Difco, Detroit, MI) (Appendix 1) supplemented with 5% defibrinated sheep blood (Beckton Dickinson). The seeded plate was allowed to dry for 5 min and the antibiotic-impregnated disks were then applied to the surface of the plate by means of a multiple disk dispenser. A 'pre-diffusion' period of 15 to 20 min for the antibiotics was allowed before the plates were inverted and incubated at 37°C for 18 to 24 h. The

diameters of the zones of inhibition of bacterial growth were measured in millimetres (mm) and compared with 'zone diameter interpretive standards' (Barry & Thornsberry, 1980). The test strains were thus determined to be resistant (R) or sensitive (S) to each antibiotic.

2.6. Plasmid DNA Preparation, Purification and Analysis.

2.6.1. Small-scale preparation (miniprep) of plasmid DNA.

Miniprep plasmid DNA was isolated according to the modified alkaline-lysis method of Birnboim & Doly (1979) as described by 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. Cells from 1 ml of this culture were harvested by centrifugation at 15,000xg in a bench-top microcentrifuge (Biofuge A, Heraeus Sepatech, FRG). The pellet was drained on tissue paper after removing the supernate and resuspended in 100 µl of ice-cold lysis buffer (TEG buffer with lysozyme) (Appendix 3) and then left for 5 min at room temperature. To each microfuge tube, 200 µl of freshly prepared alkaline-sodium dodecyl sulphate (SDS) solution (Appendix 4) was added and the contents were mixed by inverting the tube several times followed by incubation on ice for 5 min. Then 150 µl of freshly prepared ice-cold acidified potassium acetate solution (Appendix 5) was added to the tube which was vortexed in an inverted position for 10 sec and left on ice for 5 min. After centrifugation at 15,000xg for 5 min at 4°C, the supernate was removed to a fresh tube to which 400 µl of buffer-saturated phenol:chloroform (1:1) (Appendix 6) was added. The contents were mixed by vortexing, centrifuged at 15,000xg for 5 min and the supernate was removed

to a fresh tube, avoiding the material at the interface. Chloroform (400 µl) was then added. The two phases were mixed by brief vortexing, centrifuged for 2 min at 15,000xg and the upper phase was removed to a fresh tube to which 800 µl of ethanol (Hayman, Witham, UK) was added. The mixture was gently vortexed, left for 2 to 5 min at room temperature and the plasmid DNA was pelleted by centrifugation for 10 min at 15,000xg in a microfuge. The DNA pellet was saved by carefully pouring off the supernate, rinsed with 70% (v/v in H_2O) alcohol and then freeze-dried (5 min) or air-dried (20 min). The dried pellet was redissolved in a small volume of Tris-EDTA (TE) buffer, pH 8.0 (Appendix 7) and treated with 20 µg ml^{-1} pancreatic ribonuclease (RNase) A (Sigma) (Appendix 8) at 37°C for 30 min before use.

2.6.2. Large-scale preparation of plasmid DNA.

Plasmid DNA on a large scale was extracted from 500 to 1000 ml cultures by the modified alkaline-lysis method (Sambrook *et al.*, 1989) with further modifications. Cells were harvested by centrifugation at 10,000xg for 10 min at 4°C in a Sorvall GS3 rotor. The pellet was drained on a paper towel and resuspended by vortexing in 10 ml of ice-cold lysis buffer (Appendix 3) and then left for 10 min at room temperature. To each bottle containing the lysate, 20 ml of freshly prepared alkaline-SDS solution (Appendix 4) was added and the contents were mixed by gently inverting the bottle several times and left on ice for 10 min. Then 15 ml of freshly prepared ice-cold acidified potassium acetate solution (Appendix 5) was added. The contents were mixed by shaking or gently vortexing the bottle several times and left for a further 10 min on ice. The suspension was centrifuged at 12,000xg for

10 min at 4°C in a Sorvall SS-34 rotor. The supernate was filtered through tissue paper to remove suspended particles. Approximately 0.6 volume (~27 ml) of isopropanol (BDH, Poole, UK) was added and the mixture was left for 15 min at room temperature. The precipitated DNA was pelleted by centrifugation for 10 min at 15,000xg at room temperature in a Sorvall SS-34 rotor. The pellet was gently washed with 70% alcohol, freeze-dried for 10 min and resuspended in 10 ml of TE buffer (pH 8.0). An equal volume of polyethylene glycol (PEG 8000; Sigma)-NaCl solution (Appendix 9) was added and the mixture was centrifuged for 10 min at 12,000xg in an SS-34 rotor. The DNA pellet was redissolved in 10 ml of TE (pH 8.0) in a glass universal to which an equal volume of buffered-phenol:chloroform (1:1) (Appendix 6) was added and the mixture was centrifuged for 10 min at 2300xg in a bench centrifuge (MSE minor, UK). The supernate was removed to a fresh universal, avoiding debris at the interface. The extraction was repeated with 10 ml of chloroform only and the supernate was removed to a 50-ml tube to which 10 ml of 5M ammonium acetate was added and the mixture was incubated on ice for 15 min to precipitate the majority of RNA and chromosomal DNA. This was then centrifuged at 12,000xg for 10 min in an SS-34 rotor. The supernate was decanted into a clean glass beaker to which 2 volumes (~40 ml) of isopropanol were added and the mixture was left at room temperature for 10 min. The plasmid DNA was pelleted by centrifuging at 15,000xg for 10 min in an SS-34 rotor. The pellet was drained well, rinsed with 70% alcohol and freeze-dried for 15 min. The dried preparation was taken up in 500 µl of TE (pH 8.0) and treated with RNase A (20 µg ml^{-1} final concentration) at 37°C for 30 min to remove residual RNA. The plasmid DNA preparation was stored at -20°C until use.

Large-scale preparation of plasmid DNA was also carried out by using

QIAGEN-tip 500 of the plasmid maxi kit (DIAGEN GmbH, FRG), according to the manufacturer's instructions, starting from 500 ml broth cultures grown overnight.

2.6.3. Large-scale purification of CCC plasmid DNA

Plasmid DNA extracted on a large-scale, by the method described in the preceeding section, contained chromosomal DNA and/or different topological forms of plasmid DNA. These crude plasmid DNAs were purified to CCC forms by an improved and modified acid-phenol extraction method (Azad *et al.*, 1992a), based on method described by Zasloff *et al.* (1978).

To a 1.5 ml microcentrifuge tube containing a maximum of 300 µl crude plasmid DNA in TE buffer (pH8.0) was added 30 µl of 500mM sodium acetate (pH 4.0) and 33 µl of 15mM $MgCl_2$ (AnalaR®; BDH) instead of 750mM NaCl, as used by Zasloff *et al.* (1978). An equal volume of phenol (Formachem) equilibrated with 50mM sodium acetate (pH 4.0) (1:1 w/v) was added and the mixture was vortexed vigorously for 60 sec. The mixture was centrifuged at 15,000xg in a microcentrifuge (Biofuge A) for 5 min. The supernate was removed to a fresh tube and neutralized by adding 36 µl of 500mM Tris.HCl (pH 8.0) and mixed by gentle agitation. Then 396 µl of chloroform mixture (Appendix 6) was added and, after brief vortexing, the mixture was centrifuged at 15,000xg for 3 min. The supernate was removed to a fresh tube to which 39 µl of 3M sodium acetate (pH 6.0) and 860 µl of ethanol were added. The contents were mixed by gentle inversion of the tubes and then left at room temperature for 10 min. The purified CCC plasmid DNA was recovered by centrifugation at 15,000xg for 10 min, rinsed with 70% ethanol and air-dried. The dried pellet was redissolved in TE buffer (pH 8.0) or

distilled H_2O. All manipulations in the purification procedure were carried out at room temperature.

2.6.4. Electrophoresis, staining and photography of agarose gels containing plasmid DNA.

Agarose gel electrophoresis was carried out in a horizontal submarine electrophoresis apparatus (GNA-100 or 200; Pharmacia, Uppsala, Sweden). Type II-A medium EEO agarose (Sigma) was used in analytical gels, while low melting point (LMP) agarose (*ultra*Pure™; BRL, Gaithersburg, MD) was used in preparative gels. LMP agarose was also used in some analytical gels to separate and clearly visualize the smaller DNA fragments. Gels were prepared and run in either 0.5X TBE buffer (Appendix 10) (for analytical gels) or 1X TAE buffer (Appendix 11) (for preparative gels).

Gel-loading buffer (Appendix 12) was mixed with DNA samples in a ratio of 1:5 (i.e., 1 µl per 5 µl sample). Then 10 to 12 µl of the sample mix was loaded into each of 11 wells of the minigel system and 18 to 20 µl into each of 22 wells of the maxigel system. To create a bigger well, when required, teeth of the minigel comb were paired with sellotape and this allowed 40 to 50 µl DNA samples to be easily applied for both analytical and preparative purposes.

Plasmid DNA was routinely electrophoresed in 0.8% (w/v) agarose using a minigel (10.6 cm x 7.8 cm) at 100V (~6.6V/cm) for about 2 h or using a maxigel (20 cm x 20 cm) at 100V (~3.3V/cm) for about 4 h. Electrophoresis of the smaller DNA fragments up to 50 bp was carried out in agarose gels of high concentration (up to 2%) at high voltage (10V/cm) for a short time (~80 min).

Following electrophoresis, gels were stained in distilled water containing ethidium bromide (1 μg ml^{-1} final concentration) for 20 min and then rinsed with plain distilled water for at least an equal length of time. In the case of preparative gels, ethidium bromide (0.5 μg ml^{-1} final concentration) was added to the melted agarose, before pouring on to the gel-former, and also to the running buffer. However, for analytical purposes, post-electrophoresis staining was preferred to prior addition of ethidium bromide as it gave a clearer background to the agarose gel.

DNA fragments were visualized under ultraviolet illumination, using a short wave transilluminator (UVP Inc., San Gabriel, CA). Gels were photographed through an orange filter, using a Polaroid MP-4 Land camera with Polaroid 667-type Land film.

2.6.5. Standardization of plasmid DNA (size and concentration).

Sizes of the plasmid DNA fragments were estimated from a standard calibration curve obtained by plotting the relative mobility of the reference or marker DNA fragments against their logarithmic size in base pairs. Different types of DNA size markers were used, depending on the topological forms and the fragment sizes of plasmid DNA. For calculating the sizes of uncut or CCC plasmids, a supercoiled DNA ladder (11 fragments) (Gibco-BRL, Paisley, UK) was used. For sizing the digested or linear plasmid DNA fragments, either *Hind*III-digested λ DNA (8 fragments) or *Hae*III-digested φX174 RF DNA (11 fragments) or 1 kb DNA ladder (23 fragments) (all from Gibco-BRL) was used. When the linear double-stranded fragments (e.g., λ DNA/*Hind*III) were used as size markers, the supercoiled equivalent plasmid DNA size was determined by the method of Platt & Taggart

(1987). The fragment sizes of different DNA markers used in this study are given in Appendix 13.

The approximate concentration of plasmid DNA fragments was determined visually on agarose gels, by comparison of their staining intensities with those of known quantities of marker DNA of a similar size (e.g., λ DNA/ *Hind*III fragments).

2.7. Transformation.

Transformation of *E. coli* was carried out by the standard $CaCl_2$-procedure (Hanahan, 1983) and that of *P. haemolytica* by the static aerobic procedure, originally employed for encapsulated *H. influenzae* (Rowji *et al.*, 1989).

2.7.1. $CaCl_2$-mediated transformation.

A 0.2 ml portion of a fresh overnight *E. coli* culture was inoculated into 50 ml of prewarmed SOC medium (Appendix 14) in a 250-ml dimpled flask and shaken at 37°C until the culture reached an optical density $(OD)_{650}$ of 0.2 (~$5x10^7$ cells ml^{-1}). The culture was then chilled on ice for 10 min and the cells were harvested by centrifugation in sterile glass universals with a bench centrifuge (MSE) at 2000xg for 10 min (4°C). The cell pellet was resuspended in 20 ml of ice-cold 100mM $CaCl_2$, incubated on ice for 25 min and repelleted by centrifugation as before. The cells were then gently resuspended in 0.5 ml of ice-cold 100mM $CaCl_2$, transferred to a prechilled microfuge tube and left on ice overnight before use or stored at -70°C in 15% sterile glycerol.

Routinely, 1 to 2 µl of miniprep plasmid DNA (approximately 1 to 10 ng of DNA) was added to 20 µl of competent cells in a prechilled sterile

microfuge tube, which was placed on ice for 30 min. The cells were then heat-pulsed at 42°C in a water bath for 90 sec and immediately returned to ice for a further 2 min. About 80 µl of SOC medium was added to the cell suspension which was then incubated with gentle shaking (225 rpm) at 37°C for 1 h to allow expression of plasmid-encoded antibiotic resistance. The whole of the transformation culture (~100 µl) was then spread onto a pre-dried selective plate containing appropriate antibiotic(s) and the plate was incubated at 37°C overnight or longer to obtain transformant colonies.

The amounts of plasmid DNA, competent cells and SOC medium, used in the transformation process, could be scaled up, if required. In that case, the cell suspension after 1 h incubation at 37°C was centrifuged and the pellet resuspended in 100 µl of SOC medium before plating.

2.7.2. Transformation by static aerobic procedure.

A fresh overnight culture of *P. haemolytica* was diluted into BHI broth supplemented with 1mM $MgSO_4$ (1:25 dilution i.e., 0.4 ml culture into 10 ml fresh broth) and incubated in a sterile plastic petri dish (90 mm diameter) at 37°C without shaking for 6 to 8 h to allow growth to the early stationary phase (OD_{650} 0.6). Then 10 µl each of plasmid DNA preparation and of statically-grown *P. haemolytica* cells were added to a tube containing 80 µl of BHI broth plus 1mM $MgSO_4$ and the suspension was incubated for 30 min at 35°C without shaking. Pancreatic DNase was added at the concentration of 10 µg ml^{-1} to the suspension which was then incubated for a further 10 min at 35°C without shaking. The whole of the transformation culture (~100 µl) was spread onto a selective plate which was incubated at 37°C for 24 to 48 h.

2.8. Plasmid Curing.

Curing studies were performed according to Hafiz *et al.* (1979), in conjunction with the procedures of Tomas & Kay (1984).

Different concentrations of ethidium bromide (μg ml^{-1}: 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 50) and acridine orange (μg ml^{-1}: 75 and 100) were prepared in 10 ml of BHI broth in conical flasks and the media were autoclaved. A suspension of the *P. haemolytica* strain (to be cured) from a fresh 18-h culture on BHI agar was made in phosphate-buffered saline (PBS), the turbidity was matched with McFarland 0.5 standard (~10^7 cells ml^{-1}) and 100 μl of this suspension was inoculated into each dilution of ethidium bromide and acridine orange and into the control (BHI broth alone). The flasks were incubated at 37°C on a rotary shaker and the organism was subcultured by streaking onto a BHI agar plate every 20 h. The antibiotic sensitivity of representative colonies of each subculture was tested by the disk-diffusion method, using the antibiotic disks to which the organism was resistant. The effects of ethidium bromide and acridine orange on the growth and drug-sensitivity of the *P. haemolytica* strain was noted.

2.9. Conjugation.

2.9.1. Plate mating of cultures by a two- or three-way cross.

Fresh 18-h cultures of donor, recipient (*P. haemolytica* or *E. coli*) and/or helper (*E. coli* HB101 or DH5 containing plasmid pRK2013) strains, grown on selective agar, were streaked (a single stroke) individually onto a fresh BHI agar plate from different directions converging to a single point. Following overnight incubation of the plate with surface uppermost at 37°C,

a loopful of growth from the meeting-point of the streaks was suspended in 2.0 ml of sterile saline or BHI broth. Then 100 µl amounts of the resulting suspension or its dilutions were spread onto transconjugant-selection plates containing antibiotics to counter-select donor and helper strains. A control was set up by streaking separately the growth from the top of the streak of each participating strain onto a transconjugant-selection plate. All plates were incubated at 37°C for 18 to 48 h.

2.9.2. Plate mating of overnight broth cultures.

This type of plate mating was carried out by the method of Bradley *et al.* (1980), 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.

Each strain was grown overnight at 37°C on a shaker, in 10 ml BHI broth containing the appropriate antibiotic. Equal volumes (50 µl, except a double volume for the *P. haemolytica* recipient only) of the cultures of donor, recipient and/or helper strains were mixed in a microfuge tube by gentle vortexing and spread over the surface of a prewarmed BHI agar plate, which was then incubated with surface uppermost at 37°C for mating. Growth from the mating plate was harvested by washing the plate three times each with 1 ml sterile saline and appropriate dilutions were made. Then 100 µl aliquots of a neat sample or the dilutions were spread onto transconjugant-selection plates which were incubated for 18 h (*E. coli* recipient) or 48 h (*P. haemolytica* recipient).

In a further conjugation experiment, *P. haemolytica* recipient cells

were subjected to heat treatment at 42.5°C for 9 min before mating, a device adopted for plasmid transfer to coryneform bacteria (Schäfer *et al.*, 1990).

Plasmid transfer frequencies by conjugation were obtained by dividing the total number of transconjugants by the original number of recipient bacteria in the conjugation mixture.

2.10. Determination of Minimum Inhibitory Concentrations (MICs) of Beta-Lactam Antibiotics for Ap^R Strains.

MICs of β-lactam antibiotics were determined by the agar-dilution method (Washington & Sutter, 1980). The β-lactam antibiotics used were: Pe, Ap, Carb, Tic, Cex, Cet and Cer (see Section 2.3).

A MIC plate was prepared as follows: dehydrated Iso-sensitest agar medium (Oxoid) was reconstituted with 900 ml (instead of 1 litre) of distilled H_2O, dispensed in 18 ml aliquots in screw-capped universals and then autoclaved. Serial double dilutions of β-lactam antibiotics were prepared (Appendix 15), and 2 ml of the antibiotic solution of appropriate concentration was added to 18 ml melted agar (~50°C), mixed thoroughly and poured into a petri dish. Plates were allowed to dry before use.

Four to five discrete colonies of the test bacterium from a fresh overnight culture plate were suspended in 4 to 5 ml of sterile normal saline and the turbidity of the culture suspension was adjusted to that of McFarland 0.5 standard. Then 50 µl of the suspension was added to 950 µl of BHI broth (1:20 dilution) in a sterile microfuge tube, mixed by gentle vortexing and the agar surface was spot-inoculated (without spreading) with this suspension, using a wire loop calibrated to deliver 1 to 2 µl. The

plates were allowed to stand undisturbed with surface uppermost until the inoculum spots were completely absorbed and then incubated at 37°C overnight. The MIC value represented the lowest concentration of antibiotic at which complete inhibition of bacterial growth occurred. A very fine, barely visible haze or a single colony was disregarded.

2.11. Beta-Lactamase Test with Beta-Lactamase Detection Papers.

The production of β-lactamase by Ap^R strains was tested by β-lactamase detection papers (Oxoid), according to the manufacturer's instructions.

One drop of distilled water was placed on a clean microscope slide and covered with a β-lactamase test strip so that the strip was moistened but not oversaturated. Several colonies of the test organism were scrapped from a fresh overnight culture plate, using a wire loop and and streaked onto the dampened strip. The β-lactamase production by the culture was indicated by the streaked portion of the strip changing colour from violet to yellow instantly or after approximately 5 min, depending on the activity of the enzyme.

2.12. Beta-Lactamase Assay.

2.12.1. Preparation of crude extracts of *E. coli* containing β-lactamase.

Three to four colonies of a β-lactamase-containing *E. coli* strain from a fresh 18-h culture plate were inoculated into a 250-ml dimpled flask containing 50 ml BHI broth supplemented with Ap (50 $\mu g\ ml^{-1}$) and grown with shaking at 37°C for 7 to 8 h. This starter culture was then added to 1 litre of fresh Ap-supplemented BHI broth and incubated overnight at 37°C on

a shaker.

Cells were harvested by centrifugation at 12,000xg for 10 min at 4°C in a Sorvall GS3 rotor and washed once with 0.1M potassium phosphate buffer, pH 7.0 (Appendix 16). The pellet was resuspended in a minimal volume of buffer (10-20 ml) and the cells were disrupted for 3 to 4 min (4 x 30 sec with 30 sec intervals on ice) with an ultrasonic disintegrator (MSE; maximum peak setting). Supernates were collected after centrifugation at 140,000xg for 1 h at 4°C in an ultracentrifuge (Sorvall® OTD-COMBI, Dupont Co., CT), dialyzed overnight at 4°C against deionized distilled water, and concentrated by freeze-drying. Freeze-dried samples were reconstituted with 0.1M phosphate buffer (pH 7.0) and used as sources of β-lactamase enzymes.

2.12.2. Determination and inhibition of beta-lactamase activity.

Beta-lactamase activity was assayed by the macroiodometric method of Ross & O'Callaghan (1975) on crude sonic extracts of *E. coli* cells. The method was scaled down 5-fold, using a 5-ml burette graduated to 0.02 ml. Penicillin G, Ap, Carb, Oxa, Clox, Cer, Cet and Cex (see Section 2.3) were used as substrates to measure the rate of β-lactam hydrolysis.

One millilitre of β-lactam antibiotic solution (5mM in 0.1M potassium phosphate buffer), prewarmed to 37°C, was dispensed into each of two universals, followed by addition of 0.2 ml of enzyme solution (prewarmed to 37°C) to one bottle. The other bottle without enzyme was used as a control. Both test and control universals were incubated at 37°C for 30 min in a shaking waterbath, before addition of 2 ml of prewarmed iodine reagent (Appendix 17) to each container. Enzyme solution (0.2 ml) was added at this time to the control only. The reaction mixtures were left in the shaking

water bath (37°C) for a further 10 min (penicillins) or 20 min (cephalosporins) and then titrated with 0.0166N sodium thiosulphate, using one drop of 2% hydrolyzed starch as indicator. If the titration was less than 0.9 ml, the assay was repeated with diluted enzyme. Under these conditions, 1 ml of 0.0166N iodine consumed was equivalent to 2 μmoles of a penicillin or 4 μmoles of a cephalosporin destroyed. The specific activity of β-lactamases was expressed as μmol min^{-1} (mg protein)$^{-1}$ (see following section for protein estimation).

Inhibition of β-lactamase activity, either by 100mM NaCl or 0.1mM Clox, was carried out by the same method with Pe as substrate. To 0.2 ml of enzyme suspension, 0.02 ml of either 5M NaCl or 5mM Clox was added before addition of Pe.

2.12.3. Estimation of protein.

Protein content of enzyme preparations was estimated by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) (Sigma; Fraction V) as standard.

Five millilitres of Bradford reagent (Appendix 18) were added to 100 μl of standard solution of BSA containing 10 to 50 μg of protein (100 to 500 μg protein ml^{-1}), mixed well and allowed to stand for 5 to 30 min. Absorbance was then measured at 595 nm against a blank prepared from 100 μl of sample buffer and 5 ml of Bradford reagent, using a UV-240 spectrophotometer (Shimadzu Corp., Kyoto, Japan) and visible range cuvettes. Unknown protein samples were assayed by the same procedure, on 100 μl volumes of different dilutions. A standard calibration curve was

plotted and the protein concentrations (μg ml^{-1}) of unknown samples were worked out from the curve.

2.12.4. Analytical isoelectric focusing.

Analytical isoelectric focusing was done using an LKB 2117 Multiphor apparatus and Ampholine® PAG plates (pH 3.5-9.5) (Pharmacia LKB, Uppsala, Sweden), according to the manufacturer's instructions. 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. Staining and photography of the gel were performed as described by Matthew *et al.* (1975).

The gel was placed on a sample application template supported on a cooling plate maintained at 4-10°C by circulating cold water. Paraffin oil was spread uniformly over the cooling plate and the sample application template to ensure complete contact between the plate, the template and the gel respectively. The electric field was applied via two strips of paper, soaked in the appropriate anode (1M H_3PO_4) and cathode (1M NaOH) buffers and placed on two opposite edges of the gel marked by the respective electrode signs. The sample application strips (10 x 5 mm) were placed on the gel surface towards the anodic side upon which the test samples (15 to 20 μl) were applied by means of a micropipette. Isoelectric focusing was run at a constant power of 1 W with a maximum of 400 V for 3 to 4 h. The sample application strips were removed after approximately half the focusing time and the experiment was continued. The run was terminated and the gel was placed on a large glass petri dish for staining.

A sheet of Whatman no. 50 paper was soaked in a solution of a

chromogenic cephalosporin called "nitrocefin" (0.5 mg ml^{-1}) (Oxoid), laid over the surface of the gel and then removed, in one continuous rotary motion. Pink bands appeared on a yellow background, representing β-lactamase activity. Either the gel or the filter-paper blot from the gel was immediately photographed with a Polariod MP-4 camera using 665 film.

Isoelectric points (pIs) of unknown β-lactamases were determined by reference to those of known β-lactamases.

2.13. Plasmid Stability Test in *E. coli*.

An *E. coli* strain harbouring a *P. haemolytica* Ap^R plasmid was grown from a glycerol stock on LB agar containing Ap. One colony was streaked onto an antibiotic-free LB agar and grown for 18 h to single discrete colonies. Four colonies were picked, streaked separately onto LB agar plates and grown for 18 h to single discrete colonies. Sixteen colonies, four from each plate, were randomly selected and tested for the Ap^R phenotype by streaking them separately onto LB agar plates containing Ap. The β-lactamase phenotype of these colonies was concurrently tested by using the β-lactamase strips. Serial passages of the plasmid-containing *E. coli* strains could be continued in this way in the absence of antibiotic selection and the stability of plasmids monitored by the demonstrated phenotypes of the bacteria.

2.14. Amplification of Plasmid DNA in *E. coli*.

A single colony of an *E. coli* transformant, containing an Ap^R plasmid from *P. haemolytica*, from a fresh overnight culture plate was inoculated into either BHI broth, nutrient broth, LB broth (Appendix 1), Terrific broth

(Tartof & Hobbs, 1987) (Appendix 19) or 1% yeast nitrogen base (YNB) (Difco)-supplemented Terrific broth (Azad *et al.*, 1992b) and grown for 6 to 7 h to late exponential phase (OD_{600} ~0.6). A 0.05 to 0.1 volume from these cultures was inoculated into corresponding fresh broth (prewarmed) and incubated with vigorous shaking (300 rpm) at 37°C for 2.5-3.0 h (OD_{600} ~0.4). At this stage, Cm (170 μg ml^{-1} final concentration) (Clewell, 1972) or Tc (15 μg 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} was noted before the cells were used for preparation of plasmid DNA.

The same procedure was also applied for amplification in *E. coli* of the vector constructs, derived from a *P. haemolytica* Ap^R plasmid.

2.15. Restriction Endonuclease Digestion of Plasmid DNA.

Restriction endonuclease enzymes were purchased from different manufacturers and used according to the suppliers' instructions. The suppliers' names, reaction buffers and recognition sequences of 42 restriction enzymes used in this study are given in Table 7.

Restriction enzyme digestion was routinely performed in a 40 μl volume containing approximately 2 μg of DNA, 4 to 20 units of enzyme (2 μl) and reaction buffer diluted to 1X. Sterile distilled H_2O, reaction buffer (10X), plasmid DNA solution and restriction enzyme were sequentially added to a sterile microfuge tube placed on ice and mixed gently by tapping the tube. The reaction mixtures were incubated overnight at the appropriate temperatures (generally 37°C, unless otherwise specified) and analyzed by gel electrophoresis in 1 to 2% agarose. About 7 μl of gel-loading buffer

TABLE 7. RESTRICTION ENDONUCLEASES USED IN THIS STUDY

Restriction enzyme	Recognition sequence*	Reaction buffer†	Supplier§
*Acy*I	5'-GR↓CGYC-3'	Universal buffer	Stratagene
*Alu*I	5'-AG↓CT-3'	REact™1	Gibco-BRL
*Apa*I	5'-GGGCC↓C-3'	REact™4	Gibco-BRL
*Apa*LI	5'-G↓TGCAC-3'	Assay buffer 1	Stratagene
*Ava*I	5'-C↓YCGRG-3'	REact™2	Gibco-BRL
*Ava*II	5'-G↓GWCC-3'	REact™2	Gibco-BRL
*Bam*HI	5'-G↓GATCC-3'	REact™3	Gibco-BRL
*Bgl*I	5'-GCCN$_n$↓NGGC-3'	REact™2	Gibco-BRL
*Bgl*II	5'-A↓GATCT-3'	REact™3	Gibco-BRL
*Bsc*I	5'-AT↓CGAT-3'	*Bsc*I buffer	NBL
*Bsp*1286I	5'-GDGCH↓C-3'	Assay buffer 1	Stratagene
*Bss*HII	5'-G↓CGCGC-3'	Buffer A	BM
*Cfo*I	5'-GCGC-3'	REact™1	Gibco-BRL
*Cla*I	5'-AT↓CGAT-3'	REact™1	Gibco-BRL
*Dpn*I	5'-GmA↓TC-3'	REact™4	Gibco-BRL
*Dra*I	5'-TTT↓AAA-3'	REact™1	Gibco-BRL
*Eco*RI	5'-G↓AATTC-3'	REact™3	Gibco-BRL
*Eco*RV	5'-GAT↓ATC-3'	REact™2	Gibco-BRL

Continued on next page

TABLE 7 (Continued)

Restriction enzyme	Recognition sequence*	Reaction buffer†	Supplier§
*Hae*II	5'-RGCGC↓Y-3'	Buffer A	BM
*Hinc*II	5'-GTY↓RAC-3'	REact™4	Gibco-BRL
*Hind*III	5'-A↓AGCTT-3'	REact™2	Gibco-BRL
*Hinf*I	5'-G↓ANTC-3'	REact™2	Gibco-BRL
*Hpa*I	5'-GTT↓AAC-3'	REact™4	Gibco-BRL
*Hpa*II	5'-C↓CGG-3'	REact™8	Gibco-BRL
*Kpn*I	5'-GGTAC↓C-3'	REact™4	Gibco-BRL
*Mbo*I	5'-↓GATC-3'	REact™2	Gibco-BRL
*Msp*I	5'-C↓CGG-3'	REact™1	Gibco-BRL
*Nar*I	5'-GG↓CGCC-3'	Assay buffer 1	Stratagene
*Pst*I	5'-CTGCA↓G-3'	REact™2	Gibco-BRL
*Pvu*II	5'-CAG↓CTG-3'	REact™6	Gibco-BRL
*Sac*I	5'-GAGCT↓C-3'	Buffer A	BM
*Sal*I	5'-G↓TCGAC-3'	REact™10	Gibco-BRL
*Sau*3AI	5'-↓GATC-3'	REact™4	Gibco-BRL
*Sca*I	5'-AGT↓ACT-3'	REact™6	Gibco-BRL

Continued on next page

TABLE 7 (Continued)

Restrction enzyme	Recognition sequence*	Reaction buffer†	Supplier§
*Sma*I	5'-CCC↓GGG-3'	REact™4	Gibco-BRL
*Sph*I	5'-GCATG↓C-3'	REact™6	Gibco-BRL
*Stu*I	5'-AGG↓CCT-3'	REact™2	Gibco-BRL
*Taq*I	5'-T↓CGA-3'	REact™2	Gibco-BRL
*Tha*I	5'-CG↓CG-3'	REact™1	Gibco-BRL
*Xba*I	5'-T↓CTAGA-3'	REact™2	Gibco-BRL
*Xho*I	5'-C↓TCGAG-3'	REact™2	Gibco-BRL
*Xho*II	5'-R↓GATCY-3'	*Xho*II buffer	ABL

* R = A or G, Y = C or T, W = A or T, N = A or C or G or T, D = A or G or T, H = A or C or T; ↓ indicates the cleavage site within a recognition sequence.

† Reaction buffers were supplied as 10X concentrates; composition of buffers is given in Appendix 20.

§ BRL, Bethesda Research Laboratories;
NBL, Northumbria Biologics Limited;
BM, Boehringer Mannheim;
ABL, Anglian Biotec Limited.

(Appendix 12) was mixed with each restriction digest and the mixture was centrifuged briefly to remove bubbles before loading onto the gel.

2.15.1. Restriction mapping of plasmid DNA.

Restriction mapping was done by a combination of single and double digestion of DNA. Double digestion was carried out by using the combination of two enzymes and a single buffer system in either a one- or a two-step incubation, depending on the incubation temperature(s) required by the enzymes. The reaction conditions for double digestion of plasmid DNA are detailed in Table 8. A restriction map of circular plasmid was constructed, by trial-and-error search, using the relative cleavage positions of the enzymes as determined from the estimated sizes of different restriction fragments.

