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PhD thesis

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**Construction and characterisation of attenuated
derivatives of *Pasteurella multocida*
serotype B:2 strains**

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**Presented for the Degree of Doctor of Philosophy in the Faculty
of Science, University of Glasgow**

Division of Infection and Immunity

July, 2000

Construction and characterisation of
attenuated derivatives of
Pasteurella multocida
serotype B:2 strains

MOHAMMAD TABATABAEI

DECLARATION

The thesis is the original work of the author:

M. Tabatabaei

DEDICATION:

This thesis is dedicated to my wife, Vahideh and my daughters, Zahra and Rayehe, who are always sources of mental support, happiness, love and inspiration for my future.

It is also dedicated to my parents, who supported me throughout my academic career.

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ABBREVIATIONS:

A	Adenine
A: 1	<i>Pasteurella multocida</i> biotype A capsular serotype 1
A: 3	<i>Pasteurella multocida</i> biotype A capsular serotype 3
AA	Amino acid
AB	Absorbance
Ab	Antibody
AC	Adenylate cyclase
Ag	Antigen
APV	Alum precipitated vaccine
ApR	Ampicillin resistance
ApS	Ampicillin sensitive
<i>aroA</i>	Aromatic amino acid metabolism gene
ATCC	American type culture collection
ATP	Adenosine triphosphate
B	Blank
B: 2	<i>Pasteurella multocida</i> biotype B capsular serotype 2
BB	Broth bacterin
BA	Blood agar
BCG	Bacille Calmette-Guerin (attenuated derivative of <i>Mycobacterium tuberculosis</i>)
BHIA	Brain heart infusion agar

BHIB	Brain heart infusion broth
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
cAMP	3',5' cyclic adenosine monophosphate
Carb	Carbenicillin
C.F.U.	Colony forming unit
CIP	Calf intestinal phosphatase
cm	Centimetre
Cm	Chloramphenicol
CMI	Cell-mediated immunity
Conc	Concentration
cpm	Counts per minute
CPS	Capsular polysaccharide
CSPD	Disodium 3-(4-methoxy Spiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1 ^{3,7}]decan}-4-yl) phenyl phosphate
CTAB	Hexadecyl trimethyl-ammonium bromide
μCi	Micro curie
<i>cya</i>	adenylate cyclase gene
<i>crp</i>	cyclic AMP receptor protein gene
Cya	adenylate cyclase phenotype

Δ	Deletion
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
DEV	Double emulsion vaccine
dGTP	Deoxyguanine triphosphate
DIG	Digoxigenin
DH ₂ O	Distilled water
DHBA	Dihydrobenzoic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate
E: 2	<i>Pasteurella multocida</i> biotype E capsular serotype 2
EDTA	Ethylenediamine tetra-acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
fmol	Femtomol (10 ⁻¹⁵ mol)
g	Gram (s)
G	Guanine
Gm ^R	Gentamycin resistance
h	Hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hrs	Hours

H ₂ O	Distilled water
HA	Haemagglutination
HS	Haemorrhagic septicaemia
i.m.	Intramuscular
i.n.	Intranasal
Ig	Immunoglobulin
IHA	Indirect haemagglutination
IPTG	Isopropyl-B-D-thiogalactopyranoside
<i>galE</i>	Galactose metabolism gene
kb	Kilobase pair
kDa	Kilo Dalton
kg	Kilogram
Km ^R	Kanamycin resistance
Km ^S	Kanamycin sensitive
L	Litre
<i>lac</i>	Lactose operon gene
LB	Luria Bertani
LD ₅₀	Median lethal dose
LMIT	Leukocyte migration inhibition test
LPS	Lipopolysaccharide
μg	Microgram (s)
μl	Microlitre (s)

M	Molar
MCS	Multi-cloning site
Me β CD	Heptakis (2,6-O-dimethyl) β -cyclodextrin
MEV	Multi-emulsion vaccine
μ FD	Microfarad
mg	Milligram (s)
min	Minute (s)
ml	Millilitre (s)
mm	Millimetre (s)
mM	Millimolar
ng	Nanogram
nm	Nanometre
NMG	N-methyl-N-nitro-N-nitrosoguanidine
NSB	Non-specific binding
nt	Nucleotide
OAV	Oil-adjuvant vaccine
OD	Optical density
Ω	Ohm
OM	Outer membrane
OMP	Outer-membrane protein (s)
PABA	Para amino benzoic acid
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
pg	Picogram
pH	Hydrogen ion concentration
PI	Post inoculation
P. m	<i>Pasteurella multocida</i>
PMM	Pasteurella minimal medium
Pm	Polymyxin
PP	Pyrophosphate
psi	Pounds per square inch
R	Rough (lipopolysaccharide)
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
S	Smooth (lipopolysaccharide)
<i>sacB</i>	Sucrose metabolism gene
s.c.	Subcutaneous
SDS	Sodium dodecyl sulphate
Sec	Second (s)
Sm	Streptomycin
SSC	Standard saline citrate
T	Thymine
TBE	Tris borate EDTA

Tc ^R	Tetracycline resistance
TE	Tris EDTA
TMB	Tetramethylbenzidine
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris hydrochloride
U	Unit (s)
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volt (s)
<i>vacA</i>	Vacuolating toxin gene
v/v	Volume/ volume ratio
W/v	Weight/ volume ratio
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galctoside

SUMMARY:

The project was concerned with the construction of defined attenuated derivatives of *P. multocida* serotype B:2 strains, causative agents of haemorrhagic septicaemia, and attempts were made to construct defined mutations in genes such as *aroA*, *cya*, and *galE* loci that have been used to induce attenuation in other bacterial strains.

Mutants defective in the *aroA* gene were constructed by allelic exchange of the locus in the chromosome of the wild-type strains with a cloned *aroA* gene interrupted with a cassette encoding kanamycin resistance (Km^R). The *aroA* defective strains were confirmed by PCR, Southern blotting, lack of growth on minimal medium and by enzyme assay.

Km^R -inactivated *aroA* mutants JRMT1 and JRMT2 strains derived from *P. multocida* 85020 and Quetta strains, respectively, were highly attenuated in a mouse model, with an $LD_{50} \geq 10^8$ C.F.U./mouse after injection intraperitoneally (i.p.). In contrast, the wild-type strains had $LD_{50} < 50$ C.F.U./ mouse by this route. Vaccination once by the i.p. route or twice by the i.n. route with these *aroA* mutants gave complete protection to the mice against subsequent challenge i.p. with 10,000 LD_{50} of the homologous wild-type strain or 1000 LD_{50} of the heterologous wild-type strain. Vaccination with these by the s.c. route was not protective. When high doses of the attenuated strains were inoculated by the i.p. or i.n. routes, there was some spread to the internal organs but the organisms were cleared by 24 and 72 hrs respectively. In contrast, the wild-type parent strains spread rapidly and multiplied in high numbers and killed the mice by 24 and 96 hrs respectively.

Attempts were made to construct *cya* mutants, by insertion of Km^R or Ap^R cassettes into the *cya* gene by allelic exchange, but this was only partially successful. Only a single *cya*::Km^R clone that produced the correct predicted size bands by Southern blot hybridisation was obtained with the Quetta strain. This strain showed some attenuation, with an LD₅₀ about 1000 C.F.U./mouse by the i.p. route, but did not provide any protection in the mouse against challenge with the wild-type parent strain. Also, insertion of a Km^R cassette into the *galE* locus did not prove to be successful and no strains were obtained with the predicted genotype.

For construction of a marker-free *aroA* mutant, different procedures were attempted. These included selection against the vector and combinations of repeated subculturing and auxotrophic enrichment, but these were unsuccessful. Finally, for the first time in *Pasteurella* strains, the *sacB* gene was used successfully as a positive selection marker. A broad-host-range suicide plasmid pJRMT30 containing the *sacB* gene of *Bacillus subtilis* was constructed for use in the positive selection for marker-free construction in Gram-negative bacteria. *P. multocida* cells containing pJRMT30 do not grow on a medium containing 5% (w/v) sucrose and loss of the plasmid can be monitored by the capacity to grow on this medium. With this procedure, marker-free *aroA* mutants of *P. multocida* 85020 and Quetta strains, JRMT12 and JRMT13 respectively, were constructed. These two deleted *aroA* mutant strains showed attenuation and protection properties similar to the Km^R-interrupted *aroA* mutants. This strategy was not successful however for construction of a deleted *galE* mutant.

The *P. multocida* strain 85020 and its *aroA* mutant, JRMT1, were tested in a phagocytosis assay, with a macrophage-like cell line, to examine the uptake

and survival of the strains within the intracellular environment. Both strains were taken up into the macrophage-like cells but the *aroA* strain JRMT1 was able to enter more efficiently than its wild-type parent strain. Both wild-type and mutant were able to survive for at least 2 hrs in the phagocytic cells.

1. INTRODUCTION

1. 1. The family *Pasteurellaceae*

As reviewed by Bisgaard (1995), the family *Pasteurellaceae* pohl 1981 was conceived to accommodate a large group of Gram-negative chemoorganotrophic, facultatively anaerobic, and fermentative bacteria including the genera *Pasteurella*, *Actinobacillus*, *Haemophilus* and several other groups of organisms that exhibit complex phenotypic and genotypic relationships with the aforementioned genera.

1. 2. The genus *Pasteurella*

DNA:DNA hybridisations have shown that this genus consists of at least 11 species some of which have not yet been named. The *Pasteurella sensu stricto* includes *Pasteurella multocida* with 3 subspecies (*multocida*, *septica*, and *gallicida*), *P. dagmatis*, *P. gallinarum*, *P. canis*, *P. stomatis*, *P. avium*, *P. volantium*, *P. anatis*, *P. langaa* and *Pasteurella* species A and B. Species and taxa excluded from the genus include [*P.*] *aerogenes*, [*P.*] *haemolytica* biotypes A and T, *P. multocida* biotype 1, [*P.*] *pneumotropica* biotypes Heyl and Jawetz, [*P.*] *testudinis*, [*P.*] *urea*, and the SP-group. The bacteria in [*P.*] *haemolytica* biotypes A and T have been reclassified by taxonomists twice since 1990. The former T. biotype is now *P. trehalosi* and the A biotype has been placed in a new genus, *Mannheimia* (Angen *et al.*, 1999). The majority of the old *Pasteurella haemolytica* A biotype serotypes are now known as *Mannheimia haemolytica*.

In *Bergey's Manual*, the genus *Pasteurella* is listed under the heading "Gram-negative Facultatively Anaerobic Rods: Genera of Uncertain

Affiliation" as type genus of the family *Pasteurellaceae*. The type species of the genus is *P. multocida* and has now been widely accepted.

Bacteria included in the genus *Pasteurella* are commensal and occasional pathogens of many species of domestic and wild animals. The principal characteristics of members of the genus are: small, Gram-negative rods or coccobacilli with size ranging from 0.3-1.0 μm in diameter and 1.0-2.0 μm in length, non-motile, facultatively anaerobic, and they display a characteristic bipolar staining with either Leishman or methylene blue stains. The organisms do not grow on MacConkey's agar and they are non-haemolytic on blood agar. On nutrient agar they produce fine, translucent colonies with a characteristic musty odour. *P. multocida* organisms are oxidase, catalase and indole positive. They ferment sugars such as glucose, sucrose, sorbitol and mannitol with acid, but with no gas production. Lactose, maltose and salicin are not fermented, while variable fermentation results are obtained with arabinose, trehalose and xylose. Gelatin is not liquefied.

1. 3. The species *Pasteurella multocida*

Pasteurella multocida has been recognised as an important veterinary pathogen for over a century, and its importance as a human pathogen causing bite wounds, respiratory diseases and infections of the central nervous system (Fredriksen, 1989) has been increasingly recognised in the last 50 years. The organism can occur as a commensal in the naso-pharyngeal region of apparently healthy animals and it can be either a primary or secondary pathogen in disease processes of a variety of domestic and wild mammals and birds. Distributed throughout the world, it has been found among many varieties of both terrestrial and aquatic species of mammals and birds (Rimler and Rhoades, 1989).

1. 3. 1. Cell morphology and staining

P. multocida is a non-motile, non-sporogenous coccobacillus or short rod, which stains Gram-negative. Cells can occur singly, in pairs and occasionally as chains or filaments. In tissues, exudates and recently isolated cultures, the bacterium shows bipolar staining with Giemsa or Wright's stain. Cells usually measure 0.2-1.25 μm in length but after repeated subculture they tend to become pleomorphic.

Virulent strains of *P. multocida* are usually capsulated and their capsules can be seen in organisms isolated from tissues of diseased animals and laboratory cultures. The capsules are composed of carbohydrate and differ in size and chemical composition amongst the varieties of *P. multocida*. They are frequently lost upon repeated subculture. Several techniques have been used to demonstrate capsules for light microscopy. The alcian blue, Congo red and crystal violet methods have been used for direct staining while the Jasmin and Indian ink methods have been used for indirect staining (Rimler and Rhoades, 1989). Recently, the capsule has been demonstrated to be an important virulence factor (Boyce and Adler, 2000).

1. 3. 2. Colonial morphology

Generally, description of colonies is made from 18-24 hrs cultures grown aerobically at 35-37°C on enriched media. Under these conditions, colonies usually range in size from 1.0 to 3.0 mm in diameter. Ordinary cultures on agar media may develop into either or both of two principal colony forms, mucoid and smooth. Rough colony forms are encountered infrequently.

The largest smooth colonies are mucoid and they are composed of capsulated cells. The mucoid characteristic can range from cultures whose colonies are discrete, circular, and convex and have a slight mucus-like consistency to cultures whose colonies are confluent, markedly moist and

viscous. Smooth colonies are composed of capsulated or noncapsulated cells. They are discrete, circular and convex. Rough colonies, which consist of filamentous noncapsulated cells, have been described as being flat with slightly raised central papillae and flattened irregularly serrated edges or discrete, raised, circular, rough and slightly dry (Rimler and Rhoades, 1989).

Colonial morphology on transparent media observed in obliquely transmitted light with a stereomicroscope is a useful characteristic for the study of *P. multocida*. Colonies refract the light in a manner that helps distinguish variants. Except for watery mucoid colonies, which appear grey, colonies consisting of capsulated cells display a yellowish-green, bluish green or pearl-like iridescence. Composition of the medium determines the degree and type of iridescence to a certain extent. Colonies that consist primarily of noncapsulated cells are not iridescent and appear blue, greyish-blue, or grey. Dissociation, which is manifested primarily by a change from an iridescent to a blue or greyish-blue colony form, is associated with a loss of the bacterial capsule.