2.16. Southern Blotting and Hybridization.

In this study, a *Sau*3AI or an *Alu*I digest of plasmid pPH843 was used as a probe for hybridization to *Sau*3AI- or *Alu*I-digested fragments, respectively, of the prototype ROB-1 plasmid pRM3022. The transfer and hybridization of DNA fragments were essentially carried out according to Southern (1975), as described either by Sambrook *et al.* (1989) or by Ausubel *et al.* (1987).

DNA fragments generated by restriction enzyme digestion were separated by gel electrophoresis, stained in 1 μg ml^{-1} ethidium bromide and photographed under UV light. The gel was then submerged in denaturation solution (1.5M NaCl, 0.5M NaOH) and left for 15 min with constant, gentle agitation. After that the gel was rinsed in distilled water, placed in

TABLE 8. REACTION CONDITIONS FOR DOUBLE DIGESTION OF PLASMID DNA WITH RESTRICTION ENZYMES

Restriction enzymes	Reaction buffer*	First-step digestion			Single- or second-step digestion†	
		enzyme	temperature	time	temperature	time
*Pst*I+*Bsp*1286I	REact™2	...			37°C	overnight
*Pst*I+*Dra*I	REact™2	...			37°C	overnight
*Bsp*1286I+*Dra*I	REact™1	...			37°C	overnight
*Tha*I+*Pst*I	REact™1	*Tha*I	60°C	10 h§	37°C	overnight
*Tha*I+*Bsp*1286I	REact™1	*Tha*I	60°C	10 h	37°C	overnight
*Tha*I+*Dra*I	REact™1	*Tha*I	60°C	10 h	37°C	overnight
*Taq*I+*Pst*I	REact™2	*Taq*I	65°C	10 h	37°C	overnight
*Taq*I+*Bsp*1286I	REact™2	*Taq*I	65°C	10 h	37°C	overnight
*Taq*I+*Dra*I	REact™2	*Taq*I	65°C	10 h	37°C	overnight
*Tha*I+*Taq*I	REact™1	*Tha*I	60°C	10 h§	65°C	overnight
*Apa*LI+*Pst*I	REact™2	...			37°C	overnight
*Apa*LI+*Bsp*1286I	REact™1	...			37°C	overnight
*Apa*LI+*Dra*I	REact™1	...			37°C	overnight
*Ava*I+*Pst*I	REact™2	...			37°C	overnight

Continued on next page

TABLE 8 (Continued)

Restriction enzymes	Reaction buffer*	First-step digestion	Single- or second-step digestion†
		enzyme: temperature: time	temperature: time
AvaI+*Bsp*1286I	React™2	...	37°C : overnight
AvaI+*DraI*	REact™2	...	37°C : overnight
HinfI+*PstI*	REact™2	...	37°C : overnight
HinfI+*Bsp*1286I	REact™2	...	37°C : overnight
HinfI+*DraI*	REact™2	...	37°C : overnight
ScaI+*PstI*	React™6	...	37°C : overnight
ScaI+*Bsp*1286I	REact™6	...	37°C : overnight
ScaI+*DraI*	REact™6	...	37°C : overnight

* Supplier: Gibco-BRL.

† In the case of a single-step digestion, both restriction enzymes were used at the same time in the same REact™ buffer and incubated overnight at 37°C. In the case of a second-step digestion, the second restriction enzyme was added after the first-step digestion and incubated overnight at the appropriate temperature.

§ The appropriate salt concentration in the pre-existing REact™ buffer was adjusted by adding 2 µl of 1M NaCl per 40 µl reaction volume after the first-step digestion.

neutralization solution (2M NaCl, 1M Tris.HCl, pH 7.4) and left for 15 min with gentle agitation.

A DNA transfer pyramid was set up by filling a tray with 20X SSC (3M NaCl, 0.3M tri-sodium citrate, pH 7.0) and placing a wick, made from Whatman 3MM paper and soaked in 20X SSC, on a glass plate platform over the tray with both ends of the wet wick hanging over the glass plate into 20X SSC. The gel was removed from the neutralization solution and placed over the Whatman 3MM wick, avoiding trapped air bubbles beneath the gel. A sheet of Hybond-N+ membrane (Amersham, England) was cut to the exact size of the gel and soaked on one edge in distilled water followed by complete immersion in 2X SSC for at least 5 min. The wet nylon filter was then placed on top of the gel and air bubbles trapped between the filter and gel were removed by gently rolling a 10-ml pipette back and forth over the filter. Two pieces of 3MM paper, cut to exactly the same size as the filter and soaked in 2X SSC, were placed on top of the gel and wet filter, and air bubbles were smoothed out. A stack of absorbent paper towels (5-8 cm high), just smaller than the pieces of 3MM paper, was made and placed on top. A glass plate was then placed on the paper towels and a 0.75 to 1 kg weight (e.g., a bottle) was put on the glass plate. The transfer of DNA was allowed to proceed for 16 to 20 h.

After the transfer was complete, the filter was rinsed in 2X SSC for 5 min, blotted dry on a paper towel and left at room temperature for 30 min before wrapping in Saran wrap (Dow Chemical Co.). The side of the Hybond membrane with the bound DNA was exposed to a source of UV light (254 nm) for 2 min to fix the DNA.

The membrane was placed in a heat-sealable polythene bag. One hundred microlitres of denatured salmon sperm DNA (10 mg ml^{-1}) (Appendix 21) were

mixed with 10 ml of prehybridization solution (Appendix 22) and added to the bag. As many air bubbles as possible were squeezed out of the bag before sealing the open end with a heat sealer and the filter was prehybridized in a shaking water bath at 42°C overnight with gentle agitation.

Fifty microlitre volume of DNA probe was prepared by labelling the DNA fragments to high specific activity with [α-^{32}P]dATP (Amersham) by the random priming technique (Feinberg & Vogelstein, 1984) (Appendix 23). The probe was denatured by heating to 100°C in a boiling water bath for 5 min, chilled quickly in ice-water and then added to 10 ml of hybridization solution (Appendix 22). The prehybridization solution was removed from the bag and replaced by the hybridization mixture. Air bubbles were removed, the bag was re-sealed and incubated overnight at 42°C with gentle agitation.

The filter was removed from the bag and immediately washed twice in 2X SSC, 0.1%(w/v) SDS at room temperature for 1 h and twice in 1X SSC, 0.1% (w/v) SDS at 60°C for 2 h with agitation.

After the washing steps, the filter was blotted dry, wrapped in Saran wrap and then exposed to X-ray film (Kodak X-OMAT S, France) against an intensifying screen, to obtain an autoradiographic image. Autoradiographs were incubated at -70°C for varying periods of time, developed in Kodak LX24 developer and fixed in Kodak Unifix solution for 5 min each before rinsing under running tap water and then drying at room temperature.

2.17. Cloning.

2.17.1. Partial digestion of plasmid DNA.

For cloning purposes, plasmid pPH843 was subjected to partial digestion with *TaqI* restriction enzyme, according to the protocol of Ausubel *et al.* (1987).

A 100-µl reaction mixture containing DNA in 1X restriction enzyme buffer was made up and divided into 5 microfuge tubes such that tube 1 contained 30 µl, tubes 2 to 4 contained 20 µl each, and tube 5 contained 10 µl. All tubes were placed on ice. One microlitre of *TaqI* enzyme was added to tube 1 (i.e., 10 units of enzyme per 30 µl reaction volume), mixed quickly by flicking the tube, and placed back on ice. Then 10 µl portions were dispensed from tube 1 into tube 2 by using a fresh pipette tip, mixed quickly and placed back on ice. The serial dilution process was continued by successively pipetting 10 µl from tube 2 to 3, 3 to 4, and 4 to 5, such that each tube contained 20 µl and was on ice. All five tubes were then incubated for 15 min at 65°C in a water bath. The reactions were stopped by adding 0.5M EDTA, pH 8.0 (10mM final concentration) and analyzed by agarose (LMP) gel electrophoresis.

2.17.2. Dephosphorylation of linearized DNA fragments.

In order to prevent linearized vectors from self-ligation or recircularization during cloning, the 5'-phosphate groups of double-stranded DNA fragments were removed by treatment with calf intestinal phosphatase (CIP) (Pharmacia), according to the protocol of Sambrook *et al.* (1989).

Plasmid DNA (2-4 µg) was digested to completion with the appropriate restriction enzyme in a 40 µl volume. In the case of DNA fragments having protruding 5' termini, 1 µl (6.7 units) of CIP was added to the digestion mix and incubated at 37°C for 30 min. In the case of either blunt or recessed termini, 2 µl of CIP was added and incubated at 37°C for 15 min, followed by addition of another aliquot of CIP and incubation for a further 45 min at 55°C. Dephosphorylated DNA fragments were purified by LMP-agarose gel electrophoresis in TAE buffer, followed by the GENECLEAN procedure (see following section).

2.17.3. Elution and purification of DNA fragments from agarose gels.

Before proceeding to the cloning protocol, DNA fragments were eluted and purified from LMP-agarose gels (in TAE buffer) by the GENECLEAN procedure, using the GENECLEAN II® Kit (BIO 101 Inc., LaJolla, CA).

The DNA band of interest was excised from an ethidium bromide-stained agarose gel with a sharp scalpel under long wave UV light, and the gel slice was put in a pre-weighed microfuge tube. About 3 volumes of NaI stock solution were added to the gel slice in the tube and incubated at 55°C in a water bath for 5 min or more with occasional vortexing until the agarose was dissolved. GLASSMILK® tube was vortexed vertically and horizontally for ~1 min and 5 µl of the GLASSMILK suspension was added to the melted agarose, mixed well and incubated on ice for 5 min or more with occasional mixing. The GLASSMILK/DNA complex was pelleted at top speed for 5 sec in a Sarstedt microcentrifuge (Nümbrecht, FRG). The supernate was discarded by using a 1-ml micropipette and the white pellet was washed three times with ice-cold NEW (NaCl:Ethanol:Water) WASH, by adding 400 µl of NEW WASH,

resuspending the pellet by repeated pipetting, centrifuging for 5 sec and then discarding the supernate. The pellet was freeze-dried briefly, resuspended in 5 to 10 µl distilled water and then incubated at 55°C for 3 to 5 min with occasional gentle mixing. GLASSMILK was repelleted by centrifuging the suspension at top speed for 30 sec in a Sarstedt microcentrifuge and the supernate containing the purified DNA was recovered for immediate use or storage at -20°C.

2.17.4. "Filling in" or "trimming" reactions for converting incompatible DNA ends into blunt ends.

For ease of cloning, incompatible DNA ends were converted into blunt ends. This was carried out by "filling in" of 3' recessed ends (e.g. produced by *Bam*HI) or "trimming" of 3' protruding ends of DNA (e.g. produced by *Pst*I).

Plasmid DNA was digested to completion with the appropriate restriction enzyme in a 40 µl volume and fragments were separated by LMP-agarose gel electrophoresis. DNA fragments of interest were purified by the GENECLEAN procedure and taken up in distilled water in a fresh microfuge tube to which REact™4 was added as a substitute for DNA polymerase buffer. dNTP mix (a mixture of 25mM dATP, dCTP, dGTP, and dTTP; obtained from Boehringer Mannheim) was added to the tube at a final concentration of 1mM (filling reaction) or 2mM (trimming rection). To this was added 1-2 µl of Klenow fragment of *E. coli* DNA polymerase I for the filling reaction and bacteriophage T4 DNA polymerase for the trimming reaction. The reactions were incubated for 15 min at room temperature (filling) or for the same period at 12°C (trimming).

DNA polymerase enzyme or fragment of enzyme was inactivated by heating

the reaction mixture to 75°C for 10 min before proceeding with the ligation or cloning protocol.

2.17.5. Ligation, transformation, and selection of recombinant clones.

Ligation reactions were routinely performed in a 20 µl volume. Aliquots of purified 'vector' and 'insert' DNAs were combined in proportions to make up a 10 µl volume in a pre-chilled tube and stored on ice. A 2X T4 DNA ligase mixture was made up in another tube as follows:-

Content	Blunt-end ligation*	Cohesive-end ligation*
Sterile distilled H_2O	4 µl	4 µl
T4 DNA ligase buffer, 5X (Gibco-BRL) (Appendix 24)	4 µl	4 µl
10mM ATP	0 µl	1 µl
T4 DNA ligase (Gibco-BRL)	2 µl	1 µl

* Values shown are for one ligation reaction.

The mixture was stored on ice. Then 10 µl of the ice-cold 2X ligase mixture was added to 10 µl of combined DNAs, mixed by flicking the tube and incubated at 16-18°C for 12-16 h.

After the ligation reaction was over, 5 to 10 µl of the ligated products were added to 100 to 200 µl of *E. coli* competent cells for transformation (see Section 2.7.1). The remaining portions of the ligated products were analyzed by gel electrophoresis.

Recombinant clones were selected on LB+X-gal media (Appendix 25)

containing Ap and identified by their blue colour on these media. Plasmid DNA was prepared from blue clones by the miniprep procedure and analyzed by restriction enzyme digestion.

3. RESULTS

3.1. Antibiotic Resistance Profiles of *P. haemolytica* Isolates.

Sixteen antibiotics were evaluated by the disk-diffusion method to determine the antibiotic resistance patterns of *P. haemolytica* isolates (Table 9). All 35 isolates belonging to serotypes A1, A2, T4, T10, and untypable group were found to be multiply-resistant to at least 3 antibiotics. Serotype A1 isolate S/C 84/3 exhibited the widest range; being resistant to 8 of 16 antibiotics tested. All strains (100%) were resistant to Lm, Sm and Su, and sensitive to Ct, Er, Fu, Gm, Km, Neo and PB. Twenty five (71.4%) strains showed resistance to Tp, 23 (65.7%) to SxT, 6 (17.1%) to Tc, 4 (11.4%) to Ap, 2 (5.7%) to Nal, and only 1 (2.9%) strain to Cm, in combination with other antibiotics. It should be noted that the concentrations of Lm and Sm in the disks were low (Section 2.5) and resistance may thus have represented only a natural resistance of *P. haemolytica* to these antibiotics. Resistances to Tc, Ap, and Cm were only found in isolates of A1 serotype. Strain S/C 84/3 was partially resistant to Ap, as determined by an obviously poorer zone of inhibition of bacterial growth. The prevalence of individual antibiotic resistances shown by the *P. haemolytica* isolates is depicted in Figure 3.

3.2. Plasmid DNA Profiles of *P. haemolytica* Isolates and Their Relationships to Antibiotic Resistance Phenotypes.

All 35 isolates were analyzed for the presence of plasmid DNA. Plasmids were detected in 8 (22.9%) of the isolates (Figure 4). The plasmid profiles and the plasmid-associated antibiotic resistance phenotypes of these 8 strains are given in Table 10. A 4.3-kb plasmid was present in each of the

TABLE 9. ANTIBIOTIC RESISTANCE PATTERNS OF *P. HAEMOLYTICA* ISOLATES

P. haemolytica strain	Antibiotics used*															
	Ap	Cm	Ct	Er	Fu	Gm	Km	Lm	Neo	Nal	PB	Sm	Su	SxT	Tc	Tp
Serotype A1																
FA1	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
B2070	R	S	S	S	S	S	S	R	S	S	S	R	R	R	R	R
B2086	S	S	S	S	S	S	S	R	S	S	S	R	R	R	R	R
V965B	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
W629	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	S
CVL33	R	S	S	S	S	S	S	R	S	S	S	R	R	R	R	R
S/B 82/1	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	R
S/C 82/1	R	S	S	S	S	S	S	R	S	S	S	R	R	R	R	R
S/L 82/1	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
G/A 83/5	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
H/L 82/1	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
W/S 82/1	S	S	S	S	S	S	S	R	S	R	S	R	R	R	S	R
H/D 84/1	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	R
M/F 83/1	S	S	S	S	S	S	S	R	S	S	S	R	R	S	R	S
D/C 83/6	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
S/C 84/3	pR	R	S	S	S	S	S	R	S	S	S	R	R	R	R	R
G/A 83/1	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	R
W/D 83/4	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
Serotype A2																
FA2	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
B664	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R

Continued on next page

TABLE 9 (Continued)

P. haemolytica strain	Antibiotics used*															
	Ap	Cm	Ct	Er	Fu	Gm	Km	Lm	Neo	Nal	PB	Sm	Su	SxT	Tc	Tp
T844	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
Y510	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	R
C/P 84/2	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
D/I 85/1	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	S
G/T 85/15	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
Serotype T4																
FT4	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
Serotype T10																
FT10	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
Untypable																
T1262A	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
T1368	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
P709C	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
U133	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R
B1031	S	S	S	S	S	S	S	R	S	R	S	R	R	R	S	R
U300	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
U1284	S	S	S	S	S	S	S	R	S	S	S	R	R	S	S	S
U1447	S	S	S	S	S	S	S	R	S	S	S	R	R	R	S	R

KEY: S = Sensitive, R = Resistant, pR = Partially Resistant.

* Ap, ampicillin; Cm, chloramphenicol; Ct, colistin sulphate; Er, erythromycin; Fu, nitrofurantoin, Gm, gentamicin; Km, kanamycin; Lm, lincomycin; Neo, neomycin, Nal, nalidixic acid; PB, polymyxin B; Sm, streptomycin; Su, sulphonamide compound; SxT, sulphamethoxazole-trimethoprim; Tc, tetracycline; and Tp, trimethoprim.

FIGURE 3. HISTOGRAM SHOWING THE PREVALENCE OF INDIVIDUAL ANTIBIOTIC RESISTANCES EXHIBITED BY *P. HAEMOLYTICA* ISOLATES.

Lm = Lincomycin (2)

Sm = Streptomycin (10)

Su = Sulphonamide compound (3300)

Tp = Trimethoprim (1.25)

SxT = Sulphamethoxazole-trimethoprim (25)

Tc = Tetracycline (30)

Ap = Ampicillin (10)

Nal = Nalidixic acid (30)

Cm = Chloramphenicol (30).

Values in parentheses indicate the concentrations of antibiotics in μg per disk.

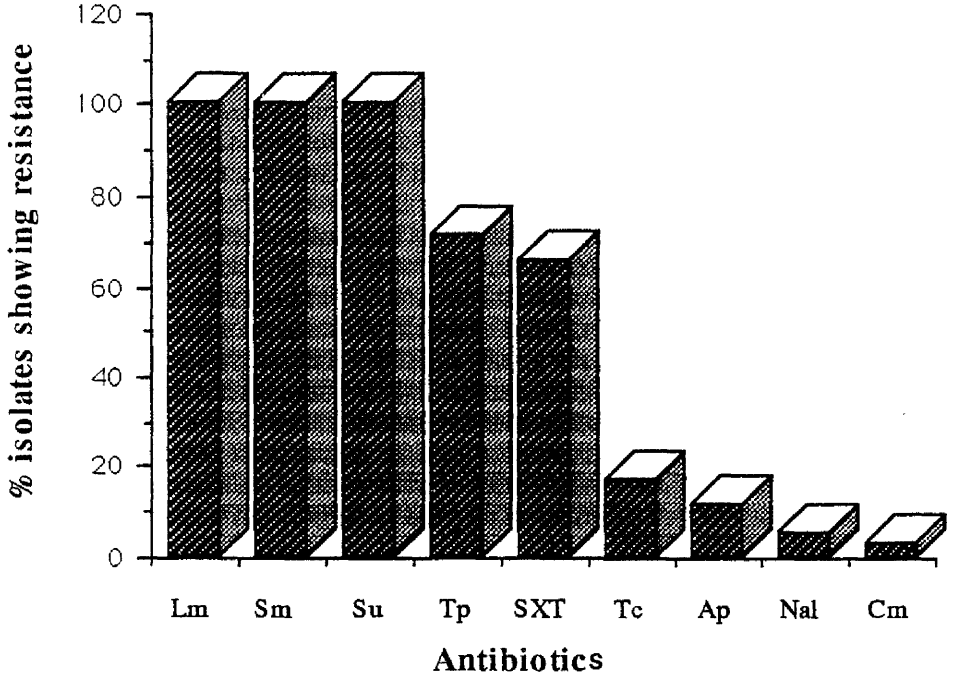

% isolates showing resistance
120
100
80
60
40
20
0
Lm
Sm
Su
Tp
SXT
Tc
Ap
Nal
Cm
Antibiotics

FIGURE 4. **AGAROSE GEL ELECTROPHORESIS OF PLASMID DNA PREPARATIONS FROM *P. HAEMOLYTICA* SEROTYPES A1, A2 AND UNTYPABLE ISOLATES.**

Key to figure:

M = A supercoiled DNA ladder used as the marker DNA.

1 = Plasmid DNA from strain B2070 (serotype A1).

2 = Plasmid DNA from strain CVL33 (,,).

3 = Plasmid DNA from strain S/C 82/1 (,,).

4 = Plasmid DNA from strain S/C 84/3 (,,).

5 = Plasmid DNA from strain B664 (serotype A2).

6 = Plasmid DNA from strain D/I 85/1 (,,).

7 = plasmid DNA from strain G/T 85/15 (,,).

8 = Plasmid DNA from strain T1368 (untypable).

The figure is a composite of three separate 0.8% (w/v) agarose 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.

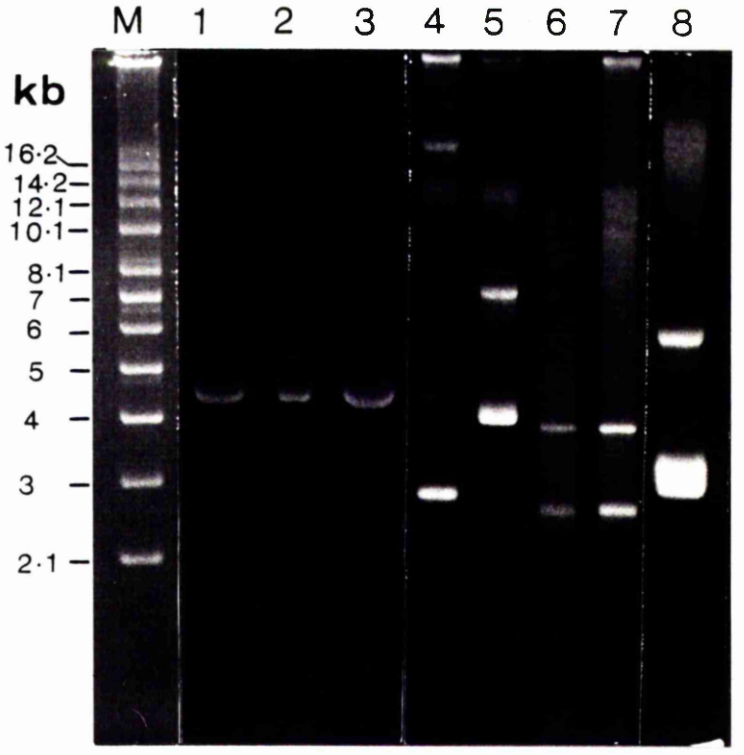

M
1
2
3
4
5
6
7
8
kb
16·2
14·2
12·1
10·1
8·1
7
6
5
4
3
2·1

TABLE 10. PLASMID PROFILES OF *P. HAEMOLYTICA* STRAINS AND THEIR ANTIBIOTIC RESISTANCE PHENOTYPES

Serotype (n = no. of strains tested)	Strain no. (positive for plasmid)	Antibiotic resistance phenotype*	Plasmid (kb)
A1 (n = 18)	B2070	Ap, Tc	4.3
	CVL33	Ap, Tc	4.3
	S/C 82/1	Ap, Tc	4.3
	S/C 84/3	Apt, Tc, Cm	~20, 4.3, 2.8
A2 (n = 7)	B664	None	7.6, 4.0
	D/I 85/1	None	3.9, 2.7
	G/T 85/15	None	3.9, 2.7
Untypable (n = 8)	T1368	None	5.9, 3.1, 2.9

* Refers only to plasmid-associated antibiotic resistance; all 35 strains (including the two T4 and T10 strains) tested by the disk-diffusion method were resistant to Lm, Sm and Su, and most of the strains were resistant to SxT and Tp.

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

four serotype A1 strains B2070, CVL33, S/C 82/1 and S/C 84/3. 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 (D/I 85/1 and G/T 85/15) had similar plasmid profiles (~4.0 and 2.7 kb) whilst in the third strain (B664) a 2.7-kb plasmid was replaced by a 7.6-kb plasmid. The untypable strain T1368 had three plasmids, all apparently different from those of the A1 and A2 strains. No plasmid DNA was detected in either the serotype T4 strain FT4 or the serotype T10 strain FT10.

Resistance to Ap, Tc and/or Cm was only found in the plasmid-bearing bovine A1 isolates. Since the Lm, Sm and Su resistances were common to all the strains and the SxT and Tp resistances were found in a majority of the strains of *P. haemolytica* irrespective of plasmid presence or absence, it was considered unlikely that these resistances were plasmid-mediated. Since these were the only resistances exhibited by the plasmid-carrying A2 and untypable strains, no resistance phenotype could be ascribed to their plasmids. Such plasmids may be of the cryptic type. 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 isolates. The resistance phenotypes of plasmid-harbouring *P. haemolytica* isolates and the possible location of the genetic determinants (chromosome or plasmid) are illustrated in Figure 5.

FIGURE 5. HISTOGRAM SHOWING INDIVIDUAL ANTIBIOTIC RESISTANCE PHENOTYPES OF PLASMID-CONTAINING *P. HAEMOLYTICA* ISOLATES AND THEIR POSSIBLE LINKAGE WITH GENETIC DETERMINANTS.

Lm = Lincomycin

Sm = Streptomycin

Su = Sulphonamide compound

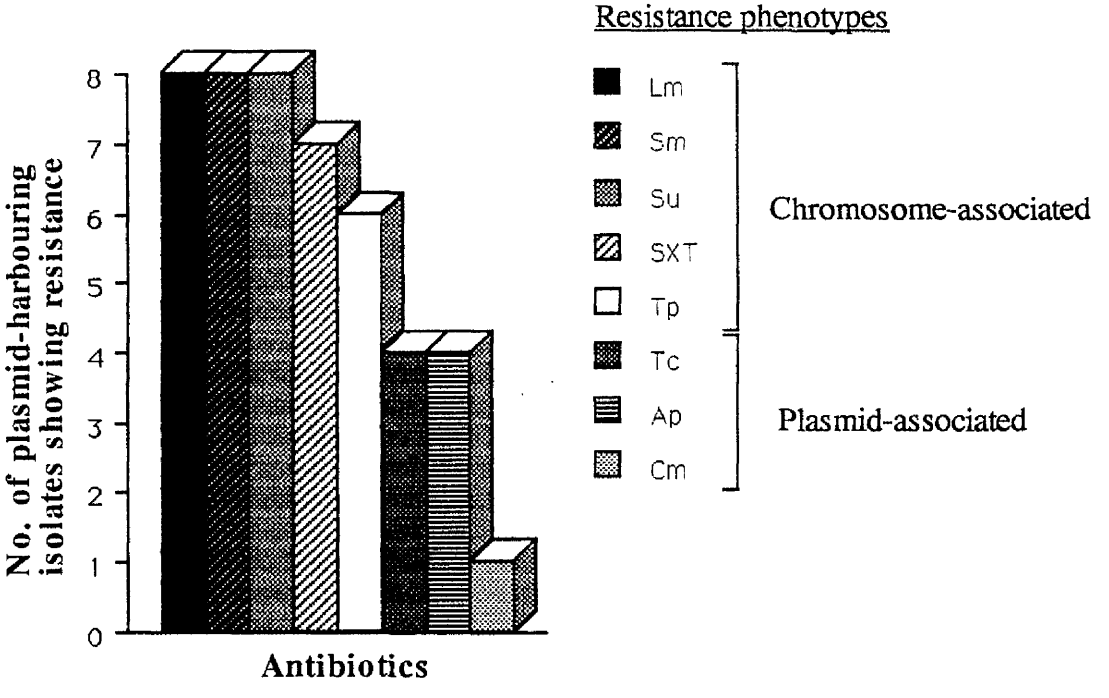
SxT = Sulphamethoxazole-trimethoprim

Tp = Trimethoprim

Tc = Tetracycline

Ap = Ampicillin

Cm = Chloramphenicol.


No. of plasmid-harbouring isolates showing resistance
0
1
2
3
4
5
6
7
8
Antibiotics
Resistance phenotypes
Lm
Sm
Su
SXT
Tp
Tc
Ap
Cm
Chromosome-associated
Plasmid-associated

3.3. Transformation of *E. coli* with Plasmid DNA Preparations from *P. haemolytica* A1 Isolates and Analysis of Transformant Clones for Plasmid DNA.

Plasmid DNA from A1 strains B2070, CVL33, S/C 82/1 and S/C 84/3, was used to transform *E. coli* strains DH1 and JM83, selecting separately for Ap^R, Tc^R and, in the case of S/C 84/3, for Cm^R transformants. Only Ap^R transformants were obtained, suggesting that Ap^R was plasmid-mediated. The lack of Tc^R and Cm^R transformants suggested that the plasmid DNA transformed did not contain determinants for Tc^R and/or Cm^R, or that these phenotypes were not expressed in *E. coli*. The frequencies of transformation to Ap^R were expressed as transformants per μg plasmid DNA and are shown in Table 11. *E. coli* JM83 showed a consistently higher transformation frequency than *E. coli* DH1. Transformation frequencies were similar with plasmid DNA preparations from strains B2070, CVL33 and S/C 82/1 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.

E. coli transformants were analyzed for the presence of plasmid DNA following a routine plasmid isolation technique. No plasmid DNA was detected on ethidium bromide-stained agarose gels and their presence in *E. coli* transformants was only confirmed by transformation of *E. coli* again to Ap^R with the plasmid DNA preparations from *E. coli*. About 40-60 transformant clones were obtained per plate, but it was not possible to deduce a transformation frequency as the amount of plasmid DNA present was not measurable. 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.

TABLE 11. TRANSFORMATION FREQUENCIES OF *E. COLI* STRAINS TO AMPICILLIN-RESISTANCE BY *P. HAEMOLYTICA* PLASMID DNA

P. haemolytica plasmid DNA source	Competent *E. coli* strain	Transformation frequency (Ap^R transformants $\mu g\ DNA^{-1}$)*
B2070	DH1	$6.8(\pm 1.2)\times 10^5$
	JM83	$1.2(\pm 0.5)\times 10^6$
CVL33	DH1	$1.3(\pm 0.2)\times 10^6$
	JM83	$2.4(\pm 0.3)\times 10^6$
S/C 82/1	DH1	$9.5(\pm 1.0)\times 10^5$
	JM83	$1.7(\pm 0.3)\times 10^6$
S/C 84/3	DH1	$4.2(\pm 0.1)\times 10^3$
	JM83	$5.2(\pm 0.2)\times 10^3$

* Each value shown is the mean(±SEM) of observations from at least three separate experiments. Transformants were selected on BHI agar containing Ap.

3.4. Mobilization of *P. haemolytica* Ap^R Plasmids from *E. coli* to *P. haemolytica* and *E. coli* by Conjugation and Plasmid DNA Analysis from Transconjugants.