A characteristic mucoid colony has been found in all serogroup A and a few serogroup D strains (See section 1. 4. 4). All strains of *P. multocida* that produce colonies of the large watery mucoid variety have been found to belong to serogroup A. Colonies of serogroup A, D and F strains display a pearl-like iridescence in oblique transmitted light, except where watery mucoid colonies of serogroup A appear grey. Serogroup B and E strains produce the smallest capsules and form smooth colonies that usually have a yellowish- or bluish-green iridescence in oblique transmittent light.

Dissociation of colonies from iridescence to non-iridescence and the concomitant loss of the capsule of a virulent strain are associated with reduction or loss of virulence (Heddleston *et al.*, 1964; Penn and Nagy, 1974). Capsule production, iridescence and virulence can sometimes be regained or enhanced by repeated passage in mice (Rimler and Rhoades, 1989).

1. 3. 3. Cellular components

1. 3. 3. 1. Capsule antigens

The capsule antigen of *P. multocida* responsible for serotype specificity is intimately associated with lipopolysaccharides (LPS), as well as non-antigenic polysaccharide material (Rimler and Rhoades, 1989). Both the capsule-specific antigen and LPS can be adsorbed onto erythrocytes from crude cell extracts (Rimler and Rhoades, 1989). However, passive haemagglutination tests, with serum containing antibodies against the capsule-specific antigen and LPS, usually show a reaction only with the capsule-specific antigen (Rimler and Rhoades, 1989). Nagy and Penn (1976) prepared capsular antigens of *P. multocida* serotypes B and E and showed that they were immunogenic and protective in cattle against serotype E challenge. However, non-capsulate and capsulate organisms of serotype A, strain X-73, were reported to be equally effective in immunising birds against fowl cholera (Heddleston *et al.*, 1964).

1. 3. 3. 2. Lipopolysaccharides

P. multocida LPS has chemical and biological properties similar to those found in many species of Gram-negative bacteria. As antigens, LPSs have been associated with immune protection of animals and are believed to be the chemical basis for the specificity of the somatic typing system (Broden and Rebers, 1978).

LPSs have been purified from many different strains of *P. multocida*. In common with LPSs from bacteria of the family *Enterobacteriaceae*, chemical analysis has shown that they contain lipid A, 2-keto-deoxyoctonate, L-glycero-D-mannoheptose, glucose and glucosamine. Other sugars encountered amongst LPSs from different strains were galactose, rhamnose, D-glycero-D-mannoheptose and galactosamine (Rimler *et al.*, 1984). Although a possible structure for the heptose region has been described, the complete structure of *P.*

multocida LPS has not been determined (Rimler and Rhoades, 1989). Long polysaccharide chains (O-antigen), like those found in S-type LPSs of *Salmonella* and other *Enterobacteriaceae*, have not been demonstrated in *P. multocida*. Chemical and physical evidence suggest that typical *P. multocida* LPSs are similar to the R-type LPSs (Rimler *et al.*, 1984; Manning *et al.*, 1986).

Purified *P. multocida* LPS is antigenic and can react with avian and mammalian antisera made against the whole organism, but has been reported to produce little or no active immune protection for mice (Schmerr and Rebers, 1979; Rebers *et al.*, 1980) or cattle (Erler *et al.*, Cited by Rimler and Rhoades, 1989) against challenge with the homologous organism but does provide protection in chickens (Rebers *et al.*, 1980). In chickens, LPSs from some serotypes produced antibodies whereas others did not (Rimler *et al.*, 1984). Wijewardana *et al.* (1990) found that an anti-LPS monoclonal antibody protected mice against homologous and heterologous challenge with serotype A strains.

Proteins of *P. multocida* are generally believed to be important immunogens. However, with the exception of an immunogenic protein toxin from a serotype D strain (see below), purification of a *P. multocida* protein antigen to homogeneity and its biochemical characterisation has not been done. Kajikava and Matsumoto (1984) partially purified proteinaceous immunogens found in extracts or culture filtrates from avian strains of *P. multocida* and they found that their purified immunogens produced protection against homologous serotype.

1. 3. 3. 4. Neuraminidase

Serogroups A, B, D and E produce neuraminidase (Scharmman *et al.*, 1970). Drzeniek *et al.* (1972) found that highest neuraminidase activity

occurred in serogroup A and D while low activity occurred in serogroup B strains. Maximum activity occurred between 6-10 hrs (log phase) in basal medium and at 50-70 hrs (decline phase) in complex medium (Drzeniek *et al.*, 1972).

Neuraminidase has been associated with virulence in other microorganisms but the association of *P. multocida* neuraminidase with a specific disease process or its role as a virulence factor is inconclusive. Drzeniek *et al.* (1972) indicated there was no correlation between virulence in mice and neuraminidase activity. Both neuraminidase positive and negative strains of serotype A were equally virulent. Muller and Krasemann (Cited by Rimler and Rhoades, 1989), in a study consisting primarily of dog and cat strains, observed that strains with rapid *in vitro* growth and high neuraminidase activity were more virulent for mice than those with low activity. Slow growing strains were less virulent than rapid growing strains.

1. 3. 3. 5. Hyaluronidases

Hyaluronidases are enzymes that have been associated with invasive mechanisms of bacteria and helminths (Rimler and Rhoades, 1994). Several Gram-positive bacteria produce hyaluronidase and it has been associated with their virulence (Rimler *et al.*, 1984). Carter and Chengappa (1980) established that serogroup B strains isolated from cases of haemorrhagic septicaemia produced hyaluronidase. Carter and Chengappa (1991) reported that all haemorrhagic septicaemia *P. multocida* strains of serotype B:2 produced a hyaluronidase, whereas strains in serogroup A, D and E did not. The role of hyaluronidase produced by type B strains in pathogenesis is as yet uncertain, especially in view of the fact that it has not been demonstrated in type E strains, which also cause HS (De Alwis, 1995). Rimler and Rhoades (1994) examined hyaluronidase production in 176 strains of *P. multocida* representing different

serotypes isolated from different animal species in different countries. They found that all 82 serotype B:2 strains had activity against hyaluronic acid.

1. 3. 3. 6. Toxins

Two main types of toxin are found in *P. multocida*, endotoxin and protein toxins. Endotoxic activity of *P. multocida* is due to LPS, the structural component of outer membrane of all strains. There is no essential difference in biological and toxic effect of *P. multocida* endotoxins and endotoxins of other Gram-negative bacteria (Rimler *et al.*, 1984).

It is well established that only some isolates of serogroup A or D produce a dermonecrotic protein toxin. Rimler and Brogden (1986) found that although toxin production occurs in different serotype of both serogroups A and D, no correlation was found between production of toxin and somatic serotype. Of these serogroups, toxin appears to occur more often in serogroup D (Foged *et al.*, 1987). Antiserum, made against the toxin from a swine serogroup D strain, neutralised the lethal effects of toxin from rabbit serogroup D and swine serogroup A and D strains from different geographic origins (Rimler and Rhoades, 1989). Consequently, the toxins of serogroups A and D strains seem to be antigenically similar. Foged *et al.* (1987) purified a proteinaceous substance produced by a toxigenic type D strain of *P. multocida* and characterised with a molecular weight about 143 kDa. The purified *P. multocida* toxin showed a dermonecrotic effect in guinea pigs, cytopathic effect in cultures of embryonic bovin lung cells and was lethal to mice. Also Bording and Foged (1991) evaluated the immunogenicity of formaldehyde detoxified *P. multocida* toxin and they found that pure toxoid was immunogenic and protected mice against challenge with *P. multocida* toxin. Chanter *et al.* (1986) purified a protein from sonicated cells of a serotype D *P. multocida* strain LFB3. The partially purified protein was cytotoxic for embryonic bovine lung cells, lethal for mice and caused turbinate atrophy in pigs; a single

intraperitoneal injection of approximately 360 ng/ kg caused 50% turbinate atrophy.