Plasmid transfer from *P. haemolytica* strains B2070, CVL33, S/C 82/1 or S/C 84/3 to either *P. haemolytica* or *E. coli* by conjugation was unsuccessful, both in the presence and 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. The *P. haemolytica* strains therefore did not appear to act as donors of their own plasmids in a conjugation system. However, plasmid DNA could be mobilized from *E. coli* (DH1 or JM83) Ap^R transformants into both *P. haemolytica* (A2S and A2N) and *E. coli* (JC3272 and C600) by using the transfer functions of pRK2013. No transconjugants were obtained in the absence of the helper plasmid, indicating that these *P. haemolytica* Ap^R plasmids were not self-transmissible (non-conjugative). The frequencies of mobilization of the plasmids were expressed as transconjugants per recipient and are shown in Table 12. Plasmids pPH2, pPH33 and pPH821 showed similar mobilization frequencies and plasmid pPH843 showed a higher frequency, both into *P. haemolytica* and into *E. coli*. The overall 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 or JM83 (Table 11). In a later conjugation experiment with these plasmids, *P. haemolytica* A1 strain was used as a recipient after spontaneously-derived Sm^R and Nal^R mutants of this serotype were isolated (see Section 2.1.1.1). Relatively higher frequencies of plasmid DNA

TABLE 12. MOBILIZATION FREQUENCIES OF *P. HAEMOLYTICA* AMPICILLIN-RESISTANCE PLASMIDS FROM *E. COLI* (DH1 AND JM83) TRANSFORMANTS TO *P. HAEMOLYTICA* AND *E. COLI* BY CONJUGATION

E. coli donor strain	Helper plasmid*	Recipient strain†		Mobilization frequency (transconjugants recipient^{-1})§
DH1(pPH2)	pRK2013	A2S	Ph	$1.2(\pm0.3)\times10^{-5}$
		JC3272	Ec	$2.8(\pm0.5)\times10^{-4}$
DH1(pPH33)	,,	A2S	Ph	$1.3(\pm0.5)\times10^{-5}$
		JC3272	Ec	$3.3(\pm0.6)\times10^{-4}$
DH1(pPH821)	,,	A2S	Ph	$1.3(\pm0.4)\times10^{-5}$
		JC3272	Ec	$3.4(\pm0.7)\times10^{-4}$
DH1(pPH843)	,,	A2S	Ph	$4.4(\pm0.4)\times10^{-4}$
		JC3272	Ec	$1.3(\pm0.2)\times10^{-1}$
JM83(pPH2)	,,	A2N	Ph	$3.9(\pm0.5)\times10^{-5}$
		C600	Ec	$4.8(\pm0.8)\times10^{-4}$
JM83(pPH33)	,,	A2N	Ph	$5.3(\pm0.6)\times10^{-5}$
		C600	Ec	$5.4(\pm0.7)\times10^{-4}$
JM83(pPH821)	,,	A2N	Ph	$8.5(\pm1.3)\times10^{-5}$
		C600	Ec	$5.4(\pm0.9)\times10^{-4}$
JM83(pPH843)	,,	A2N	Ph	$1.1(\pm0.4)\times10^{-4}$
		C600	Ec	$6.5(\pm0.5)\times10^{-4}$

* *E. coli* DH5 was used as the carrier of the helper plasmid when Sm^R recipient strains (A2S and JC3272) were used and *E. coli* HB101 was used for Nal^R recipient strains (A2N and C600). Transconjugants were selected on BHI agar with Ap+Sm in the former case and Ap+Nal in the latter case. Recipient cell numbers were calculated by plating appropriate dilutions on BHI agar plus Sm or Nal respectively.

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

§ Each value shown is the mean(±SEM) of observations from three separate experiments.

mobilization, $7x10^{-5}$ - $6x10^{-4}$ transconjugants per recipient, were obtained with these A1 strains as recipients than with the A2 recipients.

Plasmid DNA analysis of transconjugants of *P. haemolytica* A2S revealed the presence of a single predominant 4.3-kb band for all the plasmids, but a band of similar size was only visible in transconjugants of *E. coli* JC3272 containing plasmid pPH843 (Figure 6). This confirmed that the 4.3-kb plasmid present in the original strain S/C 84/3 (Table 10) encoded Ap^R and thus all four Ap^R plasmids identified in this study appeared to be identical in size. *E. coli* transconjugants showed the presence of plasmid pRK2013 (Fig. 6B), which was not shown by *P. haemolytica* transconjugants (Fig. 6A) as pRK2013 is a ColE1-based replicon which does not replicate in *P. haemolytica*.

3.5. Mobilization of Transposon-Carrying Plasmids from *E. coli* to *E. coli* and *P. haemolytica* by Conjugation.

Attempts were made to transfer the transposon Tn*5*Cm^R from *E. coli* to *P. haemolytica* strains by conjugation, using the vectors pBR322::Tn*5*Cm^R and pRK404::Tn*5*Cm^R, either in the presence or absence of the helper plasmid pRK2013. No *P. haemolytica* transconjugants exhibiting Cm^R were obtained with these *E. coli* vectors (Table 13), suggesting that Cm^R was not expressed in *P. haemolytica* or Tn*5*Cm^R was unable to transpose into the *P. haemolytica* chromosome. Other drug resistances (e.g. Tc^R, Ap^R) carried by pBR322 or pRK404 were not examined in this study, but it had previously been shown that pRK404 could not be propagated in *P. haemolytica* (Craig *et al.*, 1989) and pBR322 would not be also expected to replicate in this species. On the other hand, transposon vectors could be mobilized into *E.*

FIGURE 6. **AGAROSE GEL (0.8% W/V) SHOWING THE RECOVERY OF PLASMID DNA FROM *P. HAEMOLYTICA* A2S AND *E. COLI* JC3272 Ap^R TRANSCONJUGANTS.**

Key to figure:

M = A supercoiled DNA ladder used as the marker DNA.

Group A

1 = Plasmid DNA (pPH2) from *P. haemolytica* A2S(pPH2) transconjugant.

2 = Plasmid DNA (pPH33) from ,, A2S(pPH33) ,, .

3 = Plasmid DNA (pPH821) from ,, A2S(pPH821) ,, .

4 = Plasmid DNA (pPH843) from ,, A2S(pPH843) ,, .

Group B

1 = Plasmid DNA (pRK2013) from *E. coli* JC3272(pPH2) transconjugant.

2 = Plasmid DNA (pRK2013) from ,, JC3272(pPH33) ,, .

3 = Plasmid DNA (pRK2013) from ,, JC3272(pPH821) ,, .

4 = Plasmid DNAs (pRK2013 + pPH843) from *E. coli* JC3272(pPH843) ,, .

The upper faint band (at ~7-kb position) present in all lanes of group A probably represents an open circular (OC) form of the 4.3-kb plasmid.

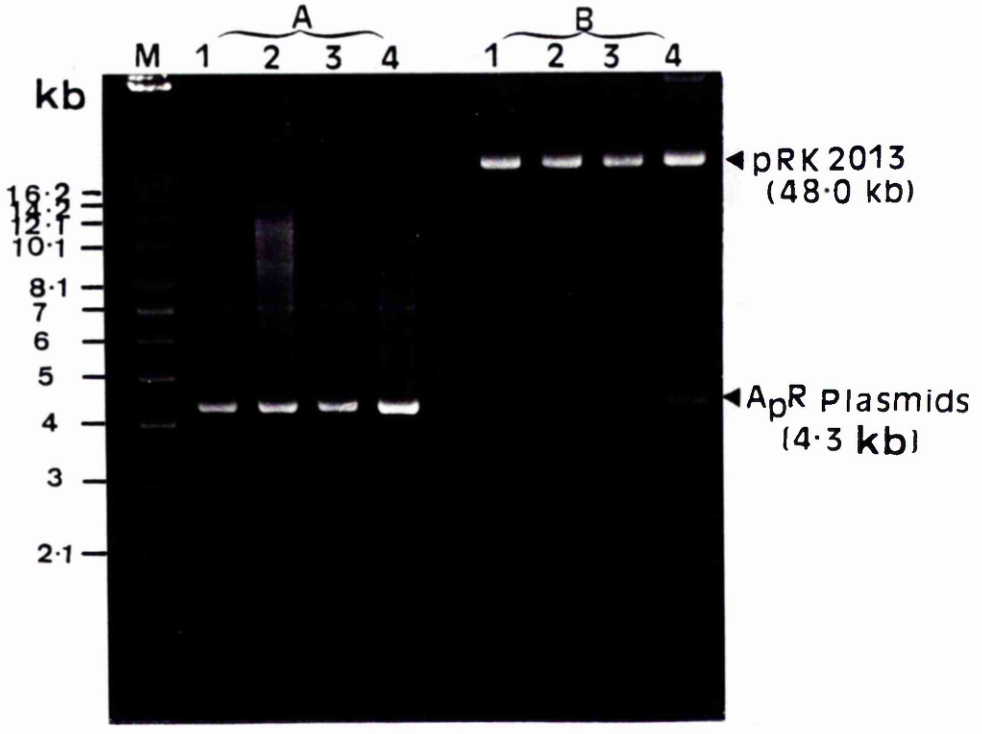

A
B
M 1 2 3 4 1 2 3 4
kb
16·2
14·2
12·1
10·1
8·1
7
6
5
4
3
2·1
pRK 2013
(48·0 kb)
ApR Plasmids
(4·3 kb)

TABLE 13. MOBILIZATION OF TRANSPOSON *5*-CARRYING PLASMIDS FROM DIFFERENT *E. COLI* DONORS TO *E. COLI* AND *P. HAEMOLYTICA* RECIPIENTS BY CONJUGATION

E. coli donor strain	Helper plasmid*	Recipient strain†		Mobilization frequency (transconjugants recipient^{-1})§
DH1(pBR322::Tn*5*Cm^R)	pRK2013	JC3272	Ec	1.1(±0.2) x 10^{-1}
		A2S	Ph	0
HB101(pBR322::Tn*5*Cm^R)	,,	C600	Ec	1.0(±0.2) x 10^{-1}
		A2N	Ph	0
C600(pRK404::Tn*5*Cm^R)	,,	JC3272	Ec	1.8(±0.5) x 10^{-1}
		A2S	Ph	0
JC3272(pRK404::Tn*5*Cm^R)	,,	C600	Ec	1.5(±0.3) x 10^{-1}
		A2N	Ph	0

* *E. coli* DH5 was used as the carrier of the helper plasmid when Sm^R recipient strains (JC3272 and A2S) were used and *E. coli* HB101 was used for Nal^R recipient strains (C600 and A2N). Transconjugants were selected on BHI agar containing Cm+Sm in the former case and Cm+Nal in the latter case. Recipient cell numbers were calculated by plating appropriate dilutions on BHI agar plus Sm or Nal respectively.

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

§ Values are the means(±SEM) of two independent determinations.

coli strains (JC3272 and C600) at a high frequency, 1.0 to 1.8 x 10^{-1} transconjugants per recipient cell (Table 13). These transposon-carrying plasmids by themselves were non-conjugative in nature, because they did not transfer to *E. coli* without the help of pRK2013.

3.6. Curing *P. haemolytica* Strain S/C 84/3 of R Factor (R Plasmid).

Since the large plasmid present in strain S/C 84/3 (Table 10; Fig. 4, lane 4) could not be demonstrated as an R factor by $CaCl_2$-mediated transformation, an attempt was made to cure strain S/C 84/3 of plasmids, especially the large one possibly associated with Cm^R. Ethidium bromide and acridine orange were used as curing agents, and an antibiogram, using six antibiotics to which the strain was resistant, was carried out every 20 h on portions of the cell culture streaked onto BHI agar (see Section 2.8) to monitor the loss of any antibiotic resistance (Table 14). The only change observed with this strain in its antibiotic resistance profile was the appearance of a clear and distinct zone of inhibition around the Ap disk after 40 h of growth in broth containing either 50 μg of ethidium bromide per ml or 75- or 100 μg of acridine orange per ml. This suggested that the Ap^R plasmid (pPH843) from strain S/C 84/3 was eliminated, but there was no loss of Cm^R. Longer incubation with the drugs caused a complete loss of cell viability.

3.7. Detection of β-Lactamase Production and Determination of MICs for Plasmid-Containing Ap^R *P. haemolytica* and *E. coli* Strains.

The four Ap^R plasmid-containing wild-type *P. haemolytica* strains, the corresponding Ap^R transconjugants of *P. haemolytica* A2S and the Ap^R

TABLE 14. CURING AND GROWTH EFFECTS OF ETHIDIUM BROMIDE AND ACRIDINE ORANGE ON *P. HAEMOLYTICA* STRAIN S/C 84/3

Concentration of drug ($\mu g\ ml^{-1}$)	Growth on BHI agar and antibiotic sensitivity of a sample of culture after incubation time		
	20h	40h	60h
Ethidium bromide			
0.0	+	+	±
0.5	+	+	−
1.0	+	+	−
1.5	+	+	−
2.0	+	+	−
2.5	+	+	−
5.0	+	+	−
50.0	+	+*	−
Acridine orange			
0.0	+	+	±
75.0	+	+*	−
100.0	+	+*	−
Antibiotic sensitivity	Ap^R, Cm^R, Tc^R, Sm^R, Su^R, Tp^R	Ap^S, Cm^R, Tc^R, Sm^R, Su^R, Tp^R	NT

KEY: +, growth; ±, poor growth; −, no growth; NT, not tested.

* Cells were completely sensitive to Ap at these concentrations of drugs after 40h.

For antibiotic designations, see footnote (*) of Table 9.

transformants of *E. coli* DH1 were tested for β-lactamase production and for MICs with seven β-lactam antibiotics. In addition, *E. coli* DH1 derivatives containing the TEM-type β-lactamase plasmid RP4 and the ROB-type β-lactamase plasmid pRM3022 were used for comparative purposes. The β-lactamase profile and the MIC values for β-lactams are given in Table 15. A good correlation was found between the β-lactamase production and the MICs of β-lactam antibiotics for the wild-type *P. haemolytica* isolates and the transconjugant strains of *P. haemolytica* A2S. Isolate S/C 84/3 was β-lactamase-negative and showed much lower MIC values for those antibiotics (penicillin group) to which the other original isolates were clearly resistant. However, strain A2S(pPH843), which contains plasmid pPH843 from strain S/C 84/3, exhibited β-lactamase and MIC profiles similar to those of the other transconjugants. The MIC values 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. 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. Plasmid-free and β-lactamase-negative *E. coli* DH1 strain showed some resistance to all the β-lactam antibiotics tested and the MIC values were noticeably higher than those exhibited by the plasmid-free *P. haemolytica* A2S strain. This suggested an inherent property of the *E. coli* K12 strain for conferring β-lactam resistance. *E. coli* strains containing plasmids pPH2, pPH33 and pPH821 were weakly positive for β-lactamase on

TABLE 15. BETA-LACTAMASE PROFILE AND MINIMUM INHIBITORY CONCENTRATIONS OF BETA-LACTAM ANTIBIOTICS FOR PLASMID-CONTAINING AMPICILLIN-RESISTANT STRAINS

Strain	β-lactamase	MIC (μg ml^{-1})						
		Pe	Ap	Carb	Tic	Cex	Cet	Cer
P. haemolytica								
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
E. coli								
DH1(plasmid-free)	−	4	2	2	1	4	2	1
DH1(pPH2), DH1(pPH33)† and DH1(pPH821)	±	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

For abbreviations of β-lactam antibiotics, see Section 2.3 of Materials and Methods. β-lactamase presence was determined as outlined in Section 2.11 and MICs as detailed in Section 2.10.

* Transconjugants, † Transformants.

±, Weakly positive.

test strips. This may be related to copy number or stability of plasmids in *E. coli* (see Table 17 and Figs. 6B & 8). Beta-lactamase production by both transformant and transconjugant strains confirmed that these β-lactamases were plasmid-encoded.

3.8. Characterization of β-Lactamases Encoded by *P. haemolytica* Plasmids pPH2, pPH33, pPH821 and pPH843.

3.8.1. Beta-lactamase assays to determine the relative rates of β-lactam hydrolysis, specific activities, and inhibition of β-lactamase activity.

The plasmid-encoded *P. haemolytica* β-lactamases were extracted from *E. coli* DH1 transformants and analyzed to compare them with known or reference β-lactamases. 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 substrate and inhibition profiles, and specific activities of the reference and the *P. haemolytica* β-lactamases are shown in Table 16. 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 16). This indicated that the *P. haemolytica* β-lactamases might belong to the ROB-1 class. These enzymes showed high activity against ampicillin and carbenicillin and low activity against cephaloridine and cephalothin as compared to the TEM-type enzymes. The ROB-1 enzyme activity was markedly inhibited by cloxacillin but not by NaCl. The β-lactamase preparation from *E. coli* containing plasmid pPH843 showed a

TABLE 16. SUBSTRATE AND INHIBITION PROFILES, AND SPECIFIC ACTIVITIES OF *E. COLI* EXTRACTS CONTAINING BETA-LACTAMASES ENCODED BY PLASMIDS pPH2, pPH33, pPH821 AND pPH843 COMPARED WITH THOSE OF TEM- AND ROB-TYPE BETA-LACTAMASES

Plasmid (β-lactamase type)	Relative rate of hydrolysis (%)*							Specific activity†	% activity in presence of§	
	Ap	Carb	Oxa	Clox	Cer	Cet	Cex		NaCl (100mM)	Clox (0.1mM)
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

Values are the means of two independent determinations.

* Activity relative to activity against penicillin G (defined as 100%). For abbreviations of β-lactam antibiotics, see Section 2.3 of Materials and Methods.

† Expressed as μmoles per min per mg protein with penicillin G as substrate.

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

higher specific activity than those prepared from *E. coli* containing the other three *P. haemolytica* plasmids (Table 16). This is probably related to plasmid copy number as pPH843 was the only plasmid DNA visible in preparations from *E. coli* (Fig. 6B, lane 4).

3.8.2. Determination of isoelectric points (pIs) of β-lactamases by analytical isoelectric focusing.

The β-lactamases contained in *E. coli* extracts were analyzed by isoelectric focusing for their pI values. The isoelectric focusing patterns of unknown β-lactamases encoded by *P. haemolytica* plasmids pPH2, pPH33, pPH821 and pPH843 and those of known β-lactamases are shown in Figure 7. The main bands of β-lactamase activity produced by the *P. haemolytica* plasmids (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 (Medeiros *et al.*, 1985) (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 variant, satellite bands and the activity of chromosomal β-lactamase of *E. coli*, respectively, as reported by Matthew *et al.* (1975) and Medeiros *et al.* (1985). The pI values confirmed that the *P. haemolytica* β-lactamases belonged to the ROB-1 class.

3.9. Stability of *P. haemolytica* Ap^R Plasmids in *E. coli*.

E. coli DH1 transformants were tested for loss of plasmids pPH2, pPH33, pPH821 and pPH843 by successive subculture on non-selective agar media. The loss or stability of these plasmids in *E. coli* was monitored after each subculture by examining their Ap^R phenotypes and β-lactamase production

FIGURE 7. COMPARATIVE ANALYTICAL ISOELECTRIC FOCUSING PATTERNS OF *E. COLI* EXTRACTS CONTAINING KNOWN β-LACTAMASES AND THOSE ENCODED BY THE *P. HAEMOLYTICA* PLASMIDS.

Key to figure:

1 = Activity and pI of the TEM-1 β-lactamase encoded by plasmid R6K.

2 = Activity and pI of the TEM-2 β-lactamase encoded by plasmid RP4.

3 = Activity and pI of the ROB-1 β-lactamase encoded by pRM3022.

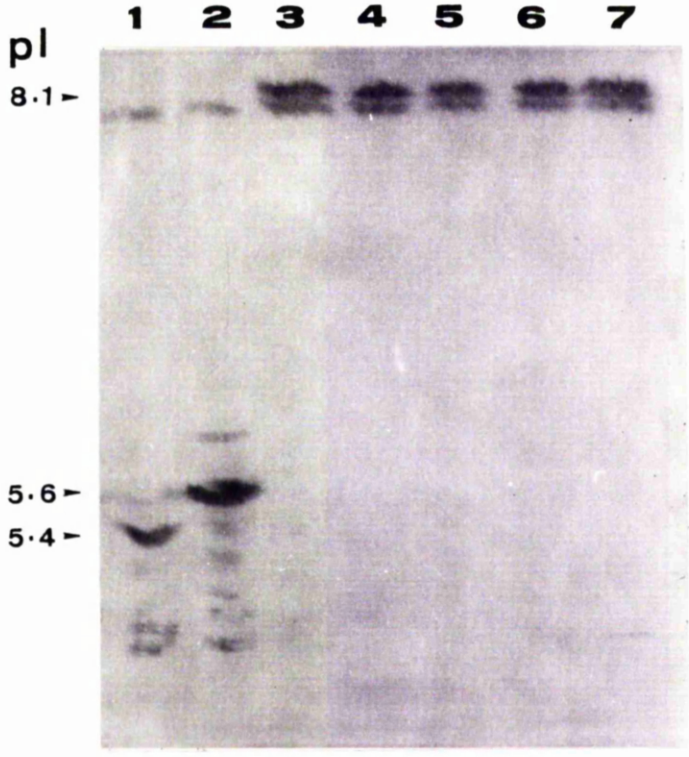
4 = Activity and pI of the β-lactamase encoded by pPH2.

5 = Activity and pI of the β-lactamase encoded by pPH33.

6 = Activity and pI of the β-lactamase encoded by pPH821.

7 = Activity and pI of the β-lactamase encoded by pPH843.

A common additional band (pI 8.0), probably representing the chromosomally-determined β-lactamase activity of *E. coli*, is seen in all lanes. Enzyme preparations from strains containing pPH2, pPH33 and pPH821 were concentrated at least 10 times compared to the preparation from strain containing pPH843, before loading onto the gel.


1
2
3
4
5
6
7
pI
8.1
5.6
5.4

(Table 17). Plasmids pPH2, pPH33 and pPH821 were very unstable in *E. coli* and were lost completely after the second passage (~50 generations) in the absence of antibiotic selection. Plasmid pPH843, on the other hand, was stable even after 10 passages (~250 generations) in the absence of selection.

3.10. Amplification in *E. coli* of Ap^R Plasmid DNA Encoding ROB-1 β-Lactamase.

As each Ap^R plasmid from *P. haemolytica* apparently had a low copy number in *E. coli*, attempts were made to amplify the plasmid DNA in *E. coli* transformants. *E. coli* 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 18). 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 18; Figure 8). 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, and Terrific broth supplemented with 1% yeast nitrogen base gave the highest yield (Table 18; Fig. 8C). 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 of these plasmids was achieved when Cm was replaced by Tc (data not shown).

Higher yields of plasmids pPH2, pPH33 and pPH821, but less of plasmid

TABLE 17. STABILITY OF *P. HAEMOLYTICA* AMPICILLIN-RESISTANCE PLASMIDS IN *E. COLI* DH1

Plasmid-containing *E. coli* strain	No. of passages on non-selective media	Approximate no. of generations*	Phenotype†	
			Ap^R	β-lactamase
DH1(pPH2), DH1(pPH33) and DH1(pPH821)	1	25	NT	±
	2	50	-§	-§
DH1(pPH843)	1	25	NT	+
	10	250	+¶	+¶

KEY: NT, not tested; ±, weakly positive; +, positive.

* One generation of *E. coli* = ~20 min.

† Ap^R and β-lactamase phenotypes were tested as described in Section 2.13 of Materials and Methods.

§ Plasmid lost.

¶ Plasmid stable.

TABLE 18. 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)

YNB, Yeast nitrogen base.

* 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.

FIGURE 8. AGAROSE GEL (0.8%) SHOWING THE YIELDS OF *P. HAEMOLYTICA* Ap^R PLASMIDS FROM *E. COLI* DH1 AFTER AMPLIFICATION PROCEDURES.

Key to figure:

M = A supercoiled DNA ladder used as the DNA size marker.

Group A

1 = DNA preparation of plasmid pPH2 from *E. coli* grown in Terrific broth alone.

2 = DNA preparation of plasmid pPH33 from *E. coli* grown in Terrific broth alone.

3 = DNA preparation of plasmid pPH821 from *E. coli* grown in Terrific broth alone.

4 = DNA preparation of plasmid pPH843 from *E. coli* grown in Terrific broth alone.

Group B

1 = Plasmid pPH2 from *E. coli* grown in Terrific broth + Cm.

2 = Plasmid pPH33 from *E. coli* grown in Terrific broth + Cm.

3 = Plasmid pPH821 from *E. coli* grown in Terrific broth + Cm.

4 = Plasmid pPH843 from *E. coli* grown in Terrific broth + Cm.

Group C

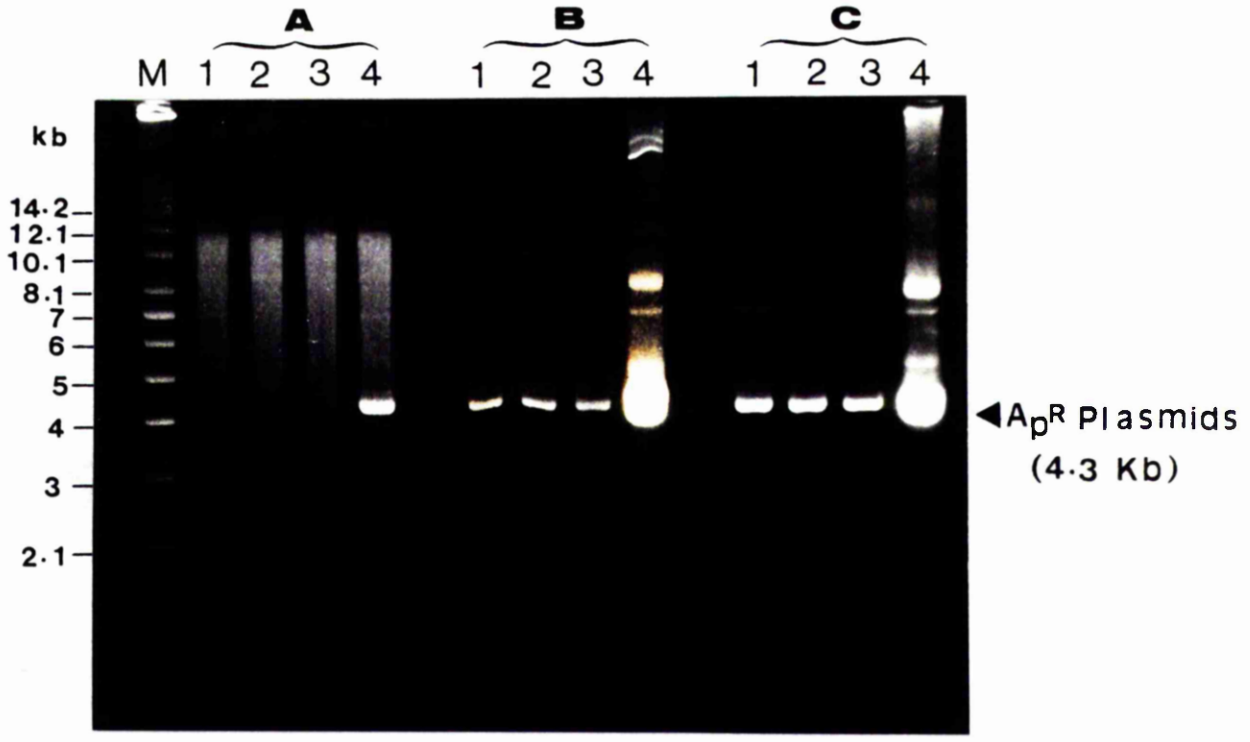
1 = Plasmid pPH2 from *E. coli* grown in Terrific broth + 1% YNB + Cm.

2 = Plasmid pPH33 from *E. coli* grown in Terrific broth + 1% YNB + Cm.

3 = Plasmid pPH821 from *E. coli* grown in Terrific broth + 1% YNB + Cm.

4 = Plasmid pPH843 from *E. coli* grown in Terrific broth + 1% YNB + Cm.

Ampicillin (50 $\mu g\ ml^{-1}$) was added to each of the above broth media used. The additional upper band(s) (groups B and C) probably represent OC forms and/or dimers of the 4.3-kb plasmid.


A
B
C
M 1 2 3 4 1 2 3 4 1 2 3 4
kb
14·2
12·1
10·1
8·1
7
6
5
4
3
2·1
◀ApR Plasmids
(4·3 Kb)

pPH843, were achieved from *E. coli* JM83 transformants than from their DH1 counterparts following identical amplification procedures (data not shown).

The prototype ROB-1 plasmid pRM3022 (4.4 kb) of *H. influenzae* origin was amplified to a similar extent to that of the *P. haemolytica* plasmid pPH843 in *E. coli* strain JM83 (Figure 9).

3.11. Development of an Improved and Rapid Method for Plasmid DNA Purification.

Since the large-scale preparation of purified plasmid DNA was required for restriction enzyme analysis, cloning and further characterization purposes, an improved and rapid procedure was developed for the large-scale purification of CCC plasmid DNA. The new protocol combined a modified alkaline-denaturation procedure with a modified version of the acid-phenol extraction (see Materials and Methods: Section 2.6.3). Major modifications were the uses of $MgCl_2$ instead of NaCl and vortexing instead of shaking in the final purification process. In addition to the smaller plasmids (4.3-4.4 kb) used in this study, some large plasmids were also employed in order to assess the effectiveness of the modified protocol for preparation of plasmids over a wide size range.

$MgCl_2$ was chosen in place of NaCl in the present protocol because Mg^{2+}, a divalent cation, is known to have a high DNA binding capacity and would more efficiently precipitate non-CCC DNA (Sander & Ts'o, 1971; Müller *et al.*, 1983) (see Discussion: Section 4.8). Several different concentrations (1-50mM) of $MgCl_2$ were used in the low-ionic strength extraction buffer, but 1.5mM $MgCl_2$ (final concentration) was found to be most effective in CCC plasmid DNA purification. Figure 10 illustrates the preparation of plasmid

FIGURE 9. **AGAROSE GEL (0.8%) SHOWING THE YIELDS OF *P. HAEMOLYTICA* PLASMID pPH843 (4.3 KB) AND *H. INFLUENZAE* PLASMID pRM3022 (4.4 KB) FROM *E. COLI* JM83 AFTER AMPLIFICATION PROCEDURES.**

Key to figure:

M = A supercoiled DNA ladder used as the DNA size marker.

Group A

1 = Plasmid pPH843 from *E. coli* JM83 grown in Terrific broth alone.

2 = Plasmid pRM3022 from *E. coli* JM83 grown in Terrific broth alone.

Group B

1 = Plasmid pPH843 from *E. coli* JM83 grown in Terrific broth + Cm.

2 = Plasmid pRM3022 from *E. coli* JM83 grown in Terrific broth + Cm.

Group C

1 = Plasmid pPH843 from JM83 grown in Terrific broth + 1% YNB + Cm.

2 = Plasmid pRM3022 from JM83 grown in Terrific broth + 1% YNB + Cm.

Ampicillin (50 $\mu g\ ml^{-1}$) was added to each of the above broth media used. The upper bands (lane 2, group C) probably represent a "step ladder" of CCC molecules with different numbers of supercoil turns (Kieser, 1984).

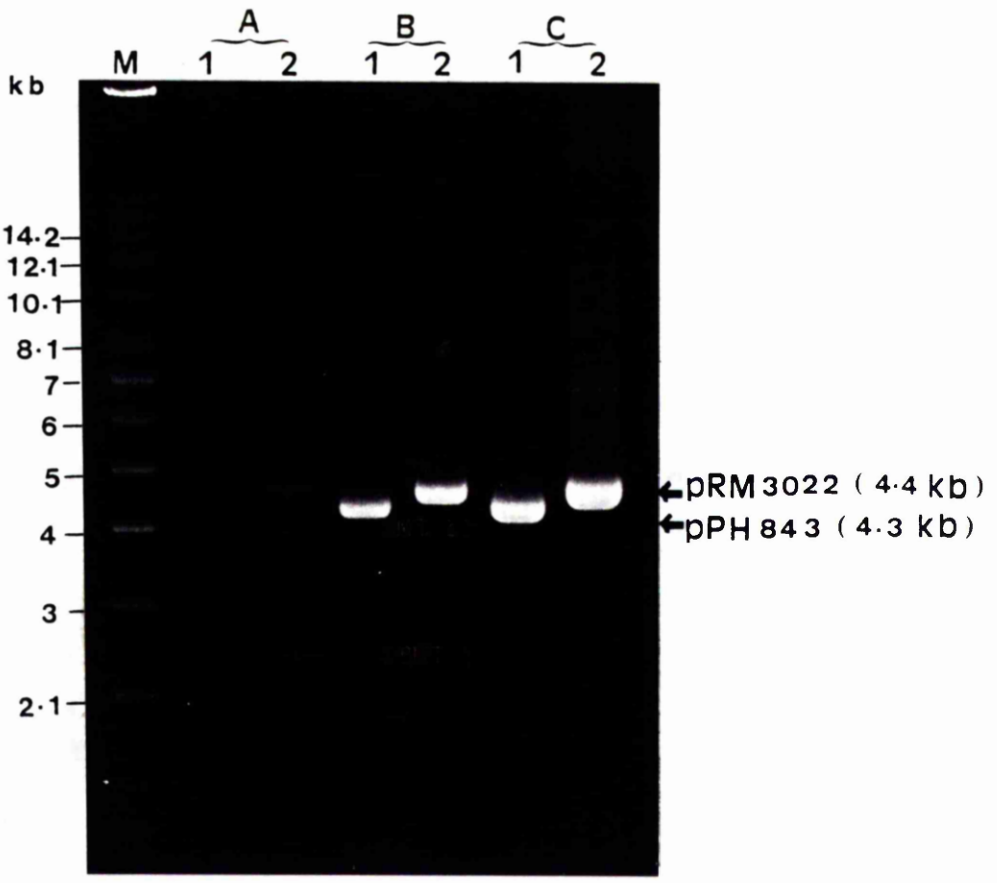

A
B
C
M
1
2
1
2
1
2
kb
14·2
12·1
10·1
8·1
7
6
5
4
3
2·1
pRM3022 (4·4 kb)
pPH843 (4·3 kb)

FIGURE 10. **AGAROSE GEL ELECTROPHORESIS OF LARGE-SCALE PLASMID DNA PREPARATIONS EXTRACTED BY THE MODIFIED ALKALINE-LYSIS PROCEDURE AND THEN FURTHER PURIFIED BY THE MODIFIED ACID-PHENOL EXTRACTION METHOD.**

Key to figure:

Group A: Plasmid pPH843 (4.3 kb)

1 = Extracted by the modified alkaline-lysis method.

2 = Purified by acid-phenol extraction using 75mM NaCl.

3 = Purified by acid-phenol extraction using 1.5mM $MgCl_2$.

Group B: Plasmid pRM3022 (4.4 kb)

1 = Extracted by the modified alkaline-lysis method.

2 = Purified by acid-phenol extraction using 75mM NaCl.

3 = Purified by acid-phenol extraction using 1.5mM $MgCl_2$.

Group C: Plasmids pRK2013 (48 kb) and pPH843 (4.3 kb)

1 = Extracted by the modified alkaline-lysis method.

2 = Purified by acid-phenol extraction using 75mM NaCl.

3 = Purified by acid-phenol extraction using 1.5mM $MgCl_2$.

Group D: Plasmid RP4 (54 kb)

1 = Extracted by the modified alkaline-lysis method.

2 = Purified by acid-phenol extraction using 75mM NaCl.

3 = Purified by acid-phenol extraction using 1.5mM $MgCl_2$.

Group E: Plasmid pUW964 (73 kb)

1 = Extracted by the modified alkaline-lysis method.

2 = Purified by acid-phenol extraction using 75mM NaCl.

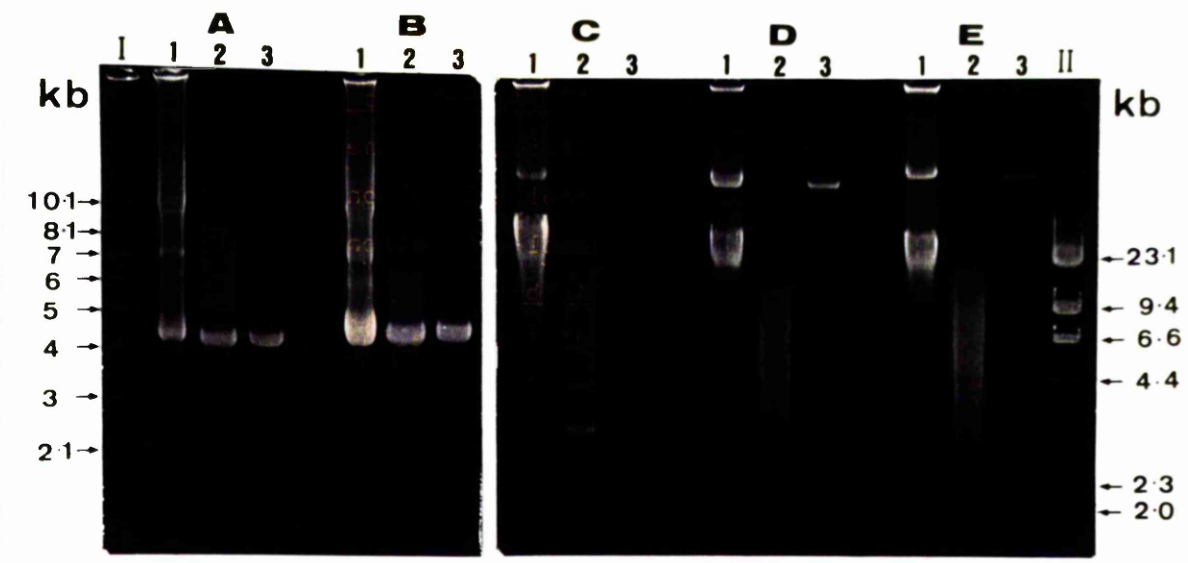
3 = Purified by acid-phenol extraction using 1.5mM $MgCl_2$.

I = A supercoiled DNA ladder used as the DNA size marker.

II = *Hind*III-digested λ DNA used as the DNA size marker.

Five to seven microlitres from a total amount of 500 µl of crude or purified plasmid DNA samples derived from one large-scale preparation were run in each lane.

When *Hind*III-digested λ DNA fragments were used as size markers, the supercoiled equivalent plasmid DNA size was determined by the method of Platt & Taggart (1987).


A
B
C
D
E
I
II
1
2
3
kb
10·1
8·1
7
6
5
4
3
2·1
23·1
9·4
6·6
4·4
2·3
2·0

DNA by the modified alkaline-lysis method (lane 1, each group) which was then purified by acid-phenol extraction (lanes 2 and 3, each group). The difference between purification of CCC plasmids with 75mM NaCl (lane 2, each group) and with 1.5mM $MgCl_2$ (lane 3, each group) can be clearly seen. The use of NaCl produced a diffuse DNA smear in each case and removed CCC plasmid DNA molecules of higher molecular weight either partially (lane 2, group C) or completely (lane 2, groups D and E). The use of $MgCl_2$, on the other hand, allowed good yields and complete purification of CCC plasmids ranging from 4.3 to 73.0 kb in size.

3.12. Comparative Restriction Endonuclease Analysis of ROB-1 β-Lactamase-Enoding Plasmids.

Plasmid DNA was extracted from *E. coli* transformants, purified by the above protocol, and subjected to digestion with 42 restriction endonuclease enzymes. Twelve enzymes were found to cut *P. haemolytica* plasmids pPH2, pPH33, pPH821 and pPH843 giving fragments of identical size (Table 19) and the calculated size of each of the plasmids was found to be 4.2 kb. These enzymes were: *Pst*I, *Apa*LI, *Ava*I, *Bsp*1286I (1 fragment each); *Hinf*I, *Sca*I (2 fragments each); *Dra*I, *Tha*I (3 fragments each); *Cfo*I, *Sau*3AI, *Taq*I (5 fragments each); and *Alu*I (10 or more fragments). The plasmids were thus cleaved by only six of the 6-bp recognition sequence enzymes used. Representative restriction digests of plasmid pPH843 are shown in Figure 11A; the unique sites for *Pst*I, *Apa*LI, *Ava*I and *Bsp*1286I (all have a 6-bp recognition sequence) were confirmed by double digestion (lanes 7-9). The other two enzymes with a 6-bp recognition sequence were *Sca*I and *Dra*I. Enzymes with a 6-bp recognition sequence which did not cut the plasmid DNA

TABLE 19. RESTRICTION ENDONUCLEASE-DIGESTION PROFILE OF THE *P. HAEMOLYTICA* AMPICILLIN-RESISTANCE PLASMIDS pPH2, pPH33, pPH821 OR pPH843 (ALL HAD AN IDENTICAL RESTRICTION PROFILE)

Restriction endonuclease enzyme*	No. of fragment(s)	Size of fragment(s) (kb)
*Pst*I	1	4.2
*Apa*LI	1	4.2
*Ava*I	1	4.2
*Bsp*1286I	1	4.2
*Hinf*I	2	4.14, 0.06
*Sca*I	2	4.14, 0.06
*Dra*I	3	3.9, 0.24, 0.06
*Tha*I	3	3.4, 0.53, 0.27
*Cfo*I	5	3.33, 0.35, 0.27, 0.18, 0.065
*Sau*3AI	5	1.84, 1.1, 0.9, 0.26, 0.1
*Taq*I	5	2.56, 1.0, 0.29, 0.23, 0.12
*Alu*I	10†	1.7, 0.53, 0.3, 0.28, 0.23, 0.2, 0.18, 0.1, 0.08, 0.06

* Recognition sequence of the enzyme is shown in Table 7 of Materials and Methods.

† The sub-total of the sizes of fragments obtained by *Alu*I (3.66 kb) indicates that one or more of the fragments were doublets, i.e. comigrated on a 2% (w/v) agarose gel. This is evident from the relative brightness of bands shown in Fig. 11B (group 10).

FIGURE 11. **RESTRICTION ENZYME DIGESTION PATTERNS OF ROB-1 β-LACTAMASE-ENCODING PLASMIDS OF *P. HAEMOLYTICA* AND *H. INFLUENZAE* ORIGIN (1.8-2% AGAROSE GEL).**

Key to figure:

A: Representative restriction digests of pPH843 with 9 enzymes

1 = *Taq*I. 2 = *Cfo*I. 3 = *Tha*I.

4 = *Hinf*I. 5 = *Sca*I. 6 = *Pst*I (or *Apa*LI or *Ava*I or *Bsp*1286).

7 = *Apa*LI+*Pst*I. 8 = *Ava*I+*Pst*I. 9 = *Bsp*1286I+*Pst*I.

M = 1 kb DNA ladder used as molecular size standard.

B: Restriction digests of pPH33, pRM3022 and pPH843 with 3 enzymes

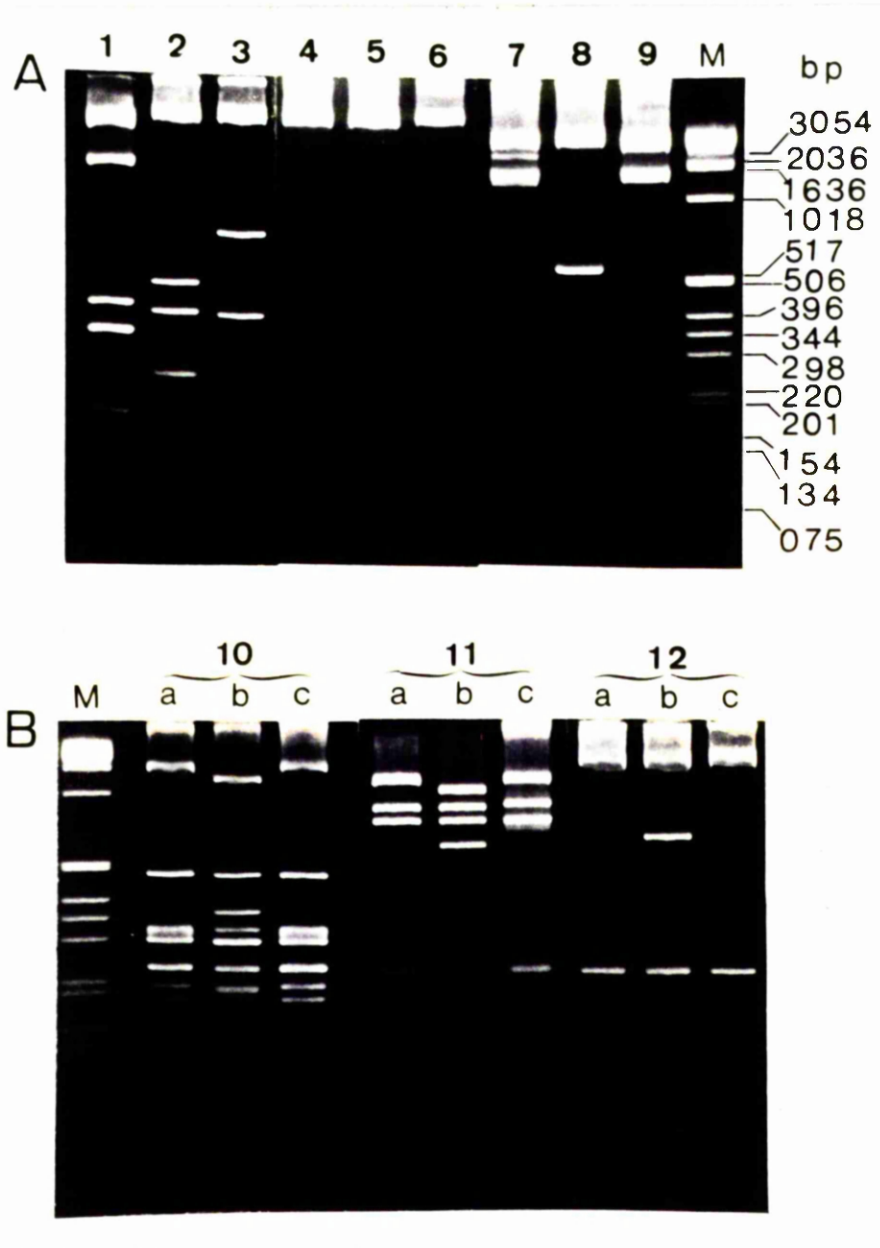
Group 10 = *Alu*I, Lane a = pPH33.

Group 11 = *Sau*3AI, Lane b = pRM3022.

Group 12 = *Dra*I, Lane c = pPH843.

M = 1 kb DNA ladder (for fragment sizes, see panel A above).

The faint high molecular-weight bands in lanes 1, 7, 10a and 11c are presumed to be partially-digested products.


A
1
2
3
4
5
6
7
8
9
M
bp
3054
2036
1636
1018
517
506
396
344
298
220
201
154
134
075
B
10
11
12
M
a
b
c
a
b
c
a
b
c

were: *Acy*I, *Apa*I, *Bam*HI, *Bgl*II, *Bsc*I, *Bss*HII, *Cla*I, *Eco*RI, *Eco*RV, *Hae*II, *Hinc*II, *Hind*III, *Hpa*I, *Kpn*I, *Nar*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Sph*I, *Stu*I, *Xba*I, *Xho*I and *Xho*II.

The prototype ROB-1 plasmid pRM3022 from *H. influenzae* presented apparently identical restriction fragment patterns to those of pPH843 with all the enzymes tested except *Alu*I, *Sau*3AI and *Dra*I. With these three enzymes slight differences were found (Figure 11B), whereby pRM3022 had two extra *Alu*I sites and an additional *Sau*3AI and *Dra*I site. Fragment sizes of pRM3022 produced by these three enzymes are given in Table 20. Two of the fragments produced by *Dra*I (0.24 and 0.06 kb), 4 of the fragments by *Sau*3AI (1.1, 0.9, 0.26, and 0.1 kb) and 9 of the fragments by *Alu*I (0.53, 0.3, 0.28, 0.23, 0.2, 0.18, 0.1, 0.08, and 0.06 kb) were common to both types of ROB-1 plasmids. This suggested that a close relationship existed between these plasmids.

3.13. DNA Hybridization between ROB-1 Plasmids.

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 ^{32}P-labelled pPH843, except one fragment (Figure 12, panel A, lane 2, arrowed), which showed only very weak hybridization (Fig. 12, panel B, 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.

TABLE 20. RESTRICTION FRAGMENTS OF THE PROTOTYPE ROB-1 BETA-LACTAMASE PLASMID pRM3022 (4.4 KB) BY THE ENZYMES *DRA*I, *SAU*3AI AND *ALU*I

Restriction enzyme	No. of fragments	Size of fragments (kb)
*Dra*I	4	3.4, 0.7, 0.24, 0.06
*Sau*3AI	6	1.35, 1.1, 0.9, 0.69, 0.26, 0.1
*Alu*I	12*	1.4, 0.53, 0.36, 0.3, 0.28, 0.23, 0.2, 0.18, 0.14, 0.1, 0.08, 0.06

* The sub-total of the sizes of fragments obtained by *Alu*I (3.86 kb) indicates that one or more of the fragments were doublets, i.e. comigrated on a 2% (w/v) agarose gel, as is evident from the relative brightness of bands shown in Fig. 11B (group 10).

FIGURE 12. **HOMOLOGY BETWEEN THE ROB-1 PLASMIDS FROM *P. HAEMOLYTICA* AND *H. INFLUENZAE*.**

Key to figure:

Panel A: Ethidium bromide-stained agarose gel (1.8% w/v)

1 = Plasmid pPH843 digested by *Sau*3AI.

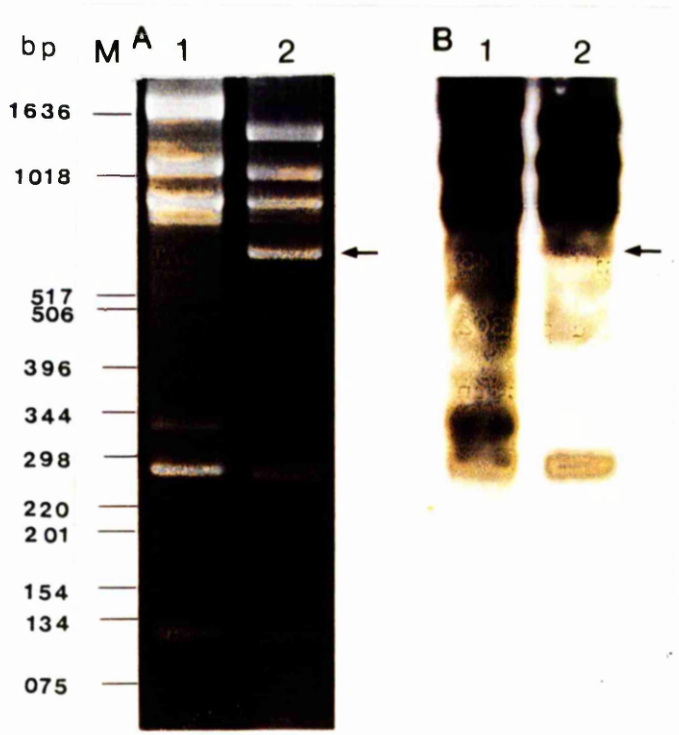
2 = Plasmid pRM3022 digested by *Sau*3AI.

M = Representation of 1 kb DNA ladder as molecular size marker.

Panel B: Autoradiograph of a Southern blot of the same gel shown in panel A

DNA was transferred to Hybond-N membrane and hybridized with ^{32}P-labelled *Sau*3AI-digested pPH843 (see Materials and Methods: Section 2.16).

Arrow (panel B, lane 2) shows a band exhibiting only faint hybridization, equivalent to the 0.69-kb fragment of pRM3022 (arrow, panel A, lane 2).


bp
M
A
1
2
B
1
2
1636
1018
517
506
396
344
298
220
201
154
134
075

3.14. Restriction Mapping of Plasmid pPH843.

A restriction map of plasmid pPH843 was constructed by using 9 of the restriction enzymes that cleaved this plasmid. The enzymes used were: *Pst*I, *Bsp*1286I, *Apa*LI, *Ava*I, *Hinf*I, *Sca*I, *Dra*I, *Tha*I and *Taq*I. Enzymes *Alu*I and *Sau*3AI were not used in mapping due to problems involving either high frequency of digestion (*Alu*I) or partial digestion (*Sau*3AI) of the plasmid DNA (see Fig. 11B, lanes 10c & 11c respectively). In addition, enzyme *Cfo*I was omitted for ease of interpretation and only *Taq*I was used as a producer of 5 fragments. DNA fragments were generated by digestion with a single enzyme or with a combination of two different enzymes and are shown in Figures 13A, 13B and 13C. Restriction fragments produced by single digestion were compared with those produced by double digestion involving the same enzyme, by running them side by side in agarose gels. The size of each fragment was estimated and the relative restriction site(s) of each enzyme was located, on a circular structure of pPH843, by a trial-and-error search. The estimated sizes of fragments of pPH843 obtained by double digestion are given in Table 21. The size of each restriction fragment fitted well into a circular map of the 4.2-kb plasmid pPH843 (Figure 14). The ampicillin-resistance (Ap^R) gene (or *bla*) region and the possible origin of replication (*ori*V) were identified in subsequent cloning experiments. All *Tha*I, *Dra*I and *Hinf*I sites were shown to be grouped together in the same region, at or near the putative *bla* gene. The segment of the map opposite to the Ap^R gene appeared to contain all the *Taq*I sites along with the unique sites for *Pst*I and *Ava*I. The unique sites located for enzymes *Bsp*1286I and *Apa*LI were very close to each other and positioned near to the Ap^R gene. The positions of the two *Sca*I sites were located on

FIGURE 13A. **AGAROSE GEL (1.8%) SHOWING DNA FRAGMENTS OF PLASMID pPH843 PRODUCED BY SINGLE OR DOUBLE DIGESTION WITH RESTRICTION ENZYME(S).**

Key to figure:

M = A mixture of φX174 RF DNA/*Hae*III fragments and λDNA/*Hind*III fragments used as molecular size standards.

pPH843 DNA fragment(s) produced by

1 = *Pst*I (or *Bsp*1286I or *Apa*LI or *Ava*I).

2 = *Pst*I + *Bsp*1286I.

3 = *Pst*I + *Dra*I.

4 = *Bsp*1286I + *Dra*I.

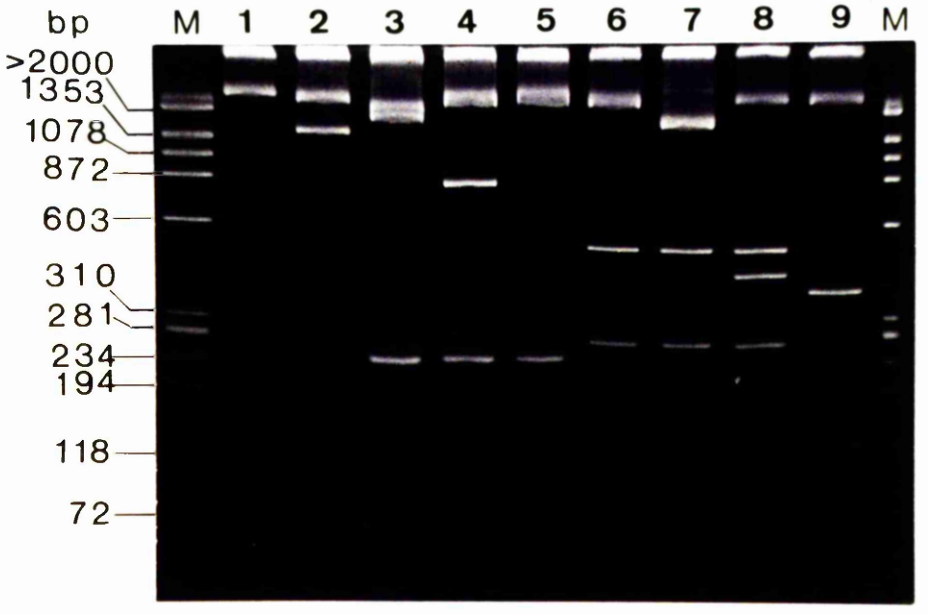
5 = *Dra*I.

6 = *Tha*I.

7 = *Pst*I + *Tha*I.

8 = *Bsp*1286I + *Tha*I.

9 = *Dra*I + *Tha*I.



A

FIGURE 13B. **AGAROSE GEL (1.8%) SHOWING DNA FRAGMENTS OF PLASMID pPH843 PRODUCED BY SINGLE OR DOUBLE DIGESTION WITH RESTRICTION ENZYME(S).**

Key to figure:

M = A mixture of φX174 RF DNA/*Hae*III fragments and λDNA/*Hind*III fragments used as molecular size standards (for fragment sizes, see Fig. 13A).

pPH843 DNA fragments produced by

10 = *Taq*I.

11 = *Taq*I + *Pst*I.

12 = *Taq*I + *Bsp*1286I.

13 = *Taq*I + *Dra*I.

14 = *Taq*I + *Tha*I.

A common faint band seen in all lanes represents an artefact and the uppermost bands in lane 14 represent a portion of either uncut or partially cut pPH843 DNA molecules.

B

FIGURE 13C. **AGAROSE GEL (1.8%) SHOWING DNA FRAGMENTS OF PLASMID pPH843 PRODUCED BY SINGLE OR DOUBLE DIGESTION WITH RESTRICTION ENZYME(S).**

Key to figure:

M = 1 kb DNA ladder used as molecular size standard.

pPH843 DNA fragments produced by

15 = *Apa*LI + *Pst*I.

16 = *Apa*LI + *Bsp*1286I.

17 = *Apa*LI + *Dra*I.

18 = *Ava*I + *Pst*I.

19 = *Ava*I + *Bsp*1286I.

20 = *Ava*I + *Dra*I.

21 = *Hinf*I.

22 = *Hinf*I + *Pst*I.

23 = *Hinf*I + *Bsp*1286I.

24 = *Hinf*I + *Dra*I.

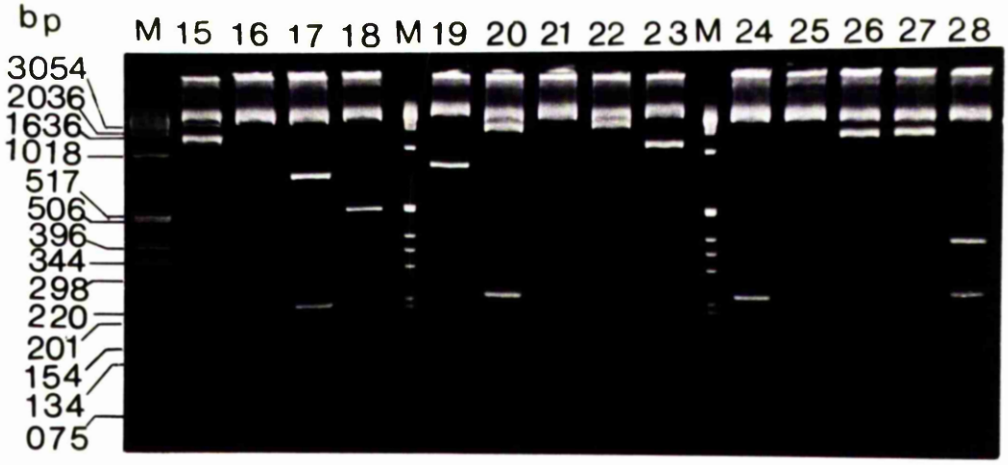
25 = *Sca*I.

26 = *Sca*I + *Pst*I.

27 = *Sca*I + *Bsp*1286I.

28 = *Sca*I + *Dra*I.

The faint bands in lanes 15 and 16 represent the products of partial digestion.


bp
M 15 16 17 18 M 19 20 21 22 23 M 24 25 26 27 28
3054
2036
1636
1018
517
506
396
344
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220
201
154
134
075

C

TABLE 21. RESTRICTION FRAGMENTS OF PLASMID pPH843 DNA GENERATED BY DOUBLE DIGESTION WITH RESTRICTION ENZYMES

Restriction enzymes	No. of fragments	Size of fragments (kb)*
*Pst*I + *Bsp*1286I	2	2.8, 1.4
*Pst*I + *Dra*I	4	2.25, 1.65, 0.24, 0.06
*Bsp*1286I + *Dra*I	4	3.05, 0.85, 0.24, 0.06
*Pst*I + *Tha*I	4	(1.85, 1.55), 0.53, 0.27
*Bsp*1286I + *Tha*I	4	2.95, 0.53, 0.45, 0.27
*Dra*I + *Tha*I	6	3.4, 0.4, 0.13, 0.11, 0.1, 0.06
*Taq*I + *Pst*I	6	2.56, 0.75, 0.29, 0.25, 0.23, 0.12
*Taq*I + *Bsp*1286I	6	1.91, 1.0, 0.65, 0.29, 0.23, 0.12
*Taq*I + *Dra*I	8	1.5, 1.0, 0.76, 0.29, (0.24, 0.23), 0.12, 0.06
*Taq*I + *Tha*I	8	1.1, 1.0, 0.66, 0.53, 0.29, 0.27, 0.23, 0.12
*Apa*LI + *Pst*I	2	2.75, 1.45
*Apa*LI + *Bsp*1286I	2†	4.15, 0.05†
*Apa*LI + *Dra*I	4	3.1, 0.8, 0.24, 0.06
*Ava*I + *Pst*I	2	3.65, 0.55

Continued on next page

TABLE 21 (Continued)

Restriction enzymes	No. of fragments	Size of fragments (kb)*
*Ava*I + *Bsp*1286I	2	3.35, 0.85
*Ava*I + *Dra*I	4	2.2, 1.7, 0.24, 0.06
*Hinf*I + *Pst*I	3	2.55, 1.59, 0.06
*Hinf*I + *Bsp*1286I	3	2.99, 1.15, 0.06
*Hinf*I + *Dra*I	5§	3.82, 0.24, (0.06, 0.06)
*Sca*I + *Pst*I	3	2.85, 1.29, 0.06
*Sca*I + *Bsp*1286I	3	2.69, 1.45, 0.06
*Sca*I + *Dra*I	5	3.54, 0.3, 0.24, (0.06, 0.06)

* Restriction fragments shown in parentheses comigrated on a 1.8% (w/v) agarose gel (Figs. 13A, 13B, 13C).

† A band of 0.05 kb was invisible on the 1.8% (w/v) agarose gel shown in Fig. 13C (lane 16), but was visible when the digest was deliberately overloaded.

§ This digest should theoretically yield 5 fragments, but 4 fragments were detected on the gel, indicating that one small fragment of about 20 bp was missing.

FIGURE 14. **A RESTRICTION MAP OF *P. HAEMOLYTICA* PLASMID pPH843.**

Key:

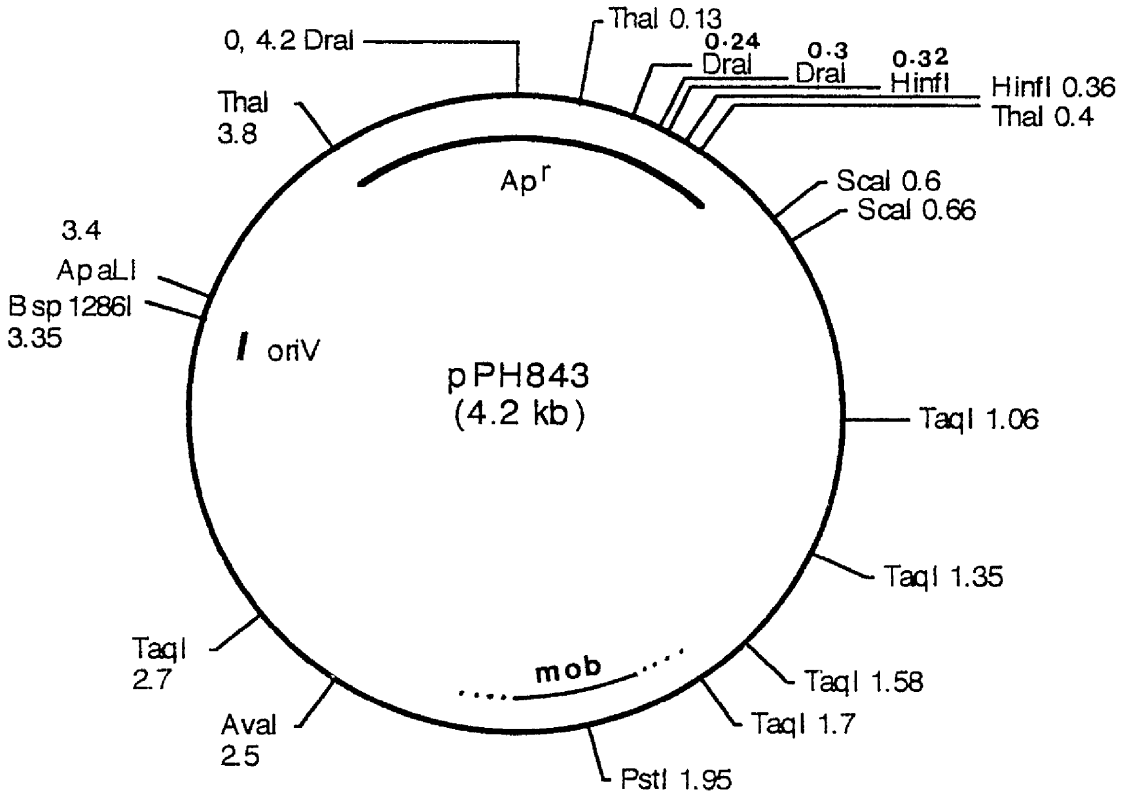
Ap^r = Ampicillin-resistance gene.

oriV = Origin of vegetative replication.

mob = Transfer mobility gene.