Recently, Shah *et al.* (1996) investigated the effect of *P. multocida* serotype B:2,5 in macrophages by *in vivo* and *in vitro* tests, and have shown that the haemorrhagic septicaemia-associated strains of *P. multocida* produced a vacuolating cytotoxin. This cell-associated and extracellular vacuolating cytotoxic activity of *P. multocida* serotypes B and E strains caused large cytoplasmic vacuoles in mouse peritoneal macrophages and in a mouse macrophage cell line, and eventually cell death. In contrast, the macrophage cell line treated with serotypes A and D strains did not show any vacuoles.

1. 3. 4. Biotypes of *P. multocida*

The species *P. multocida* incorporates a heterogenous group of types with differences in pathogenicity, host preferences, serological and antigenic characteristics, and cultural and biochemical properties. Classification is based on the capsular and somatic antigens. Five main serological typing systems of *Pasteurella multocida* have been developed, the number of serotypes identified by each method and the position of the haemorrhagic septicaemia (HS) serotypes is shown in Table 1.

Isolates from HS cases were typed as group 2 (Little and Lyon, 1943) and type I (Roberts, 1947) using slide agglutination and passive mouse protection test respectively. Carter (1955, 1961) defined HS strains as serotypes B and E. Namioka and Murata (1961 a,b), with a combination of Carter capsular types and somatic antigen typing, defined Asian and African HS strains as 6:B and 6:E respectively. Heddleston *et al.* (1972) developed an agar gel diffusion test which found HS strains to type as 2 and 5.

Serotype designation is thus based on a combination of somatic and capsular antigens. The more popular method at present is to use the Carter:Heddleston

Table 1: Methods adopted for serotyping of *Pasteurella multocida* and the position of the HS serotypes

References	Technique	No. of types	Position of HS strains
Little and Lyon (1943)	Slide agglutination and passive mouse protection tests	1, 2 and 3	2
Roberts (1947)	Passive mouse protection test	I-V	I
Carter (1955, 1961) Rimler and Rhoades (1987)	"Capsular" typing indirect hemagglutination test using heat labile (56 °C/30 min) antigen and rabbit antisera*	A,B,E and F	B and E
Namioka and Murata (1961a)	Simplified capsular typing using fresh cultures and rabbit antisera	A, B, D and E	B and E
Namioka and Murata (1961b,c) Namioka and Bruner (1963) Namioka and Murata (1964)	"Somatic" typing by agglutination test using HCl treated cells and rabbit antisera	1-11	6
Heddleston <i>et al.</i> , (1972)	"Somatic" typing by gel diffusion precipitin test using heat stable antigen and chicken antisera	1-16	2 and (5)

* Originally used fresh human 'O' erythrocytes. Subsequently modified by Carter and Rappay (1962) using formalinised cells, by Sawada *et al.*, (1982) using glutaraldehyde fixed sheep erythrocytes and by Wijewardana *et al.*, (1986) using fresh sheep erythrocytes.

system. By this system, the Asian and African HS serotypes are designated B:2 and E:2 respectively.

1. 3. 5. Diseases caused by *P. multocida*

P. multocida spp. cause septicaemia and uncomplicated or complicated respiratory diseases in domesticated and wild animal species. Two specific diseases in which this organism is the primary agent are haemorrhagic septicaemia (HS) in cattle and water buffalo, and fowl cholera in poultry. These are major bacterial diseases, of major economic and veterinary importance throughout the world. They are usually manifested as either an acute septicaemia or a chronic respiratory infection. Strains causing haemorrhagic septicaemia usually belong to serotypes B and E. Those of group A are frequently associated with fowl cholera. Serogroup D strains cause pneumonia in cattle and atrophic rhinitis in pigs (Rutter, 1985) and strains of the serogroup F vary in virulence for turkeys (Rimler and Rhoades, 1987).

1. 3. 5. 1. Haemorrhagic septicaemia (HS)

HS is an acute septicaemic disease characterised by terminal septicaemia and high mortality, principally affecting cattle and buffalo, the latter being considered more susceptible. The disease is distinctly different from some other pasteurelloses where *Pasteurellae* play only a secondary role. HS is thus defined as a specific form of pasteurellosis in cattle and buffaloes as much as Typhoid fever and Pullorum are specific forms of salmonellosis in man and poultry respectively (De Alwis, 1992). *P. multocida* serotype B:2 (6:B) and E:2 (6:E) have been found to cause HS. In Asia only serotype B: 2 strains have been associated with the disease. In Africa serotype E:2 strains have been the only serotype isolated from cases of HS, except for Sudan and Egypt where both types have been recorded (De Alwis, 1992). In North America, serotypes B:2 and B:3,4 have been reported as causative agents of HS in beef calves and bison (Rimler and Wilson, 1994). Septicaemic pasteurellosis has also been

reported in pigs in Sri Lanka, Thailand, Malaysia and India (Wijewardana, 1992). HS-causing *P. multocida* has been isolated from goats in Malaysia and India; however, these animals have been found to be very resistant to disease (Wijewardana, 1992). Septicaemic pasteurellosis in elephants with concurrent outbreaks of HS in cattle and buffalo caused by the same serotype B:2 have been reported in Sri Lanka (Wijewardana, 1992). Fatal septicaemia in horses and donkeys associated with HS-causing B:2 have been reported in India (Wijewardana, 1992). There are reports of serotype B:2, 3 being recovered from poultry, but these strains are considered either weakly or non-pathogenic to poultry (Wijewardana, 1992).

HS occurs in South East Asia, Near and Middle East, Southern Europe and in most part of Africa. Generally, North American cattle are regarded as free of the condition though it has been recorded in bison in game parks. In South America it is said to occur; however, it has not been confirmed by laboratory procedures. In Japan, the last recorded outbreak was in 1954, while Australia, Ocean, Canada and Western Europe have never recorded the disease (Carter and De Alwis, 1989). It is economically important, especially in the South Asia region where buffaloes provide the power for rice cultivation. The study of the disease may provide parallels with human diseases characterised by a septicaemic endotoxic phase.

1. 3. 5. 2. Epidemiology

Buffaloes are more prone to the disease. The incidence is directly related to animal husbandry practices, the incidence being higher in large nomadic herds compared to small intensively-reared herds (Wijewardana, 1992). In endemic areas the outbreaks are seasonal and the link between successive outbreaks is believed to be the carrier animals. The presence of the organism in the nasopharynx of healthy animals is well established (Mustafa *et al.*, 1978). The nasopharyngeal carrier state is followed by an immune state, which persists for

a long period (Wijewardana, 1992). The HS-causing organism is readily recovered from such sites as tonsils and lymph nodes associated with the head and neck, but it has been observed that recovery of the organism from the nasopharynx and other sites is intermittent. Experimental infection tests have also shown that large numbers of organisms are required to set up an infection by the intranasal or oral route.