The circular map was drawn to scale (in kb) by using the MacVector® software (Apple Computer Inc., CA). One of the *Dra*I sites was used as the zero reference point. Data indicate that all *Dra*I, *Tha*I and *Hinf*I sites are located within the Ap^r gene and the *Bsp*1286I site within *oriV* (see Sections 3.15.3 and 3.15.4).

The *Pst*I site is assumed to be located within the *mob* region (see Sections 3.15.2 and 3.15.5).


0, 4.2 DraI
ThaI 0.13
0·24
DraI
0·3
DraI
0·32
HinfI
HinfI 0.36
ThaI 0.4
ThaI
3.8
Ap r
ScaI 0.6
ScaI 0.66
3.4
ApaLI
Bsp 1286I
3.35
oriV
pPH843
(4.2 kb)
TaqI 1.06
TaqI 1.35
TaqI
2.7
mob
TaqI 1.58
TaqI 1.7
AvaI
2.5
PstI 1.95

the other side of of the Ap^R gene, as determined by the deletion experiments (Section 3.15.2).

3.15. Construction of Shuttle Cloning Vectors for *P. haemolytica*.

For the development of a shuttle vector for *P. haemolytica*, plasmid pPH843 was chosen because of its greater stability and higher capacity for amplification in *E. coli* as well as its higher mobilization frequency into *P. haemolytica*. From the restriction map (Fig. 14), a strategy was devised to convert the plasmid into a series of cloning vectors.

E. coli vector pIC20H (Marsh *et al.*, 1984), derived from pUC19, was used as a source of the α-peptide coding region (*lacZ'*) of the β-galactosidase-encoding gene and the multiple cloning site (MCS). Insertion of the *lacZ'*+MCS fragment into a small sized plasmid molecule makes it an ideal cloning vector, by providing a large number of unique restriction sites and the property of recombinant selection through histochemical screening (see Introduction: Section 1.4.4). pIC20H was preferred to the pUC19 or pUC18 vectors because of its greater range of unique cloning sites. A restriction map of pIC20H is shown in Figure 15. Eighteen different restriction enzyme sites are available in the MCS. All enzymes have unique restriction sites except *Hind*III, *Xba*I and *Sac*I which have two sites each. New restriction enzyme sites added by this portion of the pIC20H vector (compared to the pUC vectors) were: *Cla*I, *Eco*RV, *Bgl*II, *Xho*I and *Nru*I.

There were no restriction enzyme sites present on both pPH843 and pIC20H which were identical to or compatible with each other and which could have been used to cut out a fragment from pIC20H containing *lacZ'*

FIGURE 15. **A RESTRICTION MAP OF *E. COLI* PLASMID VECTOR pIC20H.**

The circular structure of the map (in bp) was adapted from that of the pUC18/pUC19 vector (Sambrook *et al.*, 1989) and the positions of the restriction enzyme sites were worked out from the computer-analyzed restriction data (GMBA08, pIC20H, Mapsort 1, 1991).

The restriction sites for the enzymes *Dra*I, *Hae*II, *Apa*LI and *Acy*I (each has 3 cleavage sites), used to cut pIC20H for cloning purposes in the present study, are shown in large print on the map.

One of the *Hae*II sites was taken as the starting or ending reference point (0 or 2722 position).

Key:

amp^r = Ampicillin-resistance gene (positions 988-1766).

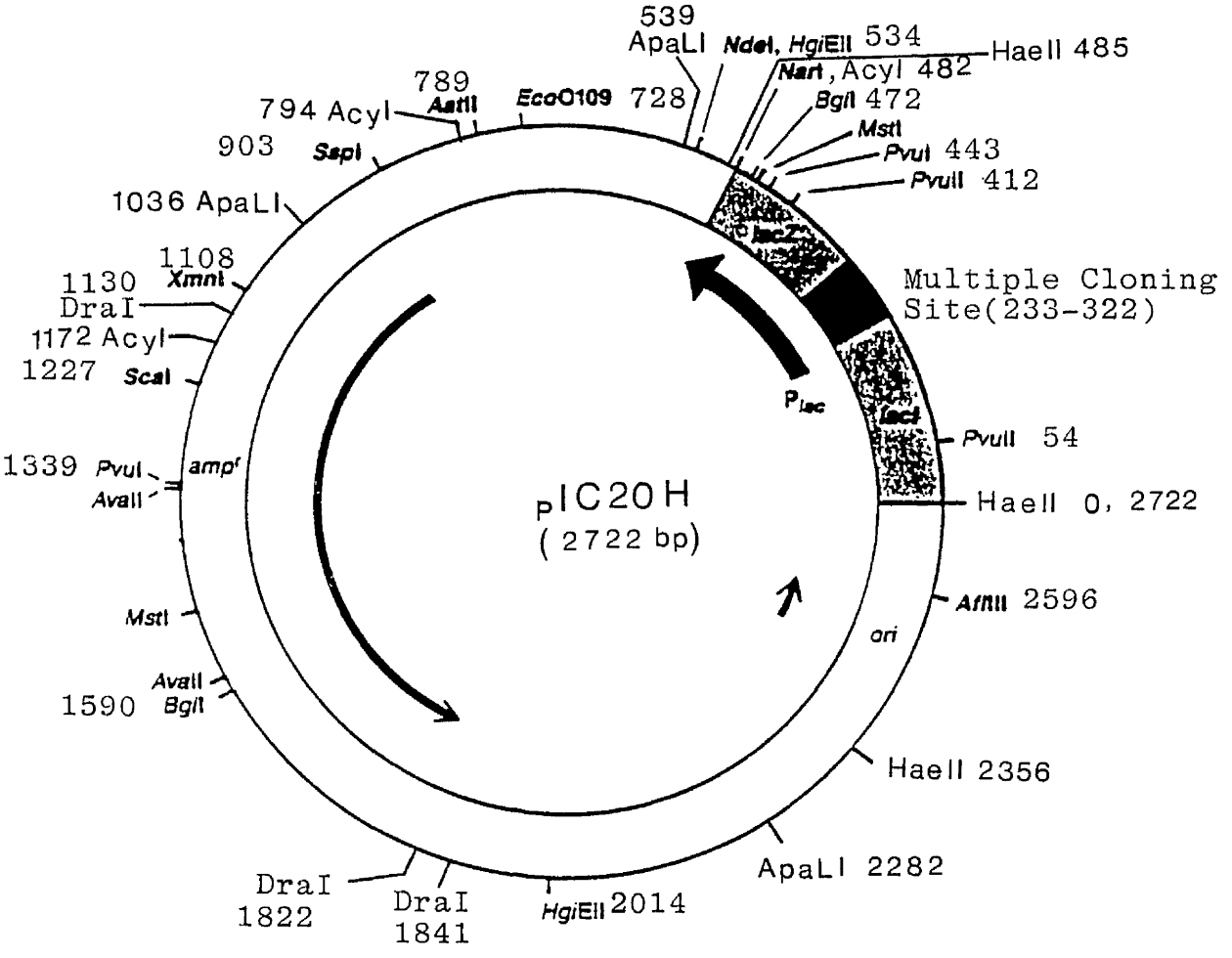
ori = Origin of replication.

P_{lac} = *lac* promoter.

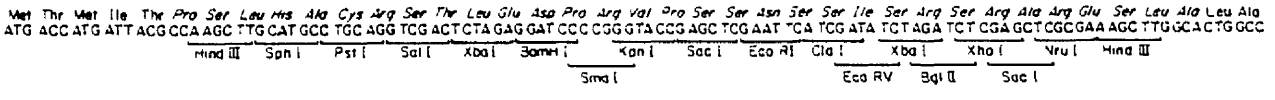
*lac*Z' = A part of the β-galactosidase-encoding gene of the *lac* operon.

*lac*I = Regulatory gene for the *lac* operon.

Arrows (→) indicate the direction of transcription.



Multiple cloning site:



and MCS for direct cloning into the corresponding sites on pPH843. Therefore, different cloning strategies were adopted to overcome this problem.

3.15.1. Cloning strategy using the large *Dra*I fragments from both pPH843 and pIC20H.

In a preliminary attempt to clone *lacZ'* and MCS (plus *lacI*) from pIC20H into the *P. haemolytica* plasmid pPH843, a 2011-bp *Dra*I fragment lacking most of the Ap^R gene from pIC20H (positions 1130-1822) (Fig. 15) was mixed with the 3.9-kb *Dra*I fragment from pPH843 (Fig. 14) and subjected to blunt-end ligation in the presence of bacteriophage T4 DNA ligase (Materials and Methods: Section 2.17.5). After overnight incubation at 16°C, the ligation mixture was transformed into *E. coli* JM83 and selection made for blue colonies on LB+Ap+X-gal agar. No transformants were obtained. Agarose gel electrophoresis of the ligated products revealed that the DNA fragments had formed oligomers to some extent (Figure 16A). The lack of transformants on the selective plate suggested that the 3.9-kb *Dra*I fragment of pPH843 is devoid of essential gene functions (perhaps Ap^R or *oriV*).

The ligation experiment was repeated following dephosphorylation of either one or other of the DNA fragments with calf intestinal phosphatase (CIP) to prevent self-ligation and to encourage recombination. The same results were achieved.

In a further experiment, the 3.9-kb *Dra*I fragment was recircularized by T4 DNA ligase and transformed into *E. coli*. Production of no transformants supported the idea that this fragment lacked at least one of the genes essential for growth on selective media.

FIGURE 16. **AGAROSE GEL (0.8% W/V) ELECTROPHORESIS OF LIGATION MIXTURE SHOWING APPARENT RECOMBINATION BETWEEN THE VECTOR AND INSERT FRAGMENTS OR SELF-LIGATION OF EITHER THE VECTOR OR THE INSERT RESULTING IN OLIGOMER FORMATION.**

Key:

A: Ligation between the 3.9-kb pPH843/*Dra*I and 2-kb pIC20H/*Dra*I fragments

M = Supercoiled DNA ladder as size marker.

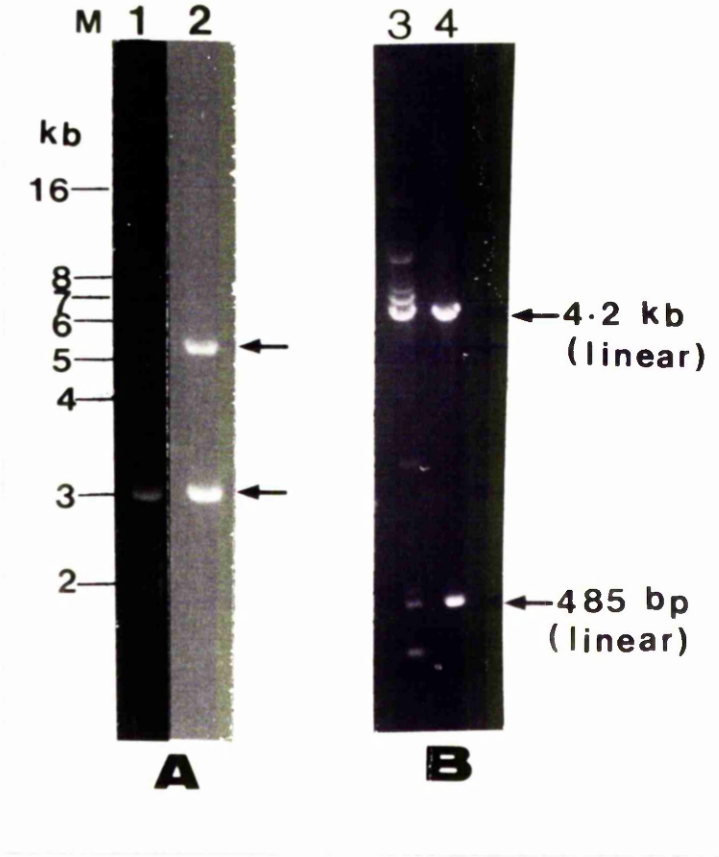
1 = Mixture of ligated (circular monomer or oligomer) and unligated (linear; arrowed) DNA products.

2 = Control mixture of linearized vector (3.9 kb) and insert (2.0 kb) DNAs (both arrowed) without T4 DNA ligase.

B: Ligation between the 4.2-kb pPH843/*Bsp*1286I and 485-bp pIC20H/*Hae*II fragments

3 = Mixture of ligated (circular recombinant or oligomer) and unligated (linear; arrowed) DNA products.

4 = Control mixture of linearized vector (4.2 kb) and insert (485 bp) DNAs (both arrowed) without T4 DNA ligase.


M 1 2
kb
16
8
7
6
5
4
3
2
A
3 4
4·2 kb
(linear)
485 bp
(linear)
B

3.15.2. Recircularization of the large fragments of pPH843 produced by endonuclease enzymes *Hinf*I, *Sca*I, *Tha*I, *Cfo*I, *Taq*I, *Sau*3AI and *Alu*I and their propagation in *E. coli* on Ap-containing media.

In the light of the previous experiments (preceeding section), it was felt necessary to recircularize the large fragments of pPH843 generated by *Hinf*I (4.14 kb), *Sca*I (4.14 kb), *Tha*I (3.4 kb), *Cfo*I (3.33 kb), *Taq*I (2.56 kb), *Sau*3AI (1.84 kb) *and Alu*I (1.7 kb) in order to check if they contained sufficient genetic material for propagation in the presence of Ap. Each of these fragments was religated and transformed into *E. coli* and selection made for Ap^R clones to see whether these deleted plasmid molecules were able to replicate and propagate in *E. coli*. Since it was very unlikely that the smaller fragments produced by these enzymes contained both the *ori*V and the Ap^R genes, only the largest fragments were selected.

Ap^R transformants were obtained only with the religated 4.14-kb *Sca*I fragment and the 2.56-kb *Taq*I fragment of pPH843. Plasmid DNA analysis of the transformants showed the presence of plasmid DNA of the predicted size. The other fragments did not yield detectable transformants, suggesting that one or more of the cleavage sites of each of the enzymes *Hinf*I, *Tha*I, *Cfo*I, *Sau*3AI and *Alu*I interferred with an essential gene function, depriving those large fragments of either *ori*V or Ap^R or both. On the other hand, the 4.14-kb *Sca*I and the 2.56-kb *Taq*I fragments apparently contained both *ori*V and Ap^R, and were thus considered suitable as the basis for a cloning vector. These two functional, but deleted, derivatives of pPH843 were designated pPH843Δ*Sca*I and pPH843Δ*Taq*I respectively. However, the plasmid pPH843Δ*Taq*I could not be mobilized with the helper plasmid pRK2013 from *E. coli* JM83 (Sm^R) to *P. haemolytica* A2N (Nal^R). This indicated that

pPH843Δ*Taq*I had lost the native mobilization (*mob*) function site or origin of transfer replication (*ori*T) and could be used as a *mob*$^-$ control in a conjugation experiment.

As seen in the pPH843 restriction map (Fig. 14), the unique sites for *Pst*I and *Ava*I lie outside the 2.56-kb *Taq*I fragment. Therefore, these two unique sites and all the *Taq*I sites appeared to be located in the remaining 1.64-kb region (positions 1.06-2.7) which was non-essential for propagation of pPH843 in the presence of Ap and might therefore be suitable for insertion of other determinants. However, this region probably contained the *mob* site. The unique sites for *Bsp*1286I and *Apa*LI were located inside the 2.56-kb *Taq*I fragment.

3.15.3. Identification of the ROB-1 β-lactamase (Ap^R) gene in plasmid pPH843.

Since pPH843 had proved to be a ROB-1 β-lactamase plasmid, the location of the β-lactamase gene was identified on the basis of recent reports on ROB-1 β-lactamase plasmids from *H. influenzae* and *P. haemolytica*, and from the findings of the deletion experiments in this study.

The ROB-1 β-lactamase gene from *H. influenzae* and *P. haemolytica* was shown to be cleaved by *Sau*3AI at 4 sites (Levesque *et al.*, 1987), by *Hin*fI at 2 sites (Livrelli *et al.*, 1991), by *Dra*I at 3 sites and by *Alu*I at a single site (Juteau & Levesque, 1990; Livrelli *et al.*, 1991). The ROB-1 β-lactamase gene from *P. haemolytica* has been sequenced recently and an open reading frame (ORF) of 915 bp was detected (Livrelli *et al.*, 1991).

The findings in the present study, as described in the preceeding section (3.15.2), were consistent with these reports. The enzymes *Dra*I,

*Tha*I and *Hinf*I (and also *Sau*3AI and *Alu*I at an unknown number of sites, not shown on the pPH843 map) appeared to cleave and thereby inactivate the β-lactamase gene of plasmid pPH843. Thus, the ROB-1 β-lactamase gene seemed to span a region of about 0.9 kb, the only region of the plasmid containing these restriction sites, as shown in Fig. 14.

3.15.4. Detection of the *ori*V region of plasmid pPH843.

Since the unique sites for *Bsp*1286I and *Apa*LI are located inside the essential region of pPH843 but outside the β-lactamase gene, they were tested for the presence of *ori*V. pPH843 DNA was cleaved by either *Bsp*1286I or *Apa*LI giving a 4.2-kb linear fragment with 3'-protruding or 3'-recessed ends respectively. The fragments were made blunt-ended by removing ("trimming") the protruding ends with bacteriophage T4 DNA polymerase or by "filling in" the recessed ends with Klenow fragment of *E. coli* DNA polymerase I in the presence of dNTPs (Materials and Methods: Section 2.17.4), and then religated with T4 DNA ligase. Transformation of *E. coli* with the religated products yielded transformant clones with the *Apa*LI fragment but not with the *Bsp*1286I fragment. This suggested that the *Bsp*1286I site was intragenic (*ori*V) and that cleavage at this site disrupted the functions of *ori*V by shifting its reading frame due to the trimming reaction. This was further confirmed, when the 485-bp *Hae*II fragment from pIC20H (containing *lac*Z'+MCS+*lac*I) was cloned into the *Bsp*1286I site of pPH843. Both the vector and the insert fragments were treated with T4 DNA polymerase in the presence of dNTPs to remove their 3'-protruding termini and were then ligated. Agarose gel electrophoresis of the ligated DNA products showed that ligation between the fragments took

place apparently accompanied by self-ligation of the individual fragment (Fig. 16B). Transformation results demonstrated that neither the recombinant nor the circularized vector and insert fragments produced detectable transformant clones (blue or white). This suggested that, since the 485-bp *Hae*II fragment did not contain *ori*V of pIC20H (Fig. 15), the vector pPH843/*Bsp*1286I(Δ3') fragment must have lost its *ori*V. Since the *Bsp*1286I and *Apa*LI sites are very close to each other, the *ori*V gene presumably spanned the *Bsp*1286I site, but was directed away from the *Apa*LI site (Fig. 14).

Taken together, these data indicated that three restriction enzyme sites on pPH843 might be suitable for cloning: the *Pst*I site for cloning in the 485-bp *Hae*II fragment of pIC20H, the *Apa*LI site for cloning in the 979-bp *Apa*LI fragment, and one of the *Taq*I sites for cloning in the 2032-bp *Acy*I fragment (*Taq*I and *Acy*I produce compatible cohesive ends) (Figs. 14 and 15).

3.15.5. Construction of vector pAKA15 and its *mob*-containing derivative pAKA15-1.

A schematic diagram showing the cloning strategies for the construction of pAKA15 and its *mob*$^+$-derivative pAKA15-1 is shown in Figure 17. The 3'-protruding ends of both the 4.2-kb *Pst*I fragment of pPH843 and the 485-bp *Hae*II fragment of pIC20H were removed by T4 DNA polymerase in the presence of dNTPs and the fragments subjected to blunt-end ligation. The ligated products were transformed into *E. coli* JM83 and selection was made for blue recombinant clones on LB+Ap+X-gal agar. Only 5 blue colonies were obtained against the background of a large number of white colonies. Plasmid DNA

FIGURE 17. **SCHEMATIC DIAGRAM SHOWING THE CONSTRUCTION OF VECTOR pAKA15 AND ITS DERIVATIVE pAKA15-1.**

Key:

Ap^r = Ampicillin-resistance gene.

Km^r = Kanamycin-resistance gene.

*lac*Z' = The α-peptide-encoding gene sequence of β-galactosidase.

*lac*I = Regulatory gene for β-galactosidase.

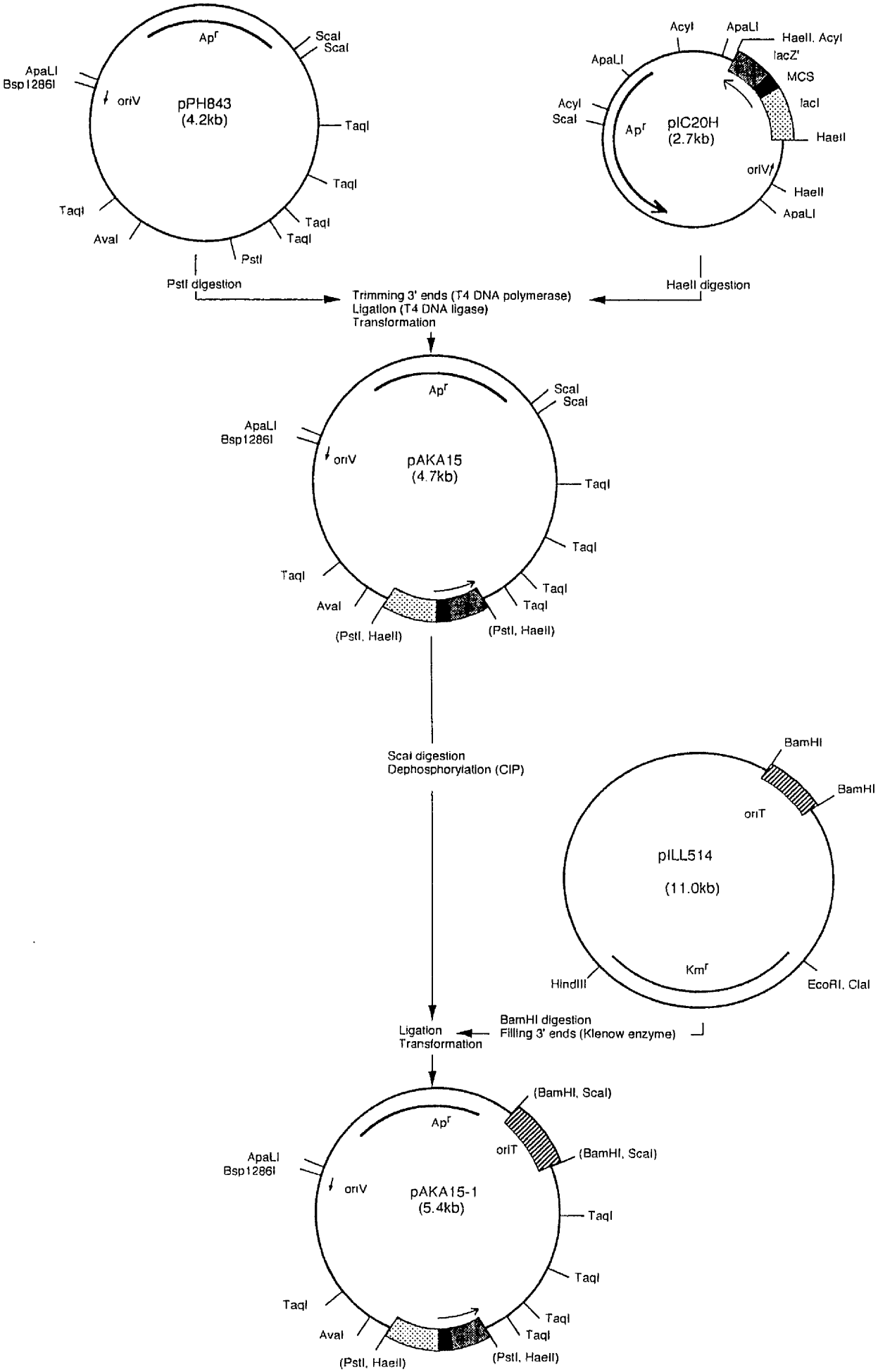
MCS = Multiple cloning site.

*ori*V = Origin of vegetative replication.

*ori*T = Origin of transfer replication.

CIP = Calf intestinal phosphatase.

The plasmids are not drawn to scale. Arrows indicate the direction of transcription. Restriction sites shown in brackets were destroyed by DNA polymerase during subcloning.


Ap^r
ScaI
ScaI
ApaLI
Bsp1286I
oriV
pPH843
(4.2kb)
TaqI
TaqI
TaqI
TaqI
TaqI
AvaI
PstI
PstI digestion
AcyI
ApaLI
HaeII, AcyI
lacZ'
MCS
lacI
ApaLI
AcyI
ScaI
Ap^r
pIC20H
(2.7kb)
HaeII
oriV
HaeII
ApaLI
HaeII digestion
Trimming 3' ends (T4 DNA polymerase)
Ligation (T4 DNA ligase)
Transformation
Ap^r
ScaI
ScaI
ApaLI
Bsp1286I
oriV
pAKA15
(4.7kb)
TaqI
TaqI
TaqI
TaqI
TaqI
AvaI
(PstI, HaeII)
(PstI, HaeII)
ScaI digestion
Dephosphorylation (CIP)
BamHI
BamHI
oriT
pILL514
(11.0kb)
Km^r
HindIII
EcoRI, ClaI
Ligation
Transformation
BamHI digestion
Filling 3' ends (Klenow enzyme)
(BamHI, ScaI)
Ap^r
oriT
(BamHI, ScaI)
ApaLI
Bsp1286I
oriV
pAKA15-1
(5.4kb)
TaqI
TaqI
TaqI
TaqI
TaqI
AvaI
(PstI, HaeII)
(PstI, HaeII)

analysis of blue colonies revealed a recombinant plasmid construct (~4.7 kb), which was designated pAKA15 (Figure 18A). The construct was confirmed by the predicted fragment sizes obtained by restriction enzyme analysis of the plasmid (Figs. 18B; 18D, lane 1). Since, there is no restriction site for *Bgl*I or *Bam*HI in pPH843, cleavage of this plasmid by *Sca*I in combination with either of these enzymes will produce one large fragment (4.14 kb) plus a minor fragment (0.06 kb) (as seen on 1.8% gel in Fig. 11A, lane 5). However, cleavage of pAKA15 by the same combination of enzymes will give rise to two larger fragments (3.2 kb & 1.4 kb for *Bgl*I as seen in Fig. 18B, lane 1; and 3.1 kb & 1.5 kb for *Bam*HI as in Fig. 18D, lane 1) plus the minor *Sca*I fragment (0.06 kb, not visible on 0.8% gel), as the insert of this construct contains unique sites for *Bgl*I and *Bam*HI. The *Pst*I and the *Hae*II sites were destroyed by DNA polymerase during the trimming reaction at the ligation points (Fig. 17). A similar restriction pattern will thus be obtained with *Pst*I when used in conjunction with *Sca*I (Fig. 18B, lane 2) because of the presence of unique *Pst*I site in the insert. The entire 485-bp insert could not be recovered from this construct by using *Hae*II, whereas a major portion (358 bp) of the insert was recovered by using *Pvu*II (Figure 19, lanes 1a and 1b) which cuts twice (at positions 54 and 412) within the 485-bp *Hae*II fragment (see Fig. 15).

A preliminary attempt to mobilize pAKA15 into *P. haemolytica* by conjugation using a triparental cross with the helper plasmid pRK2013 was unsuccessful. This indicated that cloning the insert into the *Pst*I site of pPH843 may have disrupted the *mob* functions of this plasmid. Therefore, an attempt was made to subclone the *mob* site (i.e. *ori*T) from a broad-host-range plasmid into the *Sca*I site of pAKA15 (Fig. 17), a region previously shown not to affect pPH843 functions (Section 3.15.2). The large *Sca*I

FIGURE 18. AGAROSE GELS (0.8%) SHOWING THE ANALYSIS OF MINIPREP PLASMID DNA FROM RECOMBINANT CLONES CONTAINING THE CONSTRUCTS pAKA15 AND pAKA15-1 RESPECTIVELY.

Key:

A: Construct pAKA15 from recombinant clones

M = Supercoiled DNA ladder as size marker.

O = Original plasmid pPH843 (control).

1-5 = Plasmid DNA from recombinant blue clones.

B: Confirmation of pAKA15 by restriction analysis

M = λDNA/*Hind*III fragments as size markers.

1 = pAKA15 digested by *Bgl*I + *Sca*I.

2 = pAKA15 digested by *Pst*I + *Sca*I.

C: Construct pAKA15-1 from recombinant clones

M = Supercoiled DNA ladder as size marker.

O = pAKA15 (control).

1-8 = Plasmid DNA from recombinant clones.

* (No. 7) indicates a recombinant plasmid of predicted size.

D: Confirmation of pAKA15-1 by restriction analysis

M = λDNA/*Hind*III fragments as size markers.

1 = pAKA15 digested by *Bam*HI + *Sca*I (control).

2 = pAKA15-1 digested by *Bam*HI + *Sca*I.

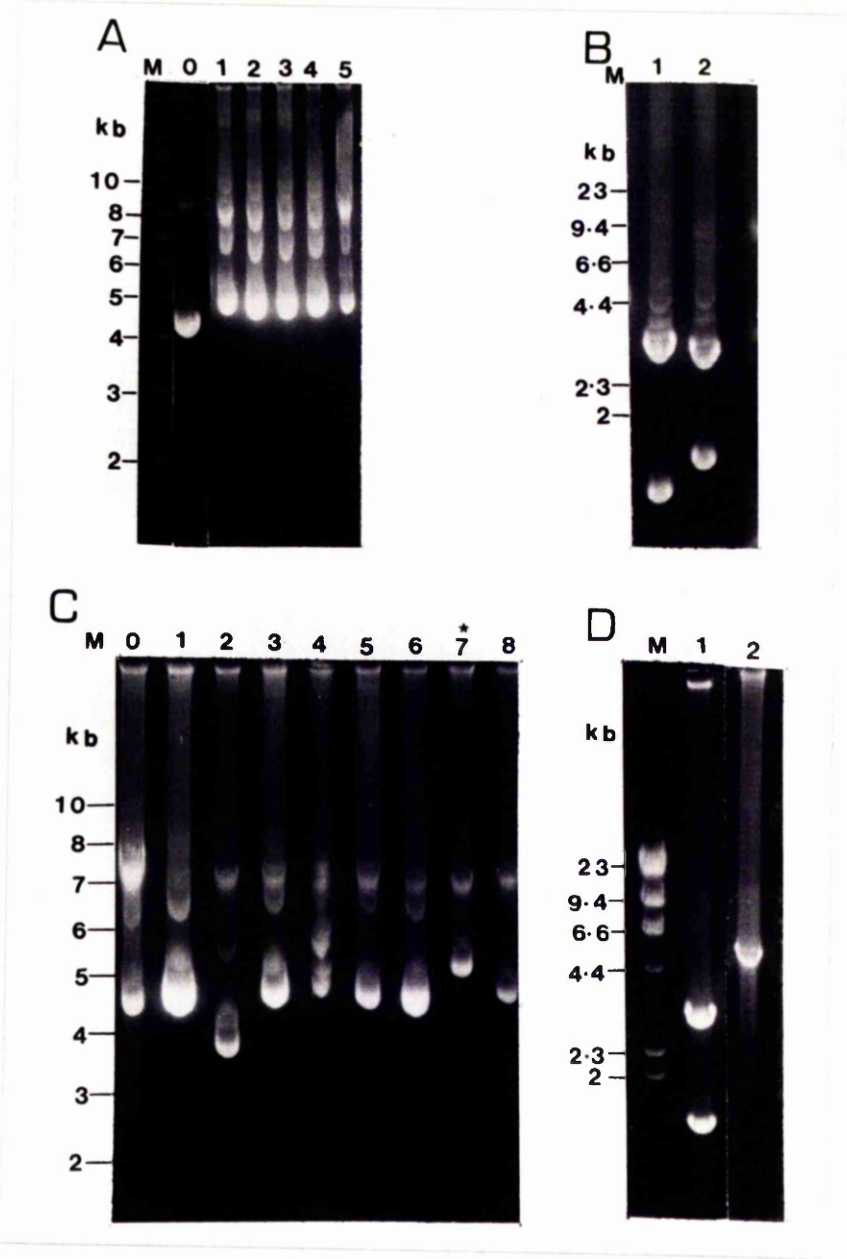

A
M 0 1 2 3 4 5
kb
10
8
7
6
5
4
3
2
B
M 1 2
kb
23
9·4
6·6
4·4
2·3
2
C
M 0 1 2 3 4 5 6 7* 8
kb
10
8
7
6
5
4
3
2
D
M 1 2
kb
23
9·4
6·6
4·4
2·3
2

FIGURE 19. **AGAROSE GEL (1.2%) SHOWING THE PARTIAL OR COMPLETE RECOVERY OF THE INSERTS FROM THE CONSTRUCTED CLONING VECTORS pAKA15, pAKA16 AND pAKA17.**

Key to figure:

1a = pAKA15 digested by *Hae*II.

1b = pAKA15 digested by *Pvu*II.

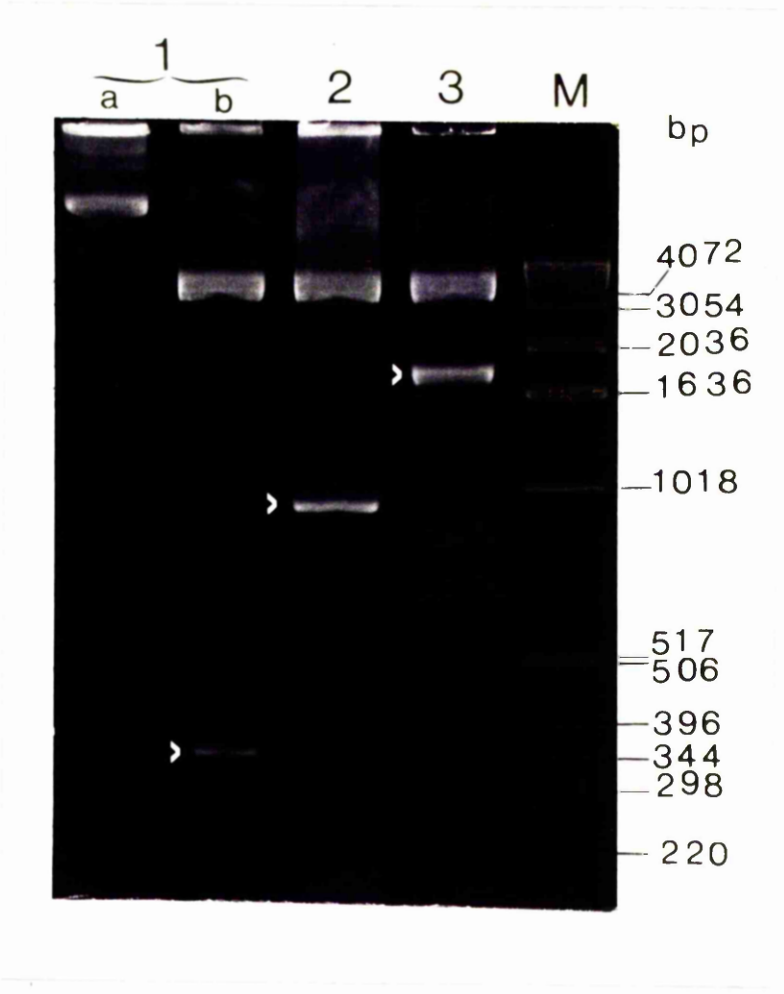
2 = pAKA16 digested by *Apa*LI.

3 = pAKA17 digested by *Pvu*I.

M = 1 kb DNA ladder used as molecular size standard.

The upper bands (lane 1a) probably represent concatemers of the undigested pAKA15 molecule.

The small white arrow (›) shows either the whole insert (lane 2: 979 bp) or a part of the insert (lane 1b: 358 bp; lane 3: 1826 bp) of the constructs.


1
a
b
2
3
M
bp
4072
3054
2036
1636
1018
517
506
396
344
298
220

fragment of pAKA15 (~4.6 kb, blunt-ended) was dephosphorylated with CIP to prevent recircularization. A 760-bp fragment containing the *ori*T of RK2 (Guiney & Yacobson, 1983) was isolated from pILL514 (see Methods and Materials: Section 2.4) by *Bam*HI digestion and the 3'-recessed ends of this fragment were filled in by Klenow polymerase in the presence of dNTPs to make them blunt-ended. After ligation of the ~4.6-kb *Sca*I fragment and the modified 760-bp *Bam*HI fragment, *E. coli* XL-1 blue (Table 6) was transformed with the ligated products yielding one dozen of blue colonies on an LB+Ap+X-gal agar plate. This *E. coli* strain was chosen for its proposed better transformation and for comparison with the routinely-used *E. coli* strain JM83. Plasmid DNA analysis of blue colonies showed the plasmid DNA molecules of different sizes and included the desired recombinant plasmid (Fig. 18C). This recombinant was digested with a combination of *Bam*HI and *Sca*I, giving a single fragment (Fig. 18D). It confirmed that both the *Bam*HI and the *Sca*I sites were lost due to the "filling in" reaction at the insertion points and that the recombinant was cleaved only at the unique *Bam*HI site present in the MCS. The *mob*$^+$-derivative of pAKA15 was designated pAKA15-1.

Conjugation experiments to mobilize pAKA15-1 from *E. coli* into *P. haemolytica*, using the routine triparental procedure with pRK2013, yielded a large number of transconjugant colonies, but these did not grow following further subculture on selective media.

3.15.6. Construction of vector pAKA16 and its *mob*-containing derivative pAKA16-1.

A schematic diagram showing the cloning strategies for the construction of pAKA16 and its *mob*$^+$-derivative pAKA16-1 is shown in Figure 20. The 4.2-kb *Apa*LI fragment of pPH843 and the 979-bp *Apa*LI fragment from pIC20H containing the *lac*Z' and MCS determinants (Fig. 15) were subjected to cohesive-end ligation using T4 DNA ligase. Transformation of *E. coli* JM83 with the ligated products produced a large number of blue transformant clones on LB+Ap+X-gal agar. A random selection of 20 blue clones were analyzed for plasmid DNA, and almost all clones showed a common DNA band equivalent to that of the expected construct (~5.2 kb) (Figure 21A). The construct was designated pAKA16 and confirmed by the predicted sizes of DNA fragments obtained by restriction analysis (Fig. 21C, lane 1). The 979-bp insert was easily recovered from pAKA16 by using *Apa*LI (Fig. 19, lane 2).

An attempt was again made to insert the *ori*T of RK2 at the *Sca*I site of pAKA16 (Fig. 20). The ~5.1-kb *Sca*I fragment of pAKA16 was dephosphorylated and the 760-bp *Bam*HI fragment (containing the *ori*T of RK2) from pILL514 was treated with Klenow enzyme. Following ligation of the two fragments, transformatiomn of *E. coli* JM83 with the ligated products generated about 100 blue clones on LB+Ap+X-gal agar. Plasmid DNA analysis of 20 clones selected at random revealed the presence of recombinant of the expected size (~5.9 kb) in several clones (Fig. 21B). Such a *mob*$^+$-recombinant derivative of pAKA16 was designated pAKA16-1. Double digestion of pAKA16-1 with *Bam*HI and *Sca*I generated only one fragment (Fig. 21C, lane 2), again indicating that the restriction sites for these two enzymes were destroyed at the ligation points in the new derivative.

FIGURE 20. **SCHEMATIC DIAGRAM SHOWING THE CONSTRUCTION OF VECTOR pAKA16 AND ITS DERIVATIVE pAKA16-1.**

Key:

Ap^r = Ampicillin-resistance gene.

Km^r = Kanamycin-rsistance gene.

*lac*Z' = The α-peptide-encoding gene sequence of β-galactosidase.

*lac*I = Regulatory gene for β-galactosidase.

MCS = Multiple cloning site.

*ori*V = Origin of vegetative replication.

*ori*T = Origin of transfer replication.

CIP = Calf intestinal phosphatase.

The plasmids are not drawn to scale. Arrows indicate the direction of transcription. Restriction sites shown in brackets were destroyed by DNA polymerase during subcloning.

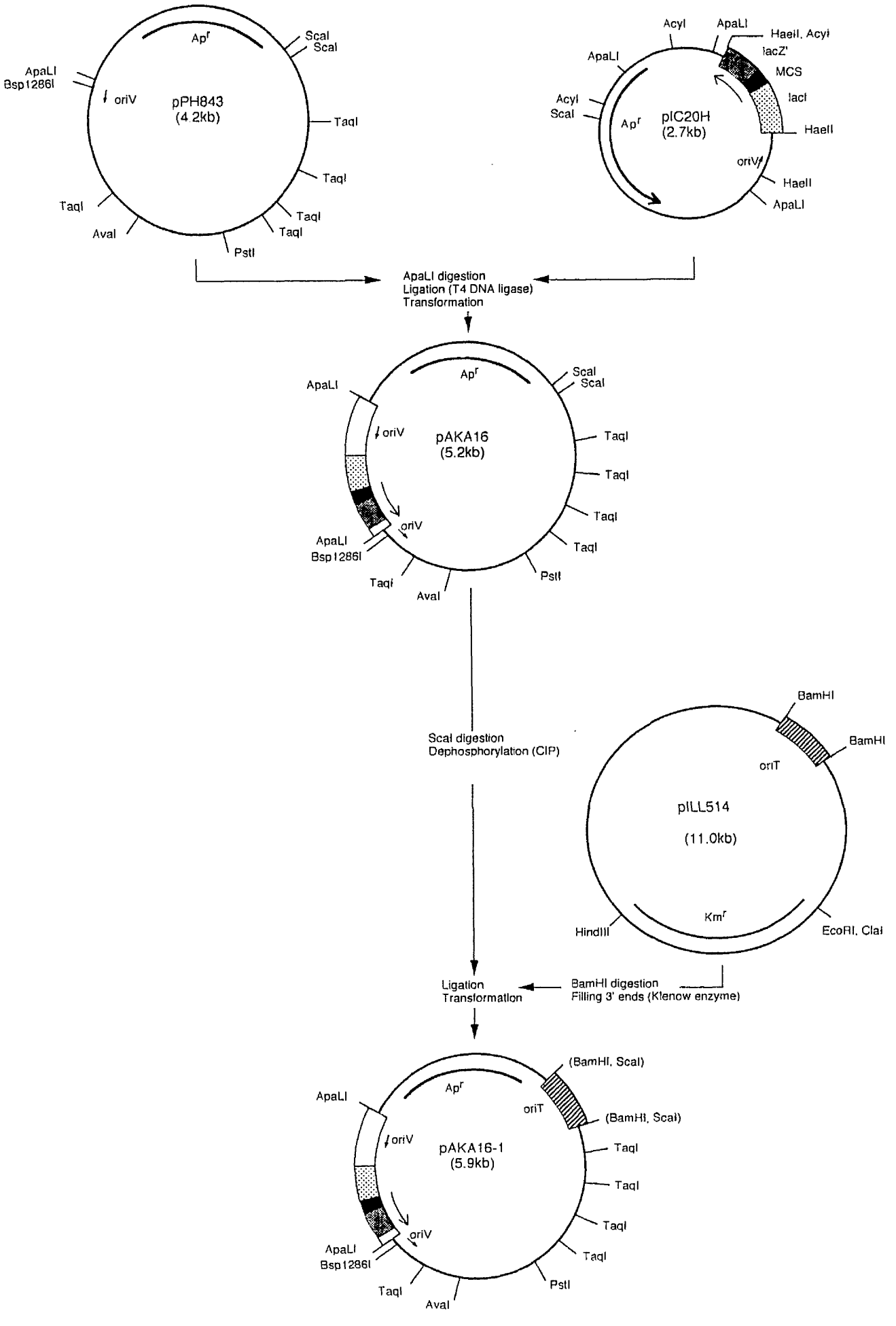

pPH843
(4.2kb)
Ap^r
ScaI
ScaI
ApaLI
Bsp1286I
oriV
TaqI
TaqI
TaqI
TaqI
AvaI
PstI
AcyI
ApaLI
HaeII, AcyI
lacZ'
MCS
lacI
HaeII
ApaLI
AcyI
ScaI
pIC20H
(2.7kb)
Ap^r
oriV
HaeII
ApaLI
ApaLI digestion
Ligation (T4 DNA ligase)
Transformation
pAKA16
(5.2kb)
Ap^r
ScaI
ScaI
ApaLI
oriV
TaqI
TaqI
TaqI
TaqI
PstI
oriV
ApaLI
Bsp1286I
TaqI
AvaI
ScaI digestion
Dephosphorylation (CIP)
pILL514
(11.0kb)
BamHI
BamHI
oriT
Km^r
HindIII
EcoRI, ClaI
Ligation
Transformation
BamHI digestion
Filling 3' ends (Klenow enzyme)
pAKA16-1
(5.9kb)
Ap^r
(BamHI, ScaI)
oriT
(BamHI, ScaI)
ApaLI
oriV
TaqI
TaqI
TaqI
TaqI
PstI
oriV
ApaLI
Bsp1286I
TaqI
AvaI

FIGURE 21. AGAROSE GELS (0.8%) SHOWING THE ANALYSIS OF MINIPREP PLASMID DNA FROM RECOMBINANT CLONES CONTAINING THE CONSTRUCTS pAKA16, pAKA16-1, AND pAKA17.

Key:

A: Construct pAKA16 from recombinant clones

M = Supercoiled DNA ladder as size marker.

O = Plasmid pPH843 (control).

1-19 = Plasmid DNA from recombinant blue clones (out of 20 analyzed).

B: Construct pAKA16-1 from recombinant clones

M = Supercoiled DNA ladder as size marker.

O = pAKA16 (control).

1-8 = Plasmid DNA from recombinant clones (out of 20 analyzed).

* (Nos. 5 & 8) indicate recombinant plasmids of predicted size.

C: Confirmation of pAKA16 and pAKA16-1 by restriction analysis

M = λDNA/*Hind*III fragments as size markers.

1 = pAKA16 digested by *Bam*I + *Sca*I.

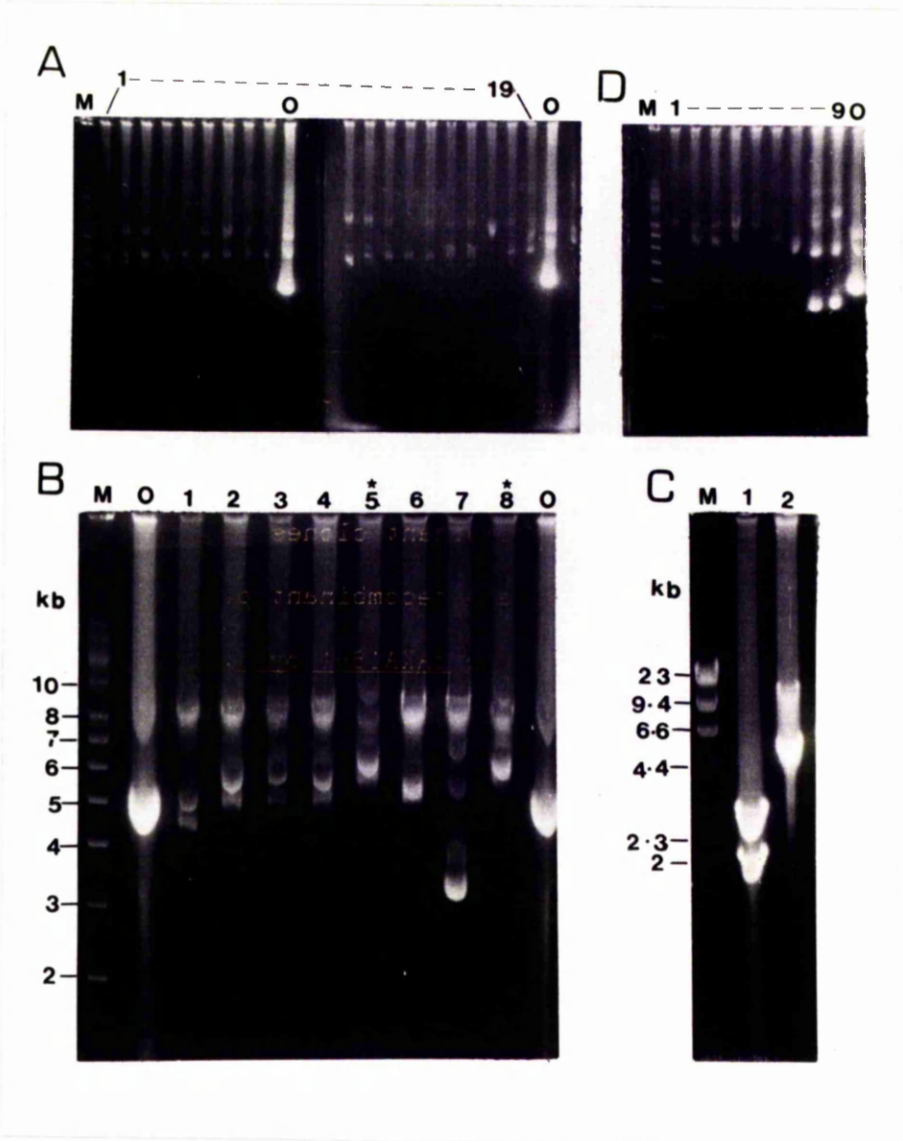
2 = pAKA16-1 digested by *Bam*HI + *Sca*I.

D: Construct pAKA17 from recombinant clones

M = Supercoiled DNA ladder as size marker.

O = plasmid pPH843 (control).

1-9 = Plasmid DNA from recombinant blue clones.


A
M
1
19
O
O
D
M 1
9 O
B
M O 1 2 3 4 *5 6 7 *8 O
kb
10
8
7
6
5
4
3
2
C
M 1 2
kb
23
9·4
6·6
4·4
2·3
2

Attempts at mobilization of pAKA16 and pAKA16-1 into *P. haemolytica* by the routine conjugation procedure produced innumerable transconjugant colonies which were again unstable on subculture on selective media.

3.15.7. Construction of vector pAKA17.

Since the large *Taq*I fragment (2.56 kb) of plasmid pPH843 was shown to lack the *mob* functions (Section 3.15.2), a partial *Taq*I digest (4.2 kb) of the whole plasmid was ligated to the large 2032-bp *Acy*I fragment (*Taq*I and *Acy*I give compatible cohesive ends) of pIC20H (Fig. 15), with the assumption that the *mob* gene of pPH843 would be retained if the plasmid was cut only at one *Taq*I site at either the 1.06 or the 2.7 position (Fig. 14). The resulting construct was designated pAKA17. A schematic diagram illustrating the construction of pAKA17 is shown in Figure 22.

The 2032-bp *Acy*I fragment of pIC20H was dephosphorylated before ligating to the *Taq*I partial digest of pPH843. If not dephosphorylated, this fragment of pIC20H was found to recircularize and regenerate the Ap^R phenotype of pIC20H which produced a high background of blue colonies on LB+Ap+X-gal agar. The ligated products were transformed into *E. coli* JM83 and 9 blue clones obtained were analyzed for the predicted size (6.2 kb) of the recombinant plasmid construct pAKA17 (Fig. 21D, lanes 1-7). Two smaller derivatives of plasmid were obtained in the analysis (Fig. 21D, lanes 8-9).

The 2032-bp insert could not be isolated from pAKA17 by digestion with *Acy*I, presumably because this enzyme site was lost due to the rearrangement of the base sequence at the *Taq*I-*Acy*I cloning site. *Taq*I was not used for the recovery of insert because this enzyme has several cleavage sites on different parts of pAKA17 including *lac*Z'. However, a major portion (1826

FIGURE 22. **DIAGRAM ILLUSTRATING THE CONSTRUCTION OF VECTOR pAKA17.**

Key:

Ap^r = Ampicillin-resistance gene.

*ori*V = Origin of replication.

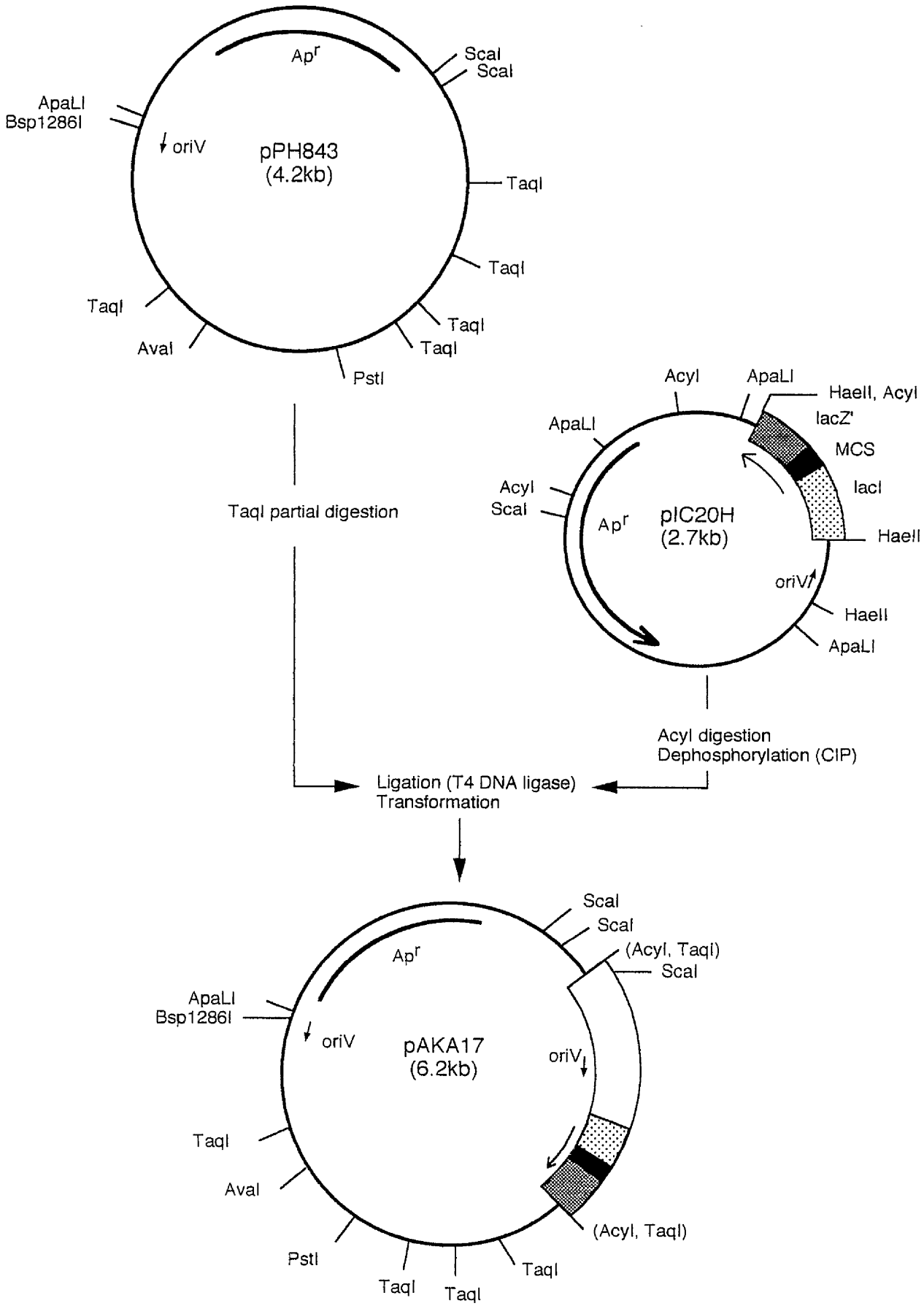
*lac*Z' = The α-peptide-encoding gene sequence of β-galactosidase.

*lac*I = Regulatory gene for β-galactosidase.

MCS = Multiple cloning site.

CIP = Calf intestinal phosphatase.

The plasmids are not drawn to scale. Arrows indicate the direction of transcription. Restriction sites shown in brackets were lost during subcloning.


Ap^r
ScaI
ScaI
ApaLI
Bsp1286I
oriV
pPH843
(4.2kb)
TaqI
TaqI
TaqI
TaqI
TaqI
AvaI
PstI
TaqI partial digestion
AcyI
ApaLI
HaeII, AcyI
lacZ'
MCS
lacI
HaeII
oriV
HaeII
ApaLI
ApaLI
AcyI
ScaI
Ap^r
pIC20H
(2.7kb)
AcyI digestion
Dephosphorylation (CIP)
Ligation (T4 DNA ligase)
Transformation
ScaI
ScaI
(AcyI, TaqI)
ScaI
Ap^r
ApaLI
Bsp1286I
oriV
pAKA17
(6.2kb)
oriV
TaqI
AvaI
PstI
TaqI
TaqI
TaqI
(AcyI, TaqI)

bp) of the insert was recovered by using *Pvu*I (Fig. 19, lane 3) which cuts twice (at positions 443 and 1339) within the 2032-bp *Acy*I fragment (Fig. 15).

A conjugation experiment to mobilize pAKA17 into *P. haemolytica* again showed innumerable transconjugant colonies, none of which grew on further subculture.

No attempt was made to subclone the *ori*T into pAKA17. Instead, mobilization of the constructs into an *E. coli* recipient (JC3272) was attempted in order to determine if the instability of *P. haemolytica* transconjugant clones was due to the host recipient. Results from this experiment gave between 500 and 2000 transconjugant colonies with pAKA16, pAKA17 and pPH843 which, when selected colonies were subcultured, grew normally on selective plate.

3.15.8. Determination of the orientation of the inserts of vector constructs pAKA15, pAKA16 and pAKA17.

Determination of the orientation of the *lacZ'* determinant would show the orientation of the insert in each construct. A *Bgl*I site is located at one end of the *lacZ'* gene within each insert (see Fig. 15) and two *Sca*I sites (in the original plasmid pPH843) are present outside the insert fragment of each construct. This allowed the orientations of the inserts in pAKA15 and pAKA16 to be readily assessed using these two enzymes. An extra *Sca*I site at one end and additional *Pst*I and *Ava*I sites in the MCS at the other end of the insert of pAKA17 could be used to orientate this insert. Double enzyme digestion of pAKA15 and pAKA16 each with *Bgl*I and *Sca*I, and single enzyme digestion of pAKA17 with either *Sca*I or *Pst*I or *Ava*I produced DNA

fragments (Figure 23) which facilitated the determination of the insert-orientation. The sizes of the *Bgl*II-*Sca*I, *Sca*I-*Sca*I, *Pst*I-*Pst*I and *Ava*I-*Ava*I fragments of the constructs were estimated and compared with the predicted sizes of those fragments in relation to two different orientations of the inserts (Table 22). The data confirmed the orientation of the inserts in pAKA15 and pAKA16, and showed that the insert of pAKA17 only fitted in one orientation into the *Taq*I-1.06 position on the pPH843 map. The orientation of the *lac*Z' and MCS determinants predicted from the data in Table 22 is as shown in Fig. 17 (pAKA15), Fig. 20 (pAKA16) and Fig. 22 (pAKA17).

FIGURE 23. **AGAROSE GEL ELECTROPHORESIS OF THE RESTRICTION ENZYME DIGESTS OF CONSTRUCTS pAKA15, pAKA16 AND pAKA17 FOR DETERMINATION OF THE ORIENTATION OF THEIR INSERTS.**

Key to figure:

M = 1 kb DNA ladder used as molecular size marker.

1 = pAKA15 digested by *Bgl*I + *Sca*I.

2 = pAKA16 digested by *Bgl*I + *Sca*I.

3 = pAKA17 digested by:

a = *Sca*I

b = *Pst*I

c = *Ava*I.

In lane 2, the 2.8- and 2.3-kb fragments comigrated on the 1.8% (w/v) agarose gel. Their sizes were estimated by running the digest in a low percentage agarose gel (0.7% w/v) shown on the right-hand panel of the figure. The 0.06-kb *Sca*I fragment is seen as a faint band in lanes 1, 2 and 3a on the 1.8% gel.

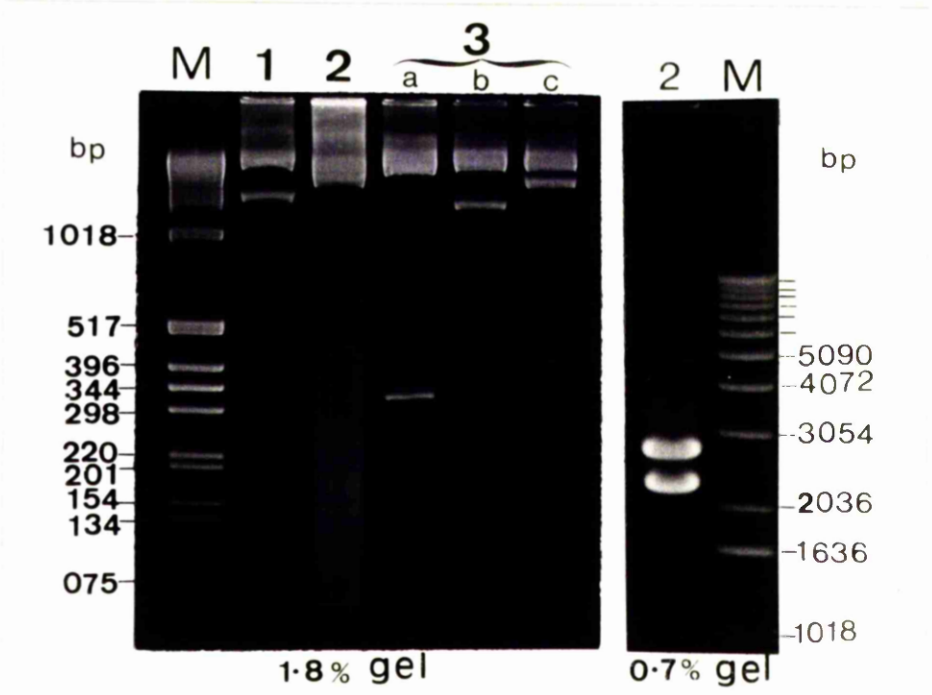

M
1
2
3
a
b
c
bp
1018
517
396
344
298
220
201
154
134
075
1·8% gel
2
M
bp
5090
4072
3054
2036
1636
1018
0·7% gel

TABLE 22. COMPARISON OF THE ESTIMATED SIZES OF FRAGMENTS OF pAKA15, pAKA16 AND pAKA17, OBTAINED BY DOUBLE OR SINGLE ENZYME DIGESTION, WITH THEIR PREDICTED SIZES IN RELATION TO TWO DIFFERENT ORIENTATIONS OF THE INSERTS

Construct	Enzyme(s)	Estimated fragment sizes (kb)	Predicted fragment sizes (kb)
pAKA15	*Bgl*II+*Sca*I	3.2, 1.4, 0.06	3.32, 1.3, 0.06
			2.86, 1.76, 0.06
pAKA16	*Bgl*II+ScaI	2.8, 2.3, 0.06	2.8, 2.3, 0.06
			3.65, 1.47, 0.06
pAKA17*	*Sca*I	5.8, 0.35, 0.06	5.72, 0.45, 0.06
			3.8, 2.4, 0.06
	*Pst*I	5.0, 1.2	5.1, 1.12
			3.54, 2.69
	*Ava*I	4.5, 1.7	4.6, 1.62
			3.29, 2.94

* The predicted sizes of fragments of pAKA17 generated by these enzymes are only shown for the insert cloned into the *Taq*I-1.06 position of pPH843 (see Figs. 14 & 22). All other *Taq*I positions did not fit with the calculated values.