It is generally believed that HS is associated with wet, humid weather and most outbreaks occur during the wet seasons. In countries where systematic epidemiological studies have been done, it has become evident that outbreaks do occur at all times of the year but those occurring during wet seasons tend to spread, presumably due to the longer survival of the organism under moist conditions (De Alwis, 1992). In the endemic areas outbreaks usually coincide with onset of rains (Francis *et al.*, 1980), which is normally preceded by a long dry period with limited food. These adverse climatic factors and accompanying nutritional deficiencies may well precipitate the activation of the carrier to become a source of infection for susceptible animals. Maternally-derived immunity wanes with age. Consequently the age group between 6 months-2 years is most susceptible, and this is the major group which contracts the disease (De Alwis, 1992). The epidemiological cycle is outlined in Figure 1.

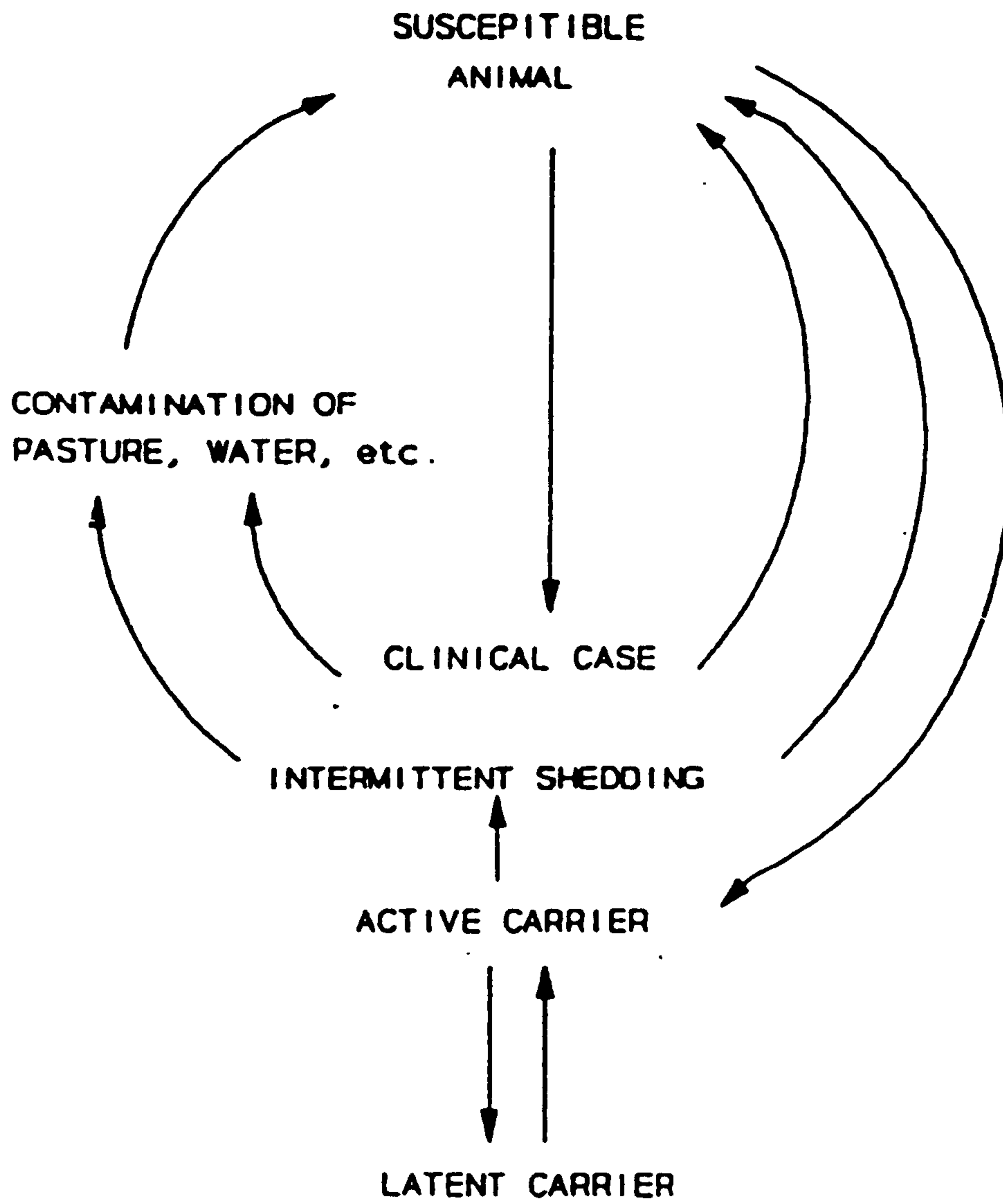
1. 3. 5. 3. Pathogenesis

The successful transmission of disease by the intranasal and oral routes, producing a syndrome with clinical signs and lesions resembling natural disease indicates that these may be the natural routes of infection (De Alwis, 1995). The organisms isolated from clinically normal animals even in the latent carrier state, have been found to be as virulent as those from clinical cases. Also it was established that carrier animals possessed high levels of humoral immunity thus maintaining a host-pathogen balance. It appears that after entry into an unexposed susceptible animal, the initial site of multiplication is the

Fig 1: The presumptive epidemiological cycle in haemorrhagic septicaemia

(From De Alwis, 1995)

Fig 1:



tonsillar tissue. Thus on the one hand the pathogen will multiply *in vivo* and on the other the host defence mechanisms both specific and nonspecific will interact and, depending on which process dominates, will lead either to clinical disease or arrested infection. The dose of infecting organisms, amongst other factors, will be an important determinant of outcome. The role of bacterial adherence in the multiplication of *Pasteurella* in the respiratory tract in HS is an area that merits investigation. This phenomenon could be an important determinant of the outcome of infection. Endotoxin is likely to play an important role in HS, as similarities were found in the pathological lesions in natural HS and endotoxic shock in calves (De Alwis, 1995).

1. 3. 5. 4. Clinical features

This disease is usually manifest by a rapid course, sudden onset of fever, profuse salivation, severe depression and death in about 24 hrs. These are the usual symptoms observed in HS. Oedema in the submandibular region and in later stages the affected animals may show severe dyspnoea and alimentary tract disorders. In outbreaks, sudden death without any previous signs is usually reported. As the disease progresses, respiratory distress becomes severe and the temperature drops to subnormal levels. Finally the animal becomes recumbent and dies.

1. 3. 5. 5. Pathology

At necropsy the gross findings are usually limited to generalised petechial haemorrhages, particularly under the serosae, and oedema of the lungs and lymph nodes. Subcutaneous infiltration of gelatinous fluid may be present and in some animals there are lesions of early pneumonia and haemorrhagic gastroenteritis. The spleen may show a few haemorrhages but is not swollen, and this is a means of differentiating it from anthrax. An experimental study in Sri Lanka showed that animals dying within 24-36 hrs of infection had widespread petechial haemorrhages particularly on the base of the heart,

abdominal wall and to a lesser extent on the intestines, while lungs showed only congestion. When the illness lasted over 72 hrs, there was extensive consolidation of the lungs, with lobulation due to marked thickening of the interlobular septa. Pleurisy and pericarditis were the other necropsy lesions observed when the disease ran through a longer course (Wijewardana, 1992).

1. 3. 5. 6. Diagnosis

Two aspects of diagnosis, field and laboratory are considered as important. Field diagnosis is important because the immediate investigation of control measures to prevent spread of disease is based on a field diagnosis. A field diagnosis can be made on the basis of characteristic clinical signs, gross pathological lesions, herd history, morbidity and mortality patterns, species susceptibility, age group affected etc. Since morbidity and mortality are highly variable and are dependent on a number of factors and their interactions, these parameters must be viewed in the background of surrounding circumstances.

Laboratory confirmation of diagnosis is by isolation and identification of the agent. This is done by cultural and biochemical as well as serological methods. Direct microscopic examination of smears from material is not conclusive. *Pasteurellae* are not consistently present in nasal secretions. Thus, blood or nasal secretions from animals during the clinical phase do not constitute reliable material and negative results using these are not conclusive. From a fresh carcass, a blood sample or swab collected within a few hours of death from the heart or from the jugular vein is satisfactory. From older carcasses, a long bone is the preferred specimen. These are transported to the laboratory in a standard transport medium on ice and well packed to prevent leakage. Direct culture yields results only with fresh material. Contaminants and post mortem invaders when present overgrow the *Pasteurellae*. A small volume of the eluted blood into 2-3 ml sterile physiological saline, or bone marrow suspended in saline is injected subcutaneously or intramuscularly into a mouse. If *Pasteurella*

are present, even in small numbers, the mouse will die within 24-36 hrs. The mouse acts as a biological screen and pure cultures of the organism can be obtained from the heart blood of the mouse. The organism is identified by morphological and cultural characteristics and serological tests. A rapid slide agglutination test (Namioka and Murata, 1961a) requiring a single colony is used initially. A single colony is mixed with a drop of saline on a slide, a loopful of hyperimmune specific rabbit antiserum is added and the slide is gently warmed while agitating with the loop. This is followed by an indirect haemagglutination test for capsular typing (Carter, 1955; Sawada *et al.*, 1982) and an agglutination test with hydrochloric acid-treated cells for somatic typing (Namioka and Bruner, 1963; Schlink and Olson, 1979). For routine diagnosis using material from HS suspected animals, the procedure used is outlined in Figure 2.

1. 3. 5. 7. Treatments

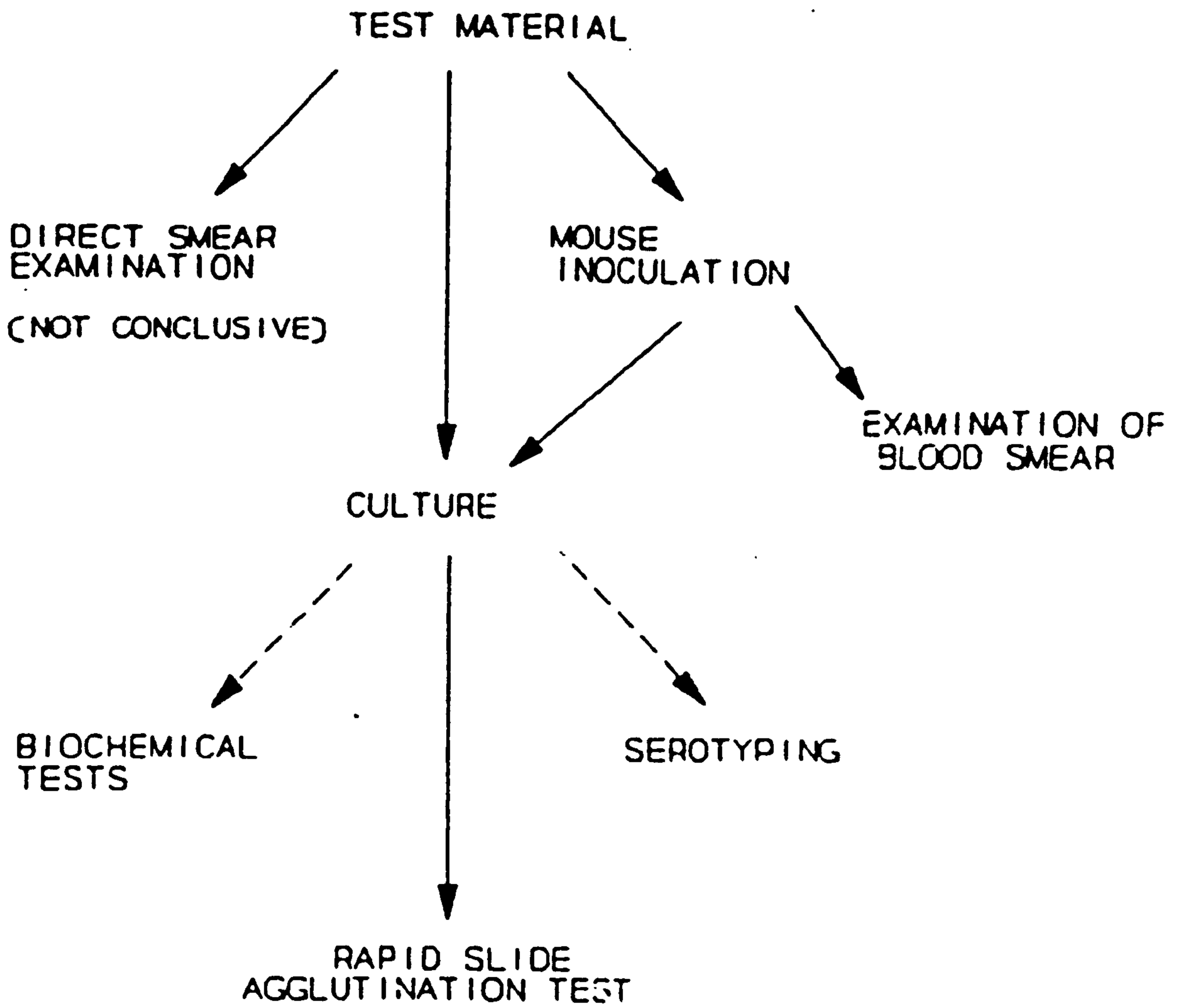
HS under field conditions is always fatal with the majority of affected animals dying without showing any previous signs. Therefore treatment is of little value once visible signs appear in the field; especially as the sudden onset and rapid course of the disease makes early detection difficult. The only practical approach in the face of an outbreak is to check the rectal temperature of all in-contact animals in diseased herds and carry out antibiotic therapy immediately. Traditionally intravenous administration of sulphadimedin or sulphamethazine (33.33%) solution has been recommended. Oxytetracycline (Francis *et al.*, 1980), tetracycline, chloramphenicol and penicillin-streptomycin combinations have been found to be useful (De Alwis, 1995).

1. 3. 5. 8. Control

Vaccination is generally accepted as the method of disease control. Several types of vaccines have been developed. These include plain bacterins, alum-precipitated vaccine, aluminium hydroxide gel vaccine and the oil-adjuvant

Fig 2: Scheme for routine laboratory diagnosis of haemorrhagic septicaemia. (From De Alwis, 1995)

Fig 2:



vaccine. Most countries use local strains as seed and fermentors for large-scale growth of the cultures. The seed is periodically passaged in a natural host in order to retain its full complement of antigens. It has been estimated that at least 1.5-2.0 mg of dry whole bacteria is required to immunise an animal. Thus vaccination needs the production of dense cultures, and enriched media are now used for this purpose. Growth-promoting additives such as yeast extract are incorporated and growth is enhanced by aeration. Finally, formalin at a final concentration of 0.3- 0.5 percent is used to inactivate the cultures (De Alwis, 1995).