4. DISCUSSION

4.1. Antibiotic Resistance in *P. haemolytica* Isolates.

In this study, typable and untypable isolates of *P. haemolytica* were examined for resistance to 16 antimicrobial drugs and each isolate showed multiple drug resistance to at least 3 antibiotics (Table 9). A common feature exhibited by this bacterial species was resistance to either Sm or Su, or to both of them. This has been observed in all previous studies (see Table 2) as well as the present one. It should be noted, however, that only resistance to a low concentration of Sm was tested and it is possible that, had higher concentrations been used, resistance may not have been so widespread. Resistance to Lm was also common to the isolates studied here, which corresponds with the findings of Allan *et al.* (1985a), Boyce & Morter (1986), Chang *et al.* (1987) and Shoo (1989) in bovine isolates of *P. haemolytica*. Resistance to β-lactam antibiotics (e.g. Ap and Pe) and Tc was only found in bovine isolates of the A1 serotype (Tables 5 and 9). It is interesting to note that Ap^R, in each case, was associated with Tc^R. This observation is consistent with the reports obtained from most of the previous studies on multiple drug resistance in *P. haemolytica* (Table 2). Resistance to these drugs may well be due to selection pressure exerted by these antibiotics, used extensively for routine treatment of cattle or in cattle feed as growth-promoters. Only one of 35 isolates was found to be resistant to Cm. Cm resistance in *Pasteurella* spp. is rare, and only one Cm^R strain each of *P. multocida* (Rossmanith *et al.*, 1991) and *P. haemolytica* (Amstutz *et al.*, 1982) has been reported. Only 2 of 141 *P. haemolytica* isolates were resistant to Cm in the study of Chang & Carter (1976). The use of this drug in food-producing animals has been banned for several years, which might have effectively removed it as an environmental

selective factor. Nevertheless, the results of some long-term studies suggest that multiple drug-resistant *P. haemolytica* has become the prevalent organism, capable of maintaining itself for long periods of time in the source population of cattle (Amstutz *et al.*, 1982; Fales *et al.*, 1982; Morter, 1983; Haghour *et al.*, 1987).

No significant differences were found in antibiotic resistance profiles between the isolates of *P. haemolytica* with regard to serotype (A1, A2, T4, T10 or untypable), animal source (bovine or ovine), health status of the animal (pneumonic or healthy), antibiotic therapy of the animal (treated or untreated) and the site of isolation (nasopharynx or lungs). This observation is similar to that of Shoo (1989), who found no differences in antimicrobial susceptibility patterns between the isolates from healthy and diseased calves or between the isolates from serotypes A1 and A2. In contrast, Allan *et al.* (1985a) observed significant differences in antibiotic susceptibilities to Pe, Ap and Tc between the nasopharyngeal isolates and the lung isolates. However, no differences in susceptibility patterns were found between the isolates from antibiotic-treated and untreated animals in their study. This may be due to the short period of time elapsed between antibiotic administration and isolation of bacteria.

4.2. Presence of Plasmids and Their Relationships to Antibiotic Resistance Phenotypes in *P. haemolytica*.

Plasmids were detected in eight isolates of *P. haemolytica* (6 of bovine and 2 of ovine origin) and all but one of the plasmid-bearing strains were isolated from pneumonic, or from animals in contact with pneumonic, cattle or sheep. Therefore, the plasmid data obtained in this study is more biased

towards the disease isolates. But the association of plasmids with disease in cattle or sheep is not clear-cut, because one *P. haemolytica* isolate (G/T 85/15), derived from a healthy bovine source, harboured an identical plasmid profile to that found in a pneumonic bovine isolate (D/I 85/1) (Table 10). It would be necessary to work with a greater number of isolates to make an assessment on the significance of plasmids with regard to disease. However, it is possible that apparently cryptic plasmids may in fact encode features that allow or sustain infections.

Plasmid DNA was identified in four serotype A1, three serotype A2 and one untypable strains of *P. haemolytica* (Table 10). The plasmid-bearing isolates of the same serotype showed similar plasmid profiles which clearly differed from the profiles exhibited by the other serotypes. Distinct plasmid profiles among different serotypes of *P. haemolytica* have been previously noted by Briggs & Frank (1984) and Boyce & Morter (1986). This study has shown some variation in plasmid pattern within the A2 isolates (Table 10), which may have been due to different animal sources (B664 was a sheep isolate and D/I 85/1 and G/T 85/15 were bovine isolates). However, the number of plasmid-containing strains studied was not sufficient to make a sound judgement on this variation relating to different animal sources.

The multiple drug-resistance profiles (Ap^R, Tc^R, Sm^R, Lm^R, Su^R) of plasmid-containing A1 isolates found in this study are very similar or identical to those of the *P. haemolytica* A1 isolates examined in other studies (Allan *et al.*, 1985a; Boyce & Morter, 1986; Haghour *et al.*, 1987; Chang *et al.*, 1987; Rossmanith *et al.*, 1991). Only plasmid-containing isolates of A1 serotype exhibited resistance to Ap, Tc and/or Cm (Table 10), which suggested that these may have been plasmid-associated phenotypes. However, only Ap^R was shown conclusively to be plasmid-mediated

by subsequent transfer studies. Serotype A1 is predominant in bovine pasteurellosis and accounts for over 80% of *P. haemolytica* isolates (Frank, 1989). Although all of the Ap^R plasmid-containing strains in the present study were from pneumonic or in-contact calves, the significance of this is difficult to assess without further examination of a larger number of isolates. Small, non-conjugative plasmids encoding 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.*, 1988a; 1991) and Germany (Schwarz *et al.*, 1989b). In the UK, multiple resistance to antibiotics, including Ap and Pe, has been reported (see Table 2) (Wray & Morrison, 1983; Allan *et al.*, 1985a; Shoo, 1989), but a detailed analysis of an Ap^R plasmid has been described only for one strain of *P. haemolytica* (Craig *et al.*, 1989). This plasmid, pPH33, is one of the four Ap^R plasmids of similar size obtained from the cattle isolates from different locations in the UK and examined here in this project.

One interesting observation is that Ap^R plasmids only occurred in *P. haemolytica* strains (Chang *et al.*, 1987; Livrelli *et al.*, 1988a; 1991; Schwarz *et al.*, 1989b; Craig *et al.*, 1989) and Sm^R plasmids in *P. multocida* strains (Berman & Hirsh, 1978; Silver *et al.*, 1979; Hirsh *et al.*, 1981; 1985; 1989; Schwarz *et al.*, 1989a). There appears to be not a single report on Ap^R plasmid from *P. multocida* and only one report of an Sm^R plasmid in a *P. haemolytica* T-type strain (Zimmerman & Hirsh, 1980). The reason for such species-specific R plasmid evolution is not understood. It seems to be relevant to mention in this connection that the possible reason for not finding in this study the Sm^R (8.3 kb) and Tc^R (8.6 kb) plasmids, reported by Zimmerman & Hirsh (1980) (see Table 4), may be due to serotype variation or the choice of antibiotics used in treatment in the two different

countries.

Although Tc^R co-existed with Ap^R in each of the plasmid-bearing A1 isolates, the resistance determinant for Tc^R was not found capable of transfer by transformation to *E. coli*. This might be due to the fact that Tc^R was not expressed in *E. coli* or that it was chromosomally-mediated. The latter possibility is supported by the observation that this phenotype was also found in two plasmid-less A1 strains (Tables 9 and 10). The occurrence of Tc^R in Ap^R plasmid-containing isolates as well as in plasmid-lacking isolates of *P. haemolytica* was also noted by Schwarz *et al.* (1989b). The unique Cm^R phenotype in strain S/C 84/3 appeared to be associated with a large plasmid (~20 kb) (Table 10). This plasmid was only found in the Cm^R strain, but its association with Cm^R could not be confirmed. No transfer of Cm^R to *E. coli* was detected by transformation. This may have been due to the high molecular weight of the plasmid, its inability to express in *E. coli* or again that it was chromosomally-mediated. The genetic determinant of Cm^R thus remains obscure.

The plasmids present in the A2 and untypable isolates (Table 10) were cryptic in nature at least with regard to antibiotic resistance. Interestingly, no R plasmids have yet been described in the A2 serotype of *P. haemolytica*, a common sheep pathogen. This phenomenon may have been due to the least amount of exposure of the A2 isolates to antibiotic selection pressure, because of the fact that sheep are not routinely treated with antibiotics (Gilmour & Gilmour, 1989) and that the A2 isolates occur in cattle pneumonia mainly as secondary pathogens (Frank, 1989).

4.3. Characterization of *P. haemolytica* Ampicillin-Resistance Plasmids.

Four Ap^R plasmids were identified and characterized in this study. All of them had a similar size (~4.3 kb), an identical restriction endonuclease profile and a gene specifying the ROB-1 β-lactamase enzyme, but plasmid pPH843 showed different behaviour from the other three plasmids in several aspects. Plasmids pPH2, pPH33 and pPH821 were essentially indistinguishable with respect to their transfer efficiencies (Tables 11 and 12), amplification properties (Table 18) and MICs with various β-lactam antibiotics (Table 15). Plasmid pPH843 had an apparently lower copy number in the wild-type *P. haemolytica* strain S/C 84/3 (Fig. 4, lane 4) which was reflected in a β-lactamase-negative phenotype on test strips and low MIC values. Transformation frequency was consistently lower with plasmid DNA preparations from strain S/C 84/3 containing pPH843, probably because of the presence in the strain of other plasmid species which were likely to interfere with pPH843 entry into competent *E. coli* during the transformation process. 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 (Table 15), presented an apparently high copy number of plasmid DNA after amplification (Fig. 8) and yielded the highest transfer frequency by conjugation (Table 12). It is possible that the co-existing plasmids in *P. haemolytica* strain S/C 84/3 suppressed the copy number of pPH843. This was substantiated by the observation that when this plasmid was transferred from the original host strain (via *E. coli*) to another *P. haemolytica* strain, A2S (serotype A2), the β-lactamase phenotype was positive and the MIC values were comparable to those for the other Ap^R plasmids in *P. haemolytica* (Table 15). 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 a plasmid was found in three plasmid-bearing A2 isolates (Table 10) or has been reported elsewhere. 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* in the absence of Ap compared to plasmid pPH843 (Table 17). The lower specific activities of β-lactamases specified by the former three plasmids (Table 16) may be due to their instability in *E. coli*. Similar instability of the ROB-1 β-lactamase-encoding plasmid (4.4 kb) of *H. influenzae* origin in *E. coli* HB101 has been observed in the absence of continued Ap selection (Medeiros *et al.*, 1986; Livrelli *et al.*, 1988b). Interestingly, plasmid pPH843 was detectable in plasmid DNA preparations from *E. coli* JC3272 transconjugants (Fig. 6B, lane 4), but not in those from *E. coli* DH1 or JM83 transformants, suggesting host strain differences in terms of plasmid content. This is in keeping with a previous report which observed that the stability of plasmids differed from *E. coli* to *E. coli* (Kumar *et al.*, 1991). When amplified by manipulation of the growth conditions, pPH843 showed a distinctly higher yield or copy number than the others (Fig. 8). Since the restriction profiles of all four plasmids were identical (Table 19), they offered no clue to the greater stability and higher amplification in *E. coli* of pPH843 compared to the other three plasmids. However, this might have been the consequence of derepression of regulatory sequences of the plasmid, taking place in the *E. coli* host.

The MIC values of β-lactam antibiotics for *E. coli*, containing the *P.*

haemolytica Ap^R plasmids, were noticeably increased over the values obtained with *P. haemolytica* hosts (Table 15). The differences are comparable to or higher in most cases than those reported by Livrelli *et al.* (1988a) for a ROB-1 β-lactamase-encoding Ap^R plasmid of similar size isolated from bovine *Pasteurella* strains and subsequently transferred to *E. coli*. Differences in MICs between the plasmid-free *P. haemolytica* and *E. coli* strains (Table 15) could be due to differences in outer-membrane permeability or the native chromosomally-encoded β-lactamase present in *E. coli* K-12 (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. The present data indicate little resistance to the cephalosporin class of β-lactams by the original and transconjugant *P. haemolytica* strains, but higher resistance by *E. coli* transformants containing the Ap^R plasmids, particularly pPH843.

E. coli DH1 transformants containing plasmids pPH2, pPH33 and pPH821 respectively were weakly positive for β-lactamase on test strips (Table 15) and showed no detectable plasmid band on gels (Fig. 8A, lanes 1-3), yet gave high MICs. Similar observations were made by Livrelli *et al.* (1988a, 1988b). These authors either failed to detect, or detected a faint band of, the ROB-1 β-lactamase plasmid in *E. coli* HB101 transformants, which still showed high MICs for β-lactam antibiotics.

4.4. Plasmid DNA Transfer to *P. haemolytica*.

Plasmid DNA transfer was not possible by the conventional $CaCl_2$-mediated transformation into *P. haemolytica* (Craig *et al.*, 1989) and into the related species, *P. multocida* (Jablonski *et al.*, 1992) and *A. pleuropneumoniae* (Lalonde *et al.*, 1989). These workers, however, introduced DNA into the respective organism by electroporation, a method designed to circumvent natural barriers to the entry of DNA by creation of holes or pores in the recipient cells. Conjugation is, however, considered as the best alternative method for plasmid DNA transfer, as it has a high frequency of transfer and permits the ready use of mobilizable shuttle vectors for transfer of cloned genes, derived from foreign sources, between *E. coli* and another host bacterium.

The Ap^R plasmids described in this study are small and not self-transmissible, but could be mobilized into *P. haemolytica* or *E. coli* from an *E. coli* source (but not from the original *P. haemolytica* strains) by conjugation, using the transfer functions (*tra*) of pRK2013. The inability to transfer plasmid DNA directly from *P. haemolytica* by this method is due to the fact that the helper plasmid pRK2013 is a ColE1-based replicon and hence unable to replicate in *P. haemolytica* donor strains. It could not therefore provide the necessary transfer apparatus (i.e. *tra*) for conjugation. In this connection, it is interesting to note that *Inc*P plasmid pRK2013 was seen to co-exist with Ap^R plasmid pPH843 (Fig. 6B, lane 4) and three other similar plasmids (Fig. 6B, lanes 1-3; not seen due to low copy number) in the same *E. coli* transconjugant strain. This indicates that the *P. haemolytica* plasmids do not belong to the *Inc*P group, but lack of time did not permit a thorough study on *Inc* grouping of the *P.*

haemolytica plasmids.

All Ap^R plasmids were obtained from *P. haemolytica* A1 isolates of bovine origin, but they were easily transferred by conjugation to strains of the A1 and A2 serotypes (FA1 and FA2 respectively) of ovine origin. This indicates that different serotypes and animal sources do not present barriers to plasmid DNA transfer among *P. haemolytica* isolates, although higher frequency of isogenic transfer was noticed within the same serotype. Thus, the plasmid transfer methods of conjugation and electroporation (Craig *et al.*, 1989) for *P. haemolytica* have been established in this laboratory.

No self-transmissible plasmids have yet been described in *P. haemolytica*. An attempt to transfer the large plasmid (~20 kb) present in *P. haemolytica* strain S/C 84/3 (Table 10) to other strains of *P. haemolytica* or *E. coli* was unsuccessful. This plasmid appeared to be associated with Cm^R, a unique phenotype shown by only S/C 84/3 within the whole collection of isolates (Table 9). The plasmid neither transferred itself under Cm selection, nor mobilized either of the two smaller plasmids present in the same strain; since one of these plasmids encodes Ap^R and would have produced Ap^R transconjugants if it had been mobilized. On the other hand, a large conjugative plasmid and a plasmid with mobilizing function have been described in *P. multocida* (Hirsh *et al.*, 1981; 1989). Plasmid DNA preparations from S/C 84/3 could not transform *E. coli* to Cm^R. The predicted nature of the large plasmid of S/C 84/3 thus remained unsolved, but might be revealed upon transfer by electroporation; which could be an objective for future experiments.

An attempt to transform *P. haemolytica* by the static aerobic procedure (see Materials and Methods: Section 2.7.2) did not succeed. This method of

DNA transfer was successfully applied to encapsulated isolates of *H. influenzae*, where the capsular material did not inhibit the transformation process (Rowji *et al.*, 1989). The failure to transform *P. haemolytica* by this procedure may have been due to differences in the capsular structures between the two types of bacteria or that competence induction could not be initiated by this method in *P. haemolytica*.

In the present work, it was not possible to effect Tn*5* mutagenesis of *P. haemolytica*, by using a suicide plasmid (pBR322) or a broad-host-range vector (pRK404) as the Tn*5* delivery vehicle in the conjugation system (Table 13). Similar observations were made with Tn*5* mutagenesis by Craig *et al.* (1989) who also failed to introduce non-*Pasteurella* broad-host-range plasmids (pRK290 and pSa4) into *P. haemolytica* even by electroporation, suggesting a lack of propagation of non-native or foreign DNA in this bacterium. This indicates that *P. haemolytica* might possess an active DNA restriction system or that the exogenous plasmids were unable either to replicate in *P. haemolytica* or to express their antibiotic resistance genes. The failure to obtain electrotransformants of *P. multocida* with non-native plasmids (pBR322 and pUC19) has also been reported (Jablonski *et al.*, 1992), whereas, Nnalue & Stocker (1989) succeeded in transfer of transposon-carrying chimeric plasmid vectors (pRKTV5 and pUW964), but not naturally-occurring broad-host-range plasmids (R388 and RP4), into this species by filter mating.

Attempts to transfer non-*Pasteurella* plasmids into *P. haemolytica* following heat treatment (42.5°C) of the recipient cells before mating, a device employed to inactivate restriction systems for enhancing plasmid transfer to Gram-positive corynebacteria (Schäfer *et al.*, 1990), did not work in this study. This may be due to loss of viability of *P. haemolytica*

recipient cells by heat treatment or the presence of a thermotolerant restriction system, if any.

Large, conjugative R plasmids (~45 kb), encoding β-lactamase, Tc resistance, or Cm-acetyltransferase, are often found in *Haemophilus* species and large, F-like indigenous cryptic plasmids have also been detected in *N. gonorrhoeae* and haemophili, which caused intra- and interspecies spread of the small R plasmids (Brunton *et al.*, 1986). It is, therefore, evident that the overall transfer capacity of *P. haemolytica* plasmids is much less prominent compared to that of the plasmids found in other related Gram-negative bacteria. This is probably due to the smaller size of most of the *P. haemolytica* plasmids, which is not sufficient for encoding both conjugation ability and conjugative pili. In spite of that, the transfer of Ap^R plasmids between *E. coli* and *P. haemolytica* by transformation and conjugation (Tables 11 and 12) suggests the potential for spread of drug resistance in nature among the bacteria *in vivo*. In the light of this, it is even more remarkable that no such Ap^R plasmids have been found in A2 serotypes. Perhaps without continued antibiotic selection, these plasmids are unstable and do not persist in these strains. However, these plasmids specifying the ROB-1 β-lactamase (see following section) may be regarded as useful markers for epidemiological studies on *P. haemolytica* A1 infections.

4.5. Characterization of *P. haemolytica* β-Lactamases.

Beta-lactamase production in penicillin- or ampicillin-resistant *P. haemolytica* isolates was reported in the UK (Wray & Morrison, 1983; Shoo, 1989), Germany (Schwarz *et al.*, 1989b) and USA (Zimmerman & Hirsh, 1980),

but not characterized in detail. However, the β-lactamase produced by a bovine isolate of *P. haemolytica* A1 (Wray & Morrison, 1983), which is one of the four Ap^R strains used in this study (CVL33), was attributed to have a high isoelectric point (pI), high activity against penicillin and low activity against cephaloridine and carbenicillin. These findings are in reasonable agreement with the results obtained here for the β-lactamase characterization (Fig. 7; Table 16).

The ROB- and TEM-type β-lactamases in Gram-negative bacteria are generally plasmid-mediated (Medeiros, 1984), and the sensitivity and substrate profiles of these two types of enzymes have been reported to be similar (Gutmann *et al.*, 1988), which was confirmed in the present study (Tables 15 and 16). The ROB-1 β-lactamase has been differentiated from the TEM-1 enzyme by its higher pI and its ability to hydrolyze ampicillin more rapidly and cephaloridine more slowly (Rubin *et al.*, 1981). The β-lactamases elaborated by *P. haemolytica* were found to be encoded by plasmids (Table 15) and the substrate profiles and isoelectric focusing data (Table 16 and Fig. 7) confirmed that these enzymes were indistinguishable from the ROB-1 β-lactamase. The ROB-1 enzyme was first discovered in *H. influenzae* of human origin (Rubin *et al.*, 1981) and later in *A. pleuropneumoniae* of animal (porcine) origin (Medeiros *et al.*, 1986) in the United States. The ROB-1 enzyme in *Pasteurella* species was first reported in France (Livrelli *et al.*, 1988a). This novel enzyme was further identified in clinical isolates of *H. influenzae* (Daum *et al.*, 1988) and Thai isolates of *H. ducreyi* (Maclean *et al.*, 1992) in the United States, and in isolates of *H. influenzae* (Livrelli *et al.*, 1988b) and *P. haemolytica* (Livrelli *et al.*, 1991) in France. This study presents the first report in Britain of the ROB-1 β-lactamase detected in *P.*

haemolytica. These reports indicate that the ROB-1 enzyme has so far been identified only in members of the family *Pasteurellaceae* (Table 23). The ROB-1 β-lactamase may thus be used as an identifying marker for ampicillin-resistant species of the *Pasteurellaceae*.

The present findings in the UK confirm the widespread nature of ROB-1 β-lactamase-producing strains among the clinical isolates of *P. haemolytica* A1 serotype. The fact that the ROB-1 β-lactamase plasmids examined here could be easily mobilized by conjugation (from *E. coli*) (Table 12) may explain the spread of such plasmid-encoded enzymes. But it does not explain the apparent absence of these plasmids in serotypes other than A1.

As reported by Livrelli *et al.* (1988a), the original ROB-1-producing *H. influenzae* strain F990 was isolated in 1980, whereas, *P. multocida* LNPB 9 (producing plasmid-encoded ROB-1) was isolated in 1978 and *P. aerogenes* ATCC 27883 (producing chromosomally-encoded ROB-1) was isolated before 1973. These authors have, therefore, suggested that the ROB-1 β-lactamase may have evolved in *Pasteurella* strains of animal origin. Recently, the ROB-1 enzyme has been analyzed at the molecular level and its predicted amino acid sequence showed a high degree of homology with class A β-lactamases from Gram-positive bacteria (Juteau & Levesque, 1990; Livrelli *et al.*, 1991). This enzyme therefore could be an evolutionary link between the β-lactamases of Gram-negative and Gram-positive organisms.

TABLE 23. PLASMID-MEDIATED ROB-1 BETA-LACTAMASES FOUND IN DIFFERENT MEMBERS OF THE *PASTEURELLACEAE*

Reference	Bacterial species	Source	Plasmid*	
			Designate	Size (kb)
Rubin *et al.* (1981)	*H. influenzae*	Human	R_{ROB}	4.4
Medeiros *et al.* (1986)	*A. pleuropneumoniae*	Porcine	pVM105	5.4
	H. influenzae	Human	pMG301	4.4
Livrelli *et al.* (1988a)	*P. haemolytica*	Bovine	...	4.4
	P. multocida	Bovine	...	4.4
Livrelli *et al.* (1988b)	*H. influenzae*	Human	pCFF242	4.4
Daum *et al.* (1988)	*H. influenzae*	Human	(NI)	(NI)
Livrelli *et al.* (1991)	*P. haemolytica*	Bovine	...	4.1/4.4
Maclean *et al.* (1992)	*H. ducreyi*	Human	pTH126	5.4
Azad *et al.* (1992b)	*P. haemolytica*	Bovine	pPH843	~4.3

* (NI) = Not investigated.

4.6. Molecular Characterization and Relationships of the Beta-Lactamase (ROB-1)-Encoding Plasmids from *P. haemolytica* and *H. influenzae*.

The ROB-1 β-lactamase-specifying Ap^R plasmids found in members of the family *Pasteurellaceae* were consistently small in size (Table 23) and similar plasmids from *P. haemolytica* were estimated at 4.1-4.5 kb in different studies (see Table 4) (Livrelli *et al.*, 1988a; 1991; Schwarz *et al.*, 1989b; Craig *et al.*, 1989). The size of the β-lactamase plasmids in the present study appeared to be 4.3 kb, but this was eventually recalculated to be 4.2 kb in the course of their molecular analysis. It is likely that all the Ap^R plasmids described here and previously are of the same size and distributed widely among isolates of *P. haemolytica*. Differences in isolation techniques of plasmid DNA, different experimental conditions and molecular-weight markers may have accounted for the variation in calculated plasmid sizes (see Table 4).

The four ROB-1 β-lactamase plasmids used in this study were all identical by restriction endonuclease fragment patterns. Similarly, β-lactamase plasmids from nine German isolates (Schwarz *et al.*, 1989b) and those from five French isolates (Livrelli *et al.*, 1991) of *P. haemolytica* were shown to be identical to each other by restriction analysis and/or Southern blot hybridization.

The restriction profile of the Ap^R plasmids (Table 19), particularly the restriction map of the representative plasmid pPH843 (Fig. 14), revealed a low frequency of common restriction enzyme sites and aggregation of a relatively larger number of sites in the Ap^R gene on the map. Such a phenomenon has been reported by Mayer & Robbins (1983), who established a map of the 7.1-kb β-lactamase-encoding plasmid from *N. gonorrhoeae* and

observed a higher frequency of restriction sites in the *bla* gene, derived from Tn*A*. Surprisingly, only a very few and relatively uncommon enzymes with a 6-nucleotide recognition sequence cleaved both types of plasmid DNA. However, the number of restriction enzymes (12) shown to cleave the plasmid DNA in the present study was greater than those (7, also used here) used by Livrelli *et al.* (1991) to cut similar ROB-1 β-lactamase-encoding plasmids from *P. haemolytica*. Enzymes cutting such plasmids not reported by Livrelli *et al.* (1991) were: *Pst*I, *Apa*LI, *Bsp*1286I, *Tha*I and *Cfo*I.

Restriction enzyme analysis and hybridization experiments in this study demonstrated that the prototype ROB-1 β-lactamase-encoding plasmid pRM3022 from *H. influenzae* and those isolated from *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 (4.2 kb) and this was reflected in the presence of extra *Alu*I, *Sau*3AI and *Dra*I sites (Fig. 11B) and only weak hybridization of one 0.69-kb *Sau*3AI fragment to pPH843 (Fig. 12, 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. Livrelli *et al.* (1991) have recently reported homology between the ROB-1 β-lactamase plasmids from eight *Pasteurella* isolates in France and two *Haemophilus* strains (one was French and the other was the original ROB-1-producing isolate) by restriction endonuclease and hybridization analyses, using only *Sau*3AI fragments. These authors isolated 4.1-kb plasmids from seven *Pasteurella* strains and 4.4-kb plasmids from two *H. influenzae* and one *P. haemolytica* strains, and observed multiple homologous fragments common to these plasmids. However, they did not report the lack of hybridization of any *Sau*3AI fragment (as has been observed here), because the relatively larger

Haemophilus plasmid (R_{RDB}/ pRM3022) (4.4 kb) was used as the hybridization probe in the study of Livrelli *et al.* (1991), as opposed to *P. haemolytica* plasmid (pPH843) (4.2 kb) used as a probe in the present study.

The available data from this and previous studies strongly suggest that a close relationship exists between the ROB-1 β-lactamase-producing plasmids from two different species and that one plasmid might have evolved from the other by deletion, or by recombination with pre-existing cryptic plasmids in the respective strains. Relationships have also been established between the ROB-1 plasmids from *H. influenzae*, *H. ducreyi* (human) and *A. pleuropneumoniae* (animal) strains isolated in the USA, France and Thailand (Medeiros *et al.*, 1986; Livrelli *et al.*, 1988b; Maclean *et al.*, 1992). These observations confirm the close relationships among ROB-1 β-lactamase plasmids, regardless of source, species and geographical origin, and signify their epidemiological importance. Similarly, a TEM-type β-lactamase-encoding plasmid from *H. influenzae* isolated in Germany was found to be closely related (65%-100% DNA homology) to various R plasmids from the same species obtained from a broad geographical range (Laufs & Kaulfers, 1977).

Sizes of ROB-1 β-lactamase plasmids were shown to be 4.4 kb from *H. influenzae* (Rubin *et al.*, 1981; Medeiros *et al.*, 1986; Livrelli *et al.*, 1988b), 4.1 to 4.4 kb from *Pasteurella* spp. (Livrelli *et al.*, 1988a; 1991), and 5.4 kb from *A. pleuropneumoniae* (Medeiros *et al.*, 1986) and *H. ducreyi* (Maclean *et al.*, 1992) (see also Table 23). A value of 4.2 kb was obtained for the *P. haemolytica* plasmids in this study. The possibility that the ROB-1-encoding genetic element (probably a transposon) might have been inserted by transposition into the resident cryptic plasmid pool of the individual bacterial species is supported by the report that the TEM-1 β-

lactamase plasmids from *Haemophilus parainfluenzae, H. influenzae, H. ducreyi* and *N. gonorrhoeae* arose by insertion of a transposon from the Tn*A* family into small cryptic plasmids (Brunton *et al.*, 1986). The ROB-1 β-lactamase gene from *P. haemolytica* was thought to be part of a transposable element (Livrelli *et al.*, 1991); but it is unlikely that this gene has been derived from Tn*A* specifying the TEM-type β-lactamase, because the ROB-1-encoding gene is not structurally related to the TEM gene (Medeiros *et al.*, 1986; Levesque *et al.*, 1987; Daum *et al.*, 1988).

It has been postulated that the ROB-1-encoding plasmid had an animal reservoir and originated in *Pasteurella* species (Livrelli *et al.*, 1988a; 1991). This might have been disseminated as a complete replicon from *Pasteurella* organisms to other members of the *Pasteurellaceae*. Such a hypothesis is strongly supported by the observation in the hybridization experiments that a portion of the DNA sequence (~0.7 kb) from the *Haemophilus* plasmid is not present in the *Pasteurella* plasmid (Fig. 12). This additional DNA sequence could be the remnants of a small resident cryptic plasmid in *H. influenzae*, which served as a foundation and received the ROB-1 determinant from the anticipated source. The ROB-1 gene in *Pasteurella*, in turn, might be derived, as a transposable element, from Gram-positive organisms, because the ROB-1 β-lactamase has been shown to be highly related to the β-lactamases from Gram-positive bacteria (Juteau & Levesque, 1990; Livrelli *et al.*, 1991).

4.7. Amplification of Plasmid DNA in *E. coli*.

Previous investigators have reported that the recovery of *P. haemolytica* Ap^R plasmids from *E. coli* transformants was either poor (Livrelli *et al.*, 1988a; Craig *et al.*, 1989) or non-existent (Zimmerman & Hirsh, 1980). Livrelli *et al.* (1991) analyzed plasmid DNA from wild-type *P. haemolytica* strains (especially strain LNPB 1899, which contained only one plasmid) throughout their study, presumably because of negligible yields of plasmid DNA from *E. coli*. Zimmerman & Hirsh (1980) could not conclusively correlate Ap^R or β-lactamase production with a specific plasmid owing to their failure to demonstrate the plasmid from *E. coli* transformants. However, these authors easily recovered Sm^R (8.3 kb) and Tc^R (8.6 kb) plasmids of the same *P. haemolytica* strain from an *E. coli* background. These observations suggest that such Ap^R plasmids are either unstable or replicate to a very low copy number in *E. coli* which may possess weak machinery for replication of the exogenous plasmid. Similar results were initially obtained in the present study, but all Ap^R (ROB-1) plasmids of *P. haemolytica* origin were eventually amplified in *E. coli* by manipulation of the growth conditions (see Results: Section 3.10; Table 18).

It has been known for many years that plasmids of the ColE1-type can be amplified in *E. coli* by inhibition of protein synthesis with Cm (Clewell, 1972) or amino acid starvation (Clewell & Helinski, 1972; Schroeter *et al.*, 1988). No strong evidence is yet available on the amplification of other types of plasmids by this process, except a report by Chang & Cohen (1978) who used Cm or spectinomycin (Sp) to amplify hybrid plasmids derived from the P15A replicon, which was shown to be ancestrally related to ColE1. *Pasteurella* plasmids are unlikely to be of the ColE1-type, because a

plasmid with this origin of replication (pRK2013) did not replicate in *P. haemolytica* (Fig. 6A; lanes 1-4). Surprisingly, therefore, the *P. haemolytica* ROB-1 plasmids in this study could be amplified by Cm or Tc as efficiently as the ColE1 plasmids. Sequence analysis may eventually relate these plasmids to ColE1, but since the type of replicon of the *P. haemolytica* plasmids is as yet unknown, the amplification properties suggest that these plasmids at least lack the *rop* (repression of primer) or *rop*-like gene. The *rop* gene in a ColE1 plasmid is normally located close to *oriV*, and the gene product (called Rop protein) reinforces the negative regulation of plasmid DNA replication by inhibition of RNA primer transcription and thereby controls the plasmid copy number (Cesareni *et al.*, 1982). Cm, Tc or Sp are inhibitors of protein synthesis and play similar roles in plasmid amplification. Inhibition of protein synthesis causes a gradual termination of chromosomal DNA synthesis consistent with completion of a round of replication and no initiation of new rounds. In contrast, however, plasmid DNA continues to replicate at the expense of proteins or replicative enzymes synthesized before inhibition of protein synthesis and which remain in excess in the cell, and plasmid DNA accumulates as noncomplexed, CCC molecules (Clewell, 1972; Clewell & Helinski, 1972).

More recently, amplification of plasmid pBR322 in *E. coli* was reported without blocking protein synthesis by using amino acid and Fe^{3+} supplements (Angelov & Ivanov, 1989). In all the above cases, the extent of amplification depended on the growth medium used. In this 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). LB broth or the presence of phosphates in the growth media (as in Terrific broth; see Appendix 19) has been reported to increase the stability and/or the copy number of certain plasmids (Kumar *et al.*, 1991). These authors also noted that the increase in plasmid copy number was associated with glucose and ammonia limitation in the media. Here, the highest amplification of plasmids was observed in Terrific broth as growth medium (Table 18; Fig. 8), which was specially formulated for plasmid and cosmid production (Tartof & Hobbs, 1987). The reason for obtaining the best yield of plasmids from yeast nitrogen base (YNB)-supplemented Terrific broth is not clear, but might be due to the presence of multivitamins, amino acids and metal ions, including Fe^{3+}. However, the further addition of amino acids and/or Fe^{3+} to YNB-supplemented Terrific broth reduced the amplification rate of plasmids, which contrasts with the findings of Angelov & Ivanov (1989). Use of more than 1% YNB in Terrific broth caused cell death, as was evident from clumping of apparently deformed cells suspended in the medium. This might have been the result of toxicity due to metal ions, when used in excess in the form of YNB.

The detection and the extent of amplification of the *P. haemolytica* plasmids differed among the different *E. coli* strains such as JC3272 (*recA*$^+$), DH1 (*recA*$^-$) and JM83 (*recA*$^+$) (Figs. 6B, 8 and 9). These observations are in agreement with the findings of Chang & Cohen (1978) who found a significant difference in the amplification of plasmids between *recA*$^-$ (*E. coli* JC1569) and *recA*$^+$ (*E. coli* C600) strains.

The prototype ROB-1 β-lactamase-encoding plasmid pRM3022 (see Materials and Methods: Section 2.4) showed similar behaviour in *E. coli* to the *P. haemolytica* plasmids with regard to DNA isolation, which again emphasizes the close relationship between these plasmids. pRM3022 was amplified in *E.*

coli in an almost identical manner (Fig. 9) when analyzed and compared with the other ROB-1 plasmids (Figs. 11 and 12). This work and other reports thus indicate that these small ROB-1 β-lactamase plasmids need to be amplified in *E. coli* in order to obtain a good yield of plasmid DNA for further characterization and cloning purposes. The *in vivo* amplification procedure has also facilitated the isolation of pPH843-derived constructed vectors (Results: Sections 3.15.5-7) from the *E. coli* backgrounds, and was essential for the development and analysis of the vectors. Thus the amplification procedures described here have practical implications in the molecular analysis of plasmid DNA.

4.8. Rapid Purification of Plasmid DNA by the Modified Acid-Phenol Extraction Method (A Developed Protocol).

The aim of the development of new protocol for purification of plasmid DNA was to rapidly prepare large amounts of purified DNA, which are required for restriction analysis, cloning experiments and further characterization of plasmids.

The original acid-phenol extraction method of Zasloff *et al.* (1978) was based on the selective extraction of chromosomal and non-CCC DNA molecules into the acidified phenolic phase leaving CCC forms in the upper aqueous phase. Apart from the use in molecular analysis, purification of CCC plasmid DNA also helps to identify the actual number of plasmid species present in a bacterium, because it is often unclear on gels whether a particular DNA band is the OC form of one plasmid or the CCC form of another, larger, plasmid.

Several modifications were made here to the original protocol of

Zasloff *et al.* (1978). These included the use of: i) $MgCl_2$ instead of NaCl in the low-ionic strength buffer, ii) undistilled phenol instead of distilled phenol, iii) vortexing for 1 min instead of shaking for 2-3 min, iv) only one acid-phenol extraction instead of up to four extractions, v) a single extraction with chloroform-isoamyl alcohol instead of four extractions with anhydrous ether to remove phenol, and vi) room temperature instead of 0-4°C. Furthermore, in the modified protocol, purified CCC DNA was finally precipitated in 0.3M sodium acetate (pH 6.0) and ethanol.

The roles of $MgCl_2$ and NaCl in the protocol were compared; the latter chemical was shown to generate a diffuse DNA smear in each case and also removed CCC plasmids of higher molecular weight (Fig. 10). This suggested that NaCl might allow degradation of high molecular-weight DNAs to small linear fragments, which were not efficiently extracted into phenol. This is in agreement with Zasloff *et al.* (1978) who reported that small oligo-ribonucleotides and linear DNA fragments of less than 1.5 kb could not be removed by their procedure.

The physical basis of acid-phenol extraction of CCC DNA molecules and Mg^{2+}-DNA interactions were described by Müller *et al.* (1983). Both phenol and acid pH at low ionic strength denature non-CCC DNA molecules by converting them into single-stranded linear forms which are extracted from the water phase into the phenolic phase due to their hydrophobic nature and charge compensation by Mg^{2+} ions. Moreover, it is likely that divalent Mg^{2+} ions bind to and interact with denatured DNA, which has exposed phosphate groups, more than monovalent Na^{+} ions. Mg^{2+} ions were reported to have a high affinity for binding to DNA and they were also found to be important for the stabilization of DNA conformation (Sander & Ts'o, 1971). At present, no explanation can be given for the selective loss of CCC plasmids

of higher molecular weight (Fig. 10, groups C-E, lane 2) in the presence of Na^+ ions. Vortexing was a crucial step in obtaining a good yield of purified CCC molecules, because more than one extraction with acid-phenol, as indicated by Zasloff *et al.* (1978), in the presence of NaCl resulted in further loss of CCC DNA. However, a single extraction with $MgCl_2$ in place of NaCl was sufficient for complete purification of CCC plasmid DNA forms. Crude plasmid DNA preparations at both high (Fig. 10, groups A and B, lane 1) and low (groups C-E, lane 1) concentrations, without adjustment to 10 A_{260} units/ml as suggested by Zasloff *et al.* (1978), could be purified equally well by the present protocol. A plasmid of 73 kb (group E, lane 3) was readily purified by this procedure, whereas a plasmid of 38 kb was the largest purified by Zasloff *et al.* (1978).

Kieser (1984) and Olsen (1990) reported improved methods for the small-scale preparation of plasmid DNA free from chromosomal DNA and suitable for restriction analysis. However, both methods used alkaline denaturation in the presence of SDS at high temperatures (56°-70°C). This is not suitable for the large-scale preparation of plasmid DNA due to the high viscosity of the lysed bacterial pellet and the fact that certain strains of *E. coli*, such as HB101, release large amounts of carbohydrate during heating and this severely interferes with the final purification process (Sambrook *et al.*, 1989). On the other hand, the protocol developed in this study is a simple, rapid and large-scale one which can offer a ready alternative, for CCC plasmid DNA purification, to either caesium chloride-ethidium bromide density gradient centrifugation (Radloff *et al.*, 1967), which is time-consuming, or commercially available purification kits, which are expensive. Furthermore, it can be reasonably expected that the involvment of $MgCl_2$ in the plasmid purification process may improve the quality of DNA

for restriction enzyme analysis, because of the fact that Mg^{2+} ions are an obligate requirement for most endonuclease activity.

4.9. Construction of Cloning Vectors for *P. haemolytica*.

Since attempts to transfer a variety of suicide or broad-host-range plasmid vectors into *P. haemolytica* strains were not successful in the present and previous work in this laboratory, the construction of an endogenous replicon-derived shuttle vector for *P. haemolytica* was considered a necessity for transfer of cloned DNA and transposon-mutagenesis studies. The stability, high amplification in *E. coli* and high transfer frequency to *P. haemolytica* of pPH843, made it an ideal plasmid for the construction of a shuttle vector. Using the restriction map data (Fig. 14), this plasmid was modified into a series of possible cloning vectors (pAKA15, pAKA15-1, pAKA16, pAKA16-1 and pAKA17) by insertion of a fragment from pIC20H which contained the *lacZ* α-peptide coding region and an MCS, and, in the case of pAKA15-1 and pAKA16-1, a fragment from pILL514 which contained the *oriT* of RK2 (Figs. 17, 20 and 22). During preliminary cloning and deletion experiments, it was found that the *Pst*I, *Apa*LI, *Taq*I and *Sca*I sites of pPH843 did not interfere with the essential functions of plasmid replication and Ap^R, and hence were used for insertion of the foreign DNA fragments. The 2.56-kb *Taq*I fragment of pPH843 (i.e. pPH843Δ*Taq*I) (Fig. 14) was found capable of replication and expression of Ap resistance in *E. coli*, but incapable of mobilization. This indicated that the remaining portion of pPH843, bounded by the *Taq*I sites (positions 1.06-2.7), might contain a part or whole of the *mob* gene. The *mob* gene was further localized to the region surrounding the *Pst*I site (Fig. 14) because insertion of the

*Hae*II fragment from pIC20H into this site in pPH843 also abolished mobilization by pRK2013. This ability was restored by insertion of the *mob* region from pILL514.

Naturally-occurring plasmid pPH843 was cleaved by only a few restriction enzymes with a 6-bp recognition sequence and the cloning vectors constructed in this project contain 16 unique restriction sites with insertional inactivation of β-galactosidase activity for recognition of recombinant clones in *E. coli*. Lalonde & O'Hanley (1989) developed a shuttle vector, pYG53, for *A. pleuropneumoniae* by ligating only the MCS from pUC19 to the wild-type plasmid pYG10 (Cm^R). Since these authors neither cloned the *lacZα*-reporter gene from pUC19 nor an additional antibiotic-resistance gene into the shuttle vector, the procedures they used for recombinant selection were not clear. Moreover, the subsequent insertion of the *oriT* into the MCS of pYG53, to construct the mobilizable derivative (pYG54), hampered the versatility of the MCS. Thus the two most desirable features of the pUC vectors, direct screening of recombinants and a versatile MCS, were lost. All the essential features of the pUC/pIC vectors were preserved in the vectors constructed for *P. haemolytica*.

Very recently, a broad-host-range expression vector, based on the RSF1010 replicon and a Cm^R gene from plasmid pSa, was developed for *A. pleuropneumoniae* and *P. haemolytica* in which the vector was introduced and propagated by electroporation (Frey, 1992). This report contrasts with the findings of Craig *et al.* (1989) who failed to propagate a plasmid of pSa replicon (pSa4) and that of RSF1010 replicon (pKT230) in *P. haemolytica* by transformation or electroporation. Lalonde & O'Hanley (1989) also made the observation that RSF1010-derived vectors could not be introduced into *A. pleuropneumoniae* or were very unstable in this host. Frey (1992), however,

demonstrated the stability of the expression vector in *A. pleuropneumoniae*, although not in *P. haemolytica*. The author made the point that the stability of the vector was associated with the use of the minimal autonomous replicon of RSF1010 which may thus not be susceptible to a restriction system present in *P. haemolytica*.

Attempts to mobilize the vector constructs from *E. coli* into *P. haemolytica* by conjugation, using the *Inc*P helper plasmid pRK2013 gave disappointing results. A large number of colonies of *P. haemolytica* recipients developed on transconjugant-selection plates in each case (except pAKA15), but they did not grow upon further subculture on Ap-containing media. The barrier associated with the failure to obtain true and stable transconjugants of *P. haemolytica* may be the possession of a restriction/ modification system by *P. haemolytica*, acting on the vector DNA derived from pIC20H or pILL514 which contains restriction enzyme sites not present in the wild-type plasmid pPH843. In later experiments, attempts were again made to mobilize the constructs into *P. haemolytica* from *E. coli* SM10, which contains the transfer functions of the *Inc*P plasmid RP4 incorporated into the chromosome (Simon *et al.*, 1983). Transconjugants were obtained for each of the constructs except pAKA15, but were again found to be unstable on subculture (Dr. J. G. Coote, personal communication). These observations are consistent with those found earlier. Transconjugants obtained from the crosses with *E. coli* as recipients were found to be stable and could be subcultured on the original selection medium used in conjugation. This strongly suggests the presence in *P. haemolytica* of a restriction system that has to be by-passed by the incoming plasmid DNA. Assaying *P. haemolytica* for the presence of restriction endonucleases would make an interesting area for further investigation. This can be done by

preparing crude extracts from the bacterium and examining them for endonuclease activity on a standard DNA (e.g. bacteriophage λ DNA) (Schleif, 1980). A similar observation with a restriction barrier was made with *C. jejuni* (Miller *et al.*, 1988), but it was shown in later experiments that successful transfer of *E. coli*-derived plasmid DNA to *C. jejuni* was strain-dependent (Wassenaar *et al.*, 1991). Similar problems with heterologous DNA transfer into *Legionella pneumophila* (Marra & Shuman, 1989) and *Erwinia carotovora* (Rella *et al.*, 1989) were overcome by isolation of restriction-deficient strains for use as recipients. Restriction mutants were derived by random screening in the former case and by chemical mutagenesis, using ethyl methanesulfonate in the latter case. Similar procedures could also be applied to *P. haemolytica* in order to isolate restriction-deficient mutants; again an area for future investigation. Finally, use of restriction- and modification-deficient *E. coli* strains for DNA cloning should also alleviate such a problem. It would be interesting to see if these vectors can be transferred to *P. multocida* and whether are stable in this species.

The molecular basis of conjugation in Gram-negative bacteria involves: i) nicking and initiation of DNA transfer at the *nic* or *oriT* site of the plasmid molecule, ii) single-stranded DNA transfer from donor to recipient with the 5' terminus leading, and iii) complementary-strand synthesis in the recipient to produce a complete replicon (Willetts & Wilkins, 1984; Derbyshire & Willetts, 1987). It was later postulated that this type of transfer should not be affected by the presence of a double-strand-specific restriction enzyme in the recipient cells (Trieu-Cuot *et al.*, 1991). In this connection, a hypothesis can be made regarding the initial growth of the unstable *P. haemolytica* transconjugants in the present study. The Ap^R

gene of the transferred plasmid DNA might be expressed, allowing growth of the transconjugants, immediately before the complete synthesis of a complementary strand forming duplex DNA. This DNA would, however, be eventually cleaved *in vivo* by the double-strand-specific restriction endonuclease possibly at a site distant from the Ap^R gene. Thus, sufficient β-lactamase would be made in the initial transconjugants to allow growth, but subsequent destruction of the β-lactamase gene or disruption of the integrity of the plasmid would prevent subculture of transconjugant cells in the presence of Ap.

It would be of interest in this regard to check if the initial transconjugants can be subcultured in the absence of Ap selection. If this is so, it means in effect that the plasmid construct is a suicide vector which may allow transfer of cloned genes and subsequent recombination with the *P. haemolytica* chromosome before it is destroyed. Initial growth of the transconjugants in the presence of Ap will thus identify clones which can then be screened for allelic exchange of cloned *P. haemolytica* DNA.

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APPENDICES

APPENDIX 1: BRAIN HEART INFUSION (BHI) BROTH OR AGAR

Brain heart infusion, dehydrated* (Oxoid) : 37 g

Distilled H_2O : 1 litre

*Formula (per litre):-

Calf brain infusion solids	– 12.5 g
Beef heart infusion solids	– 5.0 g
Proteose peptone (Oxoid L46)	– 10.0 g
NaCl	– 5.0 g
Dextrose	– 2.0 g
Na_2HPO_4 (anhydrous)	– 2.5 g
pH = 7.4	

Dissolved in distilled H_2O and sterilized by autoclaving for 20 min at 15 pounds per square inch (p.s.i.) at 121°C on liquid cycle. For solid medium, 1.2% agar (Technical No. 3, Oxoid) was added before autoclaving. The blood supplement was added to solid medium, after it was allowed to cool to 50°C.

NUTRIENT BROTH OR AGAR

Nutrient broth, dehydrated* (Oxoid) : 25.0 g

Distilled H_2O : 1 litre

*Formula (per litre):-

Lab-Lemco powder	– 1.0 g
Yeast extract	– 2.0 g
Peptone	– 5.0 g
Nacl	– 5.0 g

APPENDIX 1 (Continued)

Dissolved in distilled H_2O and sterilized by autoclaving for 20 min at 15 p.s.i. (121°C). For solid medium, 1.2% agar (Technical No. 3, Oxoid) was added before autoclaving.

LURIA-BERTANI (LB) BROTH OR AGAR

Tryptone (Oxoid)	: 10 g
Yeast extract (Oxoid)	: 5 g
NaCl (AnalaR®, BDH)	: 10 g
Distilled H_2O	: 1 litre

The solutes were dissolved in distilled H_2O by shaking and the pH was adjusted to 7.0 with NaOH. For solid medium, 1.2% agar (Technical No. 3, Oxoid) was added before autoclaving for 20 min at 15 p.s.i. (121°C).

MUELLER HINTON MEDIUM

Mueller Hinton agar, dehydrated* (Difco)	: 38.0 g
Distilled H_2O	: 1 litre

*Formula (per litre):-

Beef infusion	– 300.0 g
Bacto-casamino acids	– 17.5 g
Starch	– 1.5 g
Bacto-agar	– 17.0 g

Dissolved in distilled H_2O and autoclaved at 20 p.s.i. for 20 min. The blood supplement was added after the media was cooled to 50°C.

APPENDIX 2: PREPARATION OF ANTIBIOTIC SOLUTIONS

Stock solutions of antibiotics were made at the following concentrations (% w/v): Ap and other β-lactams (2.5%), Tc (1%), Cm (1%), Km (2.5%), Tp (4%), Sm (2.5%) and Nal (0.25%).

Ampicillin, carbenicillin and other β-lactam antibiotics were dissolved in distilled H_2O by adding a drop of 40% NaOH and filter sterilized by passing the solutions through an acrodisc of 0.45 μm pore size (Gelman Sciences). Streptomycin and kanamycin solutions were also made up in distilled H_2O and filter sterilized. Chloramphenicol was dissolved in ethanol, and tetracycline, trimethoprim and nalidixic acid were dissolved in equal volumes of ethanol-water (1:1) and were not filter sterilized. All antibiotic solutions were stored at -20°C. Nalidixic acid crystals formed during storage were dissolved by heating to 70°C in a water bath before use. Ampicillin solution was used within 2-3 days of preparation.

APPENDIX 3: LYSIS BUFFER FOR PLASMID DNA EXTRACTION

TEG buffer (per litre):-

25mM Tris	: 3.028 g
10mM EDTA	: 3.722 g
50mM glucose	: 9.008 g

Dissolved in distilled H_2O, the pH was adjusted to 8.0 with HCl, filter-sterilized or autoclaved for 15 min at 10 p.s.i. and stored at 4°C. To prepare a complete lysis buffer, 4 mg of lysozyme (Sigma) was added per ml of TEG buffer immediately before use.

APPENDIX 4: ALKALINE SDS SOLUTION

	Small-scale	Large-scale
2N NaOH :	0.2 ml	5.0 ml
20% SDS :	0.1 ml	2.5 ml
H_2O :	1.7 ml	42.5 ml

APPENDIX 5: ACIDIFIED POTASSIUM ACETATE SOLUTION

	Small-scale	Large-scale
5M K-acetate :	1.5 ml	21.0 ml
Glacial acetic acid :	288 µl	4.05 ml
H_2O :	712 µl	10.0 ml

APPENDIX 6: BUFFERED-SATURATED PHENOL/CHLOROFORM

Buffer-saturated phenol was prepared as follows:-

To crystalline phenol (Formachem, Strathhaven, UK) was added 0.1% 8-hydroxyquinoline and then an equal volume of 0.5M Tris.HCl buffer (pH 8.0) was added to saturate the phenol. The mixture was stirred on a magnetic stirrer for 15 min and the lower phenolic phase was separated and removed to a fresh bottle by using a separation funnel (Quickfit, UK). The extraction process of the phenol was repeated (~3 times) by using an equal volume of 0.1M Tris.HCl (pH 8.0) until the pH of the phenolic phase (as measured with pH paper) or of the buffer phase (as measured with a pH meter) was >7.8 (equilibrium). The phenol preparation was stored in a light-proof (brown) bottle at 4°C, with an overlay of 0.1M Tris.HCl (pH 8.0) containing 0.2% (v/v) β-mercaptoethanol.

Chloroform refers to a mixture of chloroform and isoamyl alcohol (24:1 v/v). The chloroform denatures proteins while isoamyl alcohol reduces foaming during the extraction of plasmid DNA.

APPENDIX 7: TRIS-EDTA (TE) BUFFER, pH 8.0

Per litre of distilled H_2O:-

10mM Tris : 1.212 g

1mM EDTA : 0.372 g

Dissolved in H_2O, the pH was adjusted to 8.0 with HCl, sterilized by autoclaving at 15 p.s.i. (121°C) for 20 min and stored at 4°C.

APPENDIX 8: PREPARATION OF RNase STOCK SOLUTION

Pancreatic RNase A (Sigma) was dissolved in TE (pH 8.0) at a concentration of 10 mg per ml (1% w/v) and heated to 100°C for 15 min in a boiling water bath to destroy any DNase. The solution was allowed to cool slowly to room temperature, dispensed into aliquots and stored at -20°C.

APPENDIX 9: PEG-NaCl SOLUTION

Per litre of distilled H_2O:-

13% (w/v) polyethylene glycol 800 (Sigma) : 130.0 g

1.6M NaCl : 93.5 g

Dissolved in H_2O by using a magnetic stirrer, filter-sterilized and stored at room temperature.

APPENDIX 10: TBE BUFFER

Per litre of distilled H_2O:-

	5X stock solution
89mM Tris base	: 53.9 g
89mM boric acid	: 27.5 g
2mM EDTA	: 20.0 ml of 0.5M EDTA (pH 8.0)

Dissolved in distilled H_2O by gently stirring with a magnetic stirrer and stored at room teperature.

APPENDIX 11: TAE BUFFER

Per litre of distilled H_2O:-

	10X stock solution
40mM Tris base	: 48.4 g
40mM Glacial acetic acid	: 11.4 ml
1mM EDTA	: 20.0 ml of 0.5M EDTA (pH 8.0)

Dissolved in distilled H_2O by using a magnetic stirrer and stored at room temperature.

APPENDIX 12: GEL-LOADING BUFFER

40% (w/v) sucrose

0.25% (w/v) bromophenol blue

Dissolved and mixed well in sterile distilled H_2O and stored in aliquots at 4°C.

APPENDIX 13: FRAGMENT SIZES OF DNA MARKERS

Supercoiled DNA Ladder	λ DNA/*Hind*III Fragments	φX174 RF DNA/ *Hae*III Fragments	1 Kb DNA Ladder
16.2 kb	23.1 kb	1353 bp	12216 bp
14.2 kb	9.4 kb	1078 bp	11198 bp
12.1 kb	6.6 kb	872 bp	10180 bp
10.1 kb	4.4 kb	603 bp	9162 bp
8.1 kb	2.3 kb	310 bp	8144 bp
7.0 kb	2.0 kb	271 bp	7126 bp
6.0 kb	0.56 kb	281 bp	6108 bp
5.0 kb	0.13 kb	234 bp	5090 bp
4.0 kb		194 bp	4072 bp
3.0 kb		118 bp	3054 bp
2.1 kb		72 bp	2036 bp
			1636 bp
			1018 bp
			517 bp
			506 bp
			396 bp
			344 bp
			298 bp
			220 bp
			201 bp
			154 bp
			134 bp
			75 bp

APPENDIX 14: SOC MEDIUM

A) SOC base

Per litre of distilled H_2O:-

2% tryptone	:	20.0 g
0.5% yeast extract	:	5.0 g
10mM NaCl	:	0.58 g
2.5mM KCl	:	0.18 g

The solutes were dissolved by shaking and the pH was adjusted to 7.0 with NaOH. The broth was sterilized by autoclaving for 20 min at 15 p.s.i. and stored at 4°C.

B) SOC additive

Per 10 ml H_2O (100X concentrate):-

20mM glucose	:	2.0 g
10mM $MgCl_2, 6H_2O$	:	2.03 g
10mM $MgSO_4, 7H_2O$	:	2.46 g

The solutes were dissolved by using a magnetic stirrer, filter-sterilized (0.45 μm acrodisc) and stored at 4°C. SOC additive was added to SOC base in a ratio of 1:100 just before use.

APPENDIX 15: PREPARATION OF DOUBLE DILUTIONS OF ANTIBIOTICS FOR MIC PLATE

A 20 ml stock solution of β-lactam antibiotic was prepared at the concentration of either 20480 μg ml^{-1}, 10240 μg ml^{-1}, 5120 μg ml^{-1} or 2560 μg ml^{-1}.

(Continued on next page)

Serial double dilutions of stock solutions were made as follows:-

Antibiotic solution		+	Sterile	=	Intermediate	=	Final concentration
Volume (ml)	$\mu g\ ml^{-1}$		H_2O (ml)		concentration ($\mu g\ ml^{-1}$)		in agar plates ($\mu g\ ml^{-1}$)
10	2560		...		...		256
10	2560		10		1280		128
10	1280		10		640		64
10	640		10		320		32
10	320		10		160		16
10	160		10		80		8
10	80		10		40		4
10	40		10		20		2
10	20		10		10		1
10	10		10		5		0.5

APPENDIX 16: 0.1M POTASSIUM PHOSPHATE BUFFER, pH 7.0

1M stock solution each of K_2HPO_4 (174.18 g per litre) and KH_2PO_4 (136.09 g per litre) were prepared separately in two bottles. Then 0.1M phosphate buffer, pH 7.0 was made up by adding together 61.5 ml of 1M K_2HPO_4 and 38.5 ml of 1M KH_2PO_4 stock solutions and making the volume up to 1 litre with distilled H_2O.

APPENDIX 17: IODINE REAGENT

Iodine reagent is a mixture of:

(Continued on next page)

0.0166N iodine

0.06M potassium iodide

2M sodium acetate buffer (pH 4.0)

A 20X stock solution of 0.0166N iodine (42.13 g per litre) and 0.06M potassium iodide (199.2 g per litre) was prepared and stored in a brown bottle at room temperature. The stock solution was diluted to 1X (i.e. 1:20 v/v) with 2M Na-acetate buffer (pH 4.0) just before use.

APPENDIX 18: BRADFORD REAGENT

100 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of 95% ethanol. The solution was mixed with 100 ml of 85% (w/v) phosphoric acid, diluted with distilled H_2O to 1 litre, filtered and stored at room temperature.

APPENDIX 19: TERRIFIC BROTH

A) Basal medium

To 900 ml of distilled H_2O were added:-

Tryptone : 12 g

Yeast extract : 24 g

Glycerol : 4 ml

B) Additive

To 100 ml of distilled H_2O were added:-

0.17M KH_2PO_4 : 2.31 g

0.72M K_2HPO_4 : 12.54 g

Both basal medium and additive were made up separately, sterilized by autoclaving and mixed together when cooled to 60°C or less.

APPENDIX 20: COMPOSITION OF REACTION BUFFERS

The composition given below is 1X or the working concentration.

REact™1:	50mM Tris.HCl (pH 8.0)
	10mM $MgCl_2$
REact™2:	50mM Tris.HCl (pH 8.0)
	10mM $MgCl_2$
	50mM NaCl
React™3:	50mM Tris.HCl (pH 8.0)
	10mM $MgCl_2$
	100mM NaCl
REact™4:	20mM Tris.HCl (pH 7.4)
	5mM $MgCl_2$
	50mM KCl
REact™6:	50mM Tris.HCl (pH 7.4)
	6mM $MgCl_2$
	50mM KCl
	50mM NaCl
REact™8:	20mM Tris.HCl (pH 7.4)
	10mM $MgCl_2$
React™10:	100mM Tris.HCl (pH 7.6)
	10mM $MgCl_2$
	150mM NaCl
Assay buffer 1:	25mM Tris.HCl (pH 7.7)
(Stratagene)	10mM $MgCl_2$
	1mM DTT
	100 µg/ml BSA

APPENDIX 20 (Continued)

*Bsc*I buffer:	6mM Tris.HCl (pH 7.6)
(NBL)	150mM NaCl
	6mM $MgCl_2$
	6mM 2-mercaptoethanol
Buffer A:	33mM Tris-acetate
(Boehringer	66mM K-acetate
Mannheim)	10mM Mg-acetate
	0.5mM DTT
	pH 7.9
*Xho*II buffer:	6mM Tris.HCl (pH 8.0)
(ABL)	10mM $MgCl_2$
	1mM DTT
	100 µg /ml BSA
	0.01% TRITON X 100

APPENDIX 21: PREPARATION OF DENATURED SALMON SPERM DNA (10 mg ml^{-1})

50 mg of salmon sperm DNA (Sigma type III, Na-salt) was dissolved in 5 ml of distilled H_2O and, if necessary, stirred on a magnetic stirrer for 2-4 h at room temperature. The DNA was sheared by repeated passage (rapidly ~12 times) through a 17- or 19-gauge hypodermic needle, then boiled for 10 min and stored at -20°C.

100 µl of the DNA solution was heated to 100°C in a boiling water bath for 5 min and then chilled quickly in ice-water before use.

APPENDIX 22: PREHYBRIDIZATION/HYBRIDIZATION SOLUTION

Prehybridization or hybridization solution (10 ml) (high stringency conditions) was prepared as follows:-

Stock solution	Volume	Final concentration
20X SSC	2.5 ml	5X
50X Denhardt's solution*	1.0 ml	5X
100% deionized formamide	5.0 ml	50%
20%(w/v) SDS	0.25 ml	0.5%(w/v)
Sterile distilled H_2O	1.25 ml	To final volume.

* 50X Denhardt's solution

1%(w/v) BSA (bovine serum albumin, Fraction V; Sigma)

1%(w/v) Ficoll (Type 400, Pharmacia)

1%(w/v) PVP (polyvinylpyrrolidone, Sigma)

Dissolved in distilled H_2O, filtered through Whatman paper and stored at -20°C.

APPENDIX 23: PREPARATION OF LABELLED DNA PROBE (50 µl)

Plasmid DNA was digested with a restriction enzyme and then purified by phenol:chloroform extraction followed by ethanol precipitation. The purified DNA was redissolved in distilled H_2O and brought to volume of 34 µl, denatured by heating to 100°C in a boiling water bath for 5 min, cooled immediately in ice-water and then incubated at 37°C for 10 min. The following were then added to a fresh microfuge tube in the given order:-

APPENDIX 23 (Continued)

DNA solution	: 34 µl
OLB (Oligo-labelling buffer)*	: 10 µl
1%(w/v) BSA (Sigma)	: 2 µl
Klenow fragment of DNA polymerase I (Gibco-BRL)	: 2 µl
[α-^{32}P]dATP (10 µCi/µl) (Amersham)	: 2 µl

The reaction was mixed well and incubated overnight at room temperature.

* OLB (Oligo-labelling buffer)

OLB was made up as follows:-

Solution O: 1.25M Tris.HCl (pH 8.0)
0.125M $MgCl_2$.

Solution A: 1 ml solution O
18 µl β-mercaptoethanol
5 µl dGTP (100mM in TE)
5 µl dCTP (100mM in TE)
5 µl dTTP (100mM in TE).

Solution B: 2M Hepes (pH 6.6, using 4M NaOH).

Solution C: Hexadeoxynucleotides (Pharmacia) suspended in TE to 90 OD units per ml.

Solutions A, B and C were mixed in the ratio of 100:250:150 and stored at -20°C.

APPENDIX 24: T4 DNA LIGASE BUFFER

Working concentration (1X):-

50mM Tris.HCl (pH 7.6)

10mM $MgCl_2$

1 mM ATP

1mM DTT

5%(w/v) PEG-800

Supplied by Gibco-BRL and stored frozen at -20°C.

APPENDIX 25: PREPARATION OF LB+X-GAL MEDIA

20 mg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) was dissolved per ml in dimethyl formamide (Sigma) just before use.

200 mg of IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma) was dissolved per ml in distilled water, filter-sterilized and stored at -20°C.

1 ml of X-gal and 100 µl of IPTG (each 100 µg ml^{-1} final concentration) were added to 200 ml of melted LB agar, mixed by gentle rotation or inversion of the bottle and then poured into petri dishes (~15 plates).