Broth bacterins confer only an immunity of very short duration, and dense bacterin may cause an endotoxic shock and are useful only in the face of outbreaks. The alum-precipitated and aluminium hydroxide gel vaccines are believed to confer only 4-5 months of immunity and need be given twice yearly. These vaccines are used in the face of outbreaks in Sri Lanka (Wijewardana, 1992). Oil-adjuvant vaccines confer 6-9 months of immunity. This vaccine is traditionally prepared by emulsifying bacterin and mineral oil with lanolin to produce a pure white, thick emulsion. For prophylactic purposes, the 4-6 months old calves are vaccinated with two initial doses followed by annual revaccination to provide adequate immunity.

Considerable research has been carried out in order to produce an improved vaccine. These range from the low-viscosity double-emulsion vaccine (Verma and Jaiswal 1997) to using immunising fractions (Zhang *et al.*, 1994), purified capsular extract vaccine (Boyce and Adler, 2000), a stable, avirulent streptomycin- dependent mutant (De Alwis and Carter, 1980), and a non-HS type B:3,4 isolate from fallow deer (Myint and Carter, 1989). All these have shown varying degrees of effectiveness. However, because of certain disadvantages, they have failed to yield a practical vaccine. These types of vaccines are discussed later.

1. 3. 6. Vaccination strategies

Vaccines have been used with varying degrees of success against a number of infectious diseases. Vaccination consists of the administration of antigens, in the form of live attenuated or killed microorganisms or as purified individual components in acellular vaccines with the aim of inducing a protective immune response against the disease-causing microorganism. One of the first attempts to prevent disease in this way came when material from smallpox lesions was inoculated into non-sufferers. This so-called variolation was highly successful in preventing subsequent attacks of smallpox, but caused death from smallpox in two percent of those inoculated. The technique was improved considerably in 1796 when Jenner introduced vaccination, using cowpox rather than smallpox material. Vaccination against smallpox has been highly successful since Jenner's innovation, so that by 1979 smallpox as a disease was considered to be globally eradicated (Maskell and Dougan, 1988).

A modern vaccine, for humans or animals, must meet several requirements in order to gain general acceptance. The task of fulfilling all these requirements leads to major problems associated with developing new vaccines. A successful vaccine must elicit a sufficiently strong and long lasting response in the arms of the immune system responsible for protection. This requires that protective antigens be administered in a way that will induce the correct type of immunity. The most effective immunity is often present in individuals who have recovered from a natural infection. In the past, pathogenesis of infectious diseases has had little influence over vaccine design. Many old vaccination schemes involved the parenteral administration of inactivated whole microorganisms or specific virulence factors. With a few notable exceptions this has proved to be an inefficient approach.

The vaccine should be safe. Even killed whole organisms are often capable, following injection, of inducing serious side effects or disease. For example,

whole-cell killed typhoid vaccine contains large amounts of endotoxic lipopolysaccharide which can cause swelling and pain at the site of injection. In more serious cases typhoid vaccination can lead to encephalitis and even death. Often the risks of side effects are considered worth taking. Vaccination against smallpox carried a risk of 1/1,000,000 of death. These risks were worth taking in an attempt to eradicate a disease prevalent worldwide and with a mortality of up to 45%, but would probably not be acceptable for other milder infections (Maskell and Dougan, 1988).

In order to be effective, a vaccine must stimulate a protective immune response, antibody or cell-mediated, against the appropriate antigens at the site of infection or microbial replication. It must protect against infection by organisms following the normal route of infection. Furthermore, the ideal vaccine is one that provides complete protection for the lifetime of the host to the largest realistic challenge dose of the most virulent strains. It must also be cheap enough so that all those at risk, whether humans or animals, even in the poorest countries, can be vaccinated.

The vaccination programme against HS varies. Some countries that still use the alum-precipitated vaccine administer it annually before the rainy seasons, so that maximum protection is provided when it is required most. Vaccination before the onset of a monsoon or sometimes at the onset of an outbreak of the disease is practised in most countries. Verma and Jaiswal (1998) has reviewed vaccination programmes against HS with information on the type of vaccines, their dose, route and duration of immunity in different countries (Table 2).

1. 3. 7. Haemorrhagic septicaemia vaccines

1. 3. 7. 1. Broth bacterins

It has been over 100 years since the discovery of prophylactic vaccination of cattle against HS with killed vaccines. In the last century, different workers

Table 2: Haemorrhagic septicaemia vaccination practices in different countries

[Table based on Verma and Jaiswal (1998) review]

Country	Type of vaccine	Dose	Route	Duration of Immunity
India	Alum-precipitated vaccine	3 ml	s.c.	4- 6 months
	Aluminium hydroxide gel	3 ml	s.c.	4- 6 months
	Oil-adjuvant vaccine	3 ml	i.m.	1 year or more
Indonesia	Oil-adjuvant vaccine	3 ml	i.m.	up to 1 year
	Animals older than 5 months vaccinated every 6 months,			
Thailand	Aluminium hydroxide gel, contains 3×10^{10} C.F.U./ Dose of <i>P. multocida</i> serotype B:2	3 ml	s.c.	3-4 months
Malaysia	Alum-precipitated vaccine	5 ml	s.c.	4-6 months
	Oil-adjuvant vaccine	3 ml	i.m.	up to 1 year
Vietnam	Alum-precipitated vaccine (Two injections per year)	2 ml	s.c.	4- 6 months
	Oil-adjuvant vaccine	2 ml	i.m.	up to 1 year
Sri Lanka	Oil-adjuvant vaccine. Cattle and buffalo vaccinated at 4-6 months and given a booster 3-6 months later, followed by annual vaccination	3 ml	i.m.	up to 1 year
Cambodia	Alum-adjuvant vaccine give twice a year	2 ml	s.c.	3-6 months
Myanmar	Live vaccine contain 10^7 C.F.U./ dose of <i>P. multocida</i> serotype B:3,4	2 ml	i.n.	1 year
Phillipines	Killed alum adjuvanted vaccine booster every 6 months	5 ml	s.c.	4-6 months
Nepal	Alum- precipitated vaccine	5 ml	s.c.	6 months
Iran*	Alum- precipitated vaccine combined with Black leg vaccine give twice a year	2-3 ml	s.c.	4-6 months

* personal communication from Razi Institute of Iran

inactivated the bacteria by chemical or physical procedures and though these vaccines can induce protection against HS under field conditions, they only promote short-term protection. Chorherr (Cited by Verma and Jaiswal, 1998). studied formalin-or heat-inactivated *P. multocida* vaccines. The index of protection was higher with the heat-inactivated vaccine than the formalinised vaccine. He did not find any improvement in its protectivity by the addition of various adjuvants. A vaccine prepared from a strain isolated from cattle and buffaloes, and consisting of a suspension of lysed bacteria in 5% (v/v) saponin to provide a continuous antigenic stimulus, did not find much use due to undesirable tissue damaging effect at the vaccination site, (Verma and Jaiswal 1998). The main problem with broth bacterin is short-term immunity of about 6 weeks (Chandrasekaran, 1994_a). Also, De Alwis (1995) reported that broth bacterins confer immunity for only a few weeks and dense bacterin may cause shock reactions.

1. 3. 7. 2. Alum-precipitated and aluminium hydroxide gel vaccines

These vaccines against HS have been used for over 50 years. As described by Edelman (1980), two principal methods are used to prepare vaccines containing aluminium adjuvant. In the first method, a solution of alum [$\text{Al}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] is added into the antigen to form a precipitate of protein aluminate; the product so obtained has been termed alum-precipitated vaccine. In the second method, antigen solution is added to preformed aluminum hydroxide, aluminium phosphate, mixed aluminium hydroxide plus phosphate or gamma aluminium oxide; the resulting preparations have been called aluminium-adsorbed vaccines. These vaccines are believed to confer immunity for 4-5 months and need be given twice yearly (De Alwis, 1995). They are still used in Southern India, Central Africa and appear to be the most popular vaccine used in Asian countries. De Alwis *et al.* (Cited by Verma and Jaiswal, 1998) reported a controlled trial in Sri Lanka using a standard vaccine containing 2.5 mg of bacteria per dose and this gave protection for up to 4

months. Alum-precipitated vaccines in conjunction with levamisole have been reported to increase antibody titres significantly (Sharma *et al.*, 1980). The efficacy of alum-precipitated vaccine in protection against challenge for HS has been reported (Chandrasekaran *et al.*, 1994 a). They found that all buffaloes vaccinated with alum-precipitated vaccine were completely protected for up to 6 months post-immunisation.

1. 3. 7. 3. Oil-adjuvanted vaccines

Shaking together two immiscible liquids can form a simple emulsion with little stability, but addition of an emulsifying agent can overcome this problem. This emulsifying agent is very important in influencing the type of emulsion that is produced, for example for water-in-water emulsion, lanolin has been used and Arlacel A for water-in-oil emulsion. One of most popular materials used as an adjuvant is mineral oil. Some of these adjuvants can stimulate cellular immunity and enhance protection in cattle against a high challenge dose of virulent strains of *P. multocida*. Chandrasekaran *et al.* (1994 a) compared immunological and protective properties of broth bacterin and oil-adjuvant vaccines. The total volume of the vaccines used was 5.0 ml, containing 2.25×10^{10} C.F.U./ ml of *P. multocida* serotype 6:B strain. They found that broth bacterin protected buffaloes just for a few weeks post-vaccination against experimental challenge with *P. multocida* wild-type strain, whereas the oil-adjuvant vaccine protected the buffaloes for up to 12 months. As was found by Herbert (1968), water-in-oil emulsion releases antigens over a longer period of time than do aluminum products and they can delay absorption of antigen and stimulate mononuclear cells to produce antibody at local and distal sites. The main problems with adjuvanted vaccine seem to be the extent of local tissue inflammatory responses (Ahmed *et al.*, 1974).

Shah *et al.* (1997) evaluated immunisation against HS induced by vaccination of buffalo calves with an improved oil-adjuvant vaccine and they

found that this new vaccine conferred protection for about one year with a single dose of vaccine. Mineral oil, Marcol 52, was used as adjuvant together with Span 85 and Tween 80 as emulsifiers. The vaccine is easily injectable intramuscularly and did not show any local or systematic adverse reaction. Also the vaccine appeared to be stable for more than 3 years at room temperature in tropical areas of Pakistan.

Evaluation of various adjuvants, like aluminium hydroxide, oil adjuvant, multiple-emulsion and sodium alginate in HS vaccines in rabbit showed that the oil adjuvant and multiple emulsion were good adjuvants for protection and produced the highest agglutination titres for up to 91 days post-vaccination (Lalrinliana *et al.*, 1988).

1. 3. 7. 4. Multiple Emulsion (ME) vaccine

Although the oil-adjuvant vaccine provides a higher degree of immunity in cattle and buffaloes than other vaccines, its main disadvantage is high viscosity that can cause poor injectability and a local swelling at the site of inoculation (Verma and Jaiswal, 1997). For these reasons vaccines based on thinner emulsions preparations were evaluated. Verma and Jaiswal (1997) showed that the viscosity of oil adjuvant vaccines may be reduced by re-emulsifying it with an equal volume of Tween 80 and producing a ME vaccine. For this work a formalin-killed, oil-adjuvant vaccine was prepared from *P. multocida* cultured on agar and resuspended the bacteria in saline to 3.0×10^9 organisms per ml. The vaccine contained 15 parts of bacterial suspension, 10 parts liquid paraffin plus one part lanoline as emulsifying agent. Finally this oil adjuvant vaccine was mixed with an equal volume of 2% (v/v) Tween 80 in formol saline and blended in a mixer. This new ME vaccine was shown to be stable, of low viscosity and easy to administer to experimental animals. It overcame the problem of local reaction at the site of inoculation, which sometimes happens with oil adjuvant vaccines. The results of immunity trials in calves have shown

that ME-HS vaccine is protective for up to one year against challenge infection with wild-type *P. multocida* strains (Verma and Jaiswal, 1997).

1. 3. 7. 5. Outer membrane protein (OMP)

Lu *et al.* (1988) showed that a 37.5 kDa outer-membrane protein of *P. multocida* serotype A:3 was a promising vaccine candidate in rabbits because it was a strong immunogen and protective against homologous challenge. Protection in mice was afforded against heterologous challenge only if the challenge strain possessed the 37.5 kDa antigen. Antibodies against *P. multocida* OMP in rabbit immune sera had an inhibitory effect against *P. multocida* proliferation in mice (Lu *et al.*, 1991)

Abdullahi *et al.* (1990) investigated the outer-membrane protein profile of *P. multocida* serogroup A:3 and they attempted to define a protective immunogen by working on immunogenicity of outer-membrane proteins in a mouse septicaemia model. However, they found that the major outer-membrane proteins were not protective antigens in the mouse model of pasteurellosis.

Recently, Pati *et al.* (1996) evaluated immunogenicity of outer-membrane proteins of *P. multocida* serogroup B:2 strains in buffalo calves in comparison with a commercial oil-adjuvant vaccine. They showed that calves vaccinated with either vaccine developed the highest mean log₁₀ ELISA titre at 21 days post vaccination. Following subcutaneous challenge of the vaccinated animals with virulent *P. multocida* strain, all the 5 buffalo calves given OMP vaccine survived whereas only 2 of three buffalo calves in the HS vaccinated group survived.

1. 3. 7. 6. Anti-idiotypic antibodies

Purified LPS from Gram-negative organisms has generally been found to be poorly immunogenic (Sutherland, *et al.*, 1993). Rebers *et al.* (1980) reported that LPS of *P. multocida* was poorly immunogenic in mice in its purified form,

but protective in chicken. However, a protein-LPS complex protected mice, rabbits and chicken (Rebers and Heddleston 1974; Ganfield *et al.*, 1976). Also Philips and Rimler (1984) and Rimler and Brogden (1986) found that LPS has been recognised as a major immunogen in ribosomal vaccines against serotypes A and D respectively. The poor immunogenicity of purified LPS is probably due to it being a low molecular weight linear molecule which is a T cell independent polyclonal B cell activator (Sutherland, *et al.*, 1993).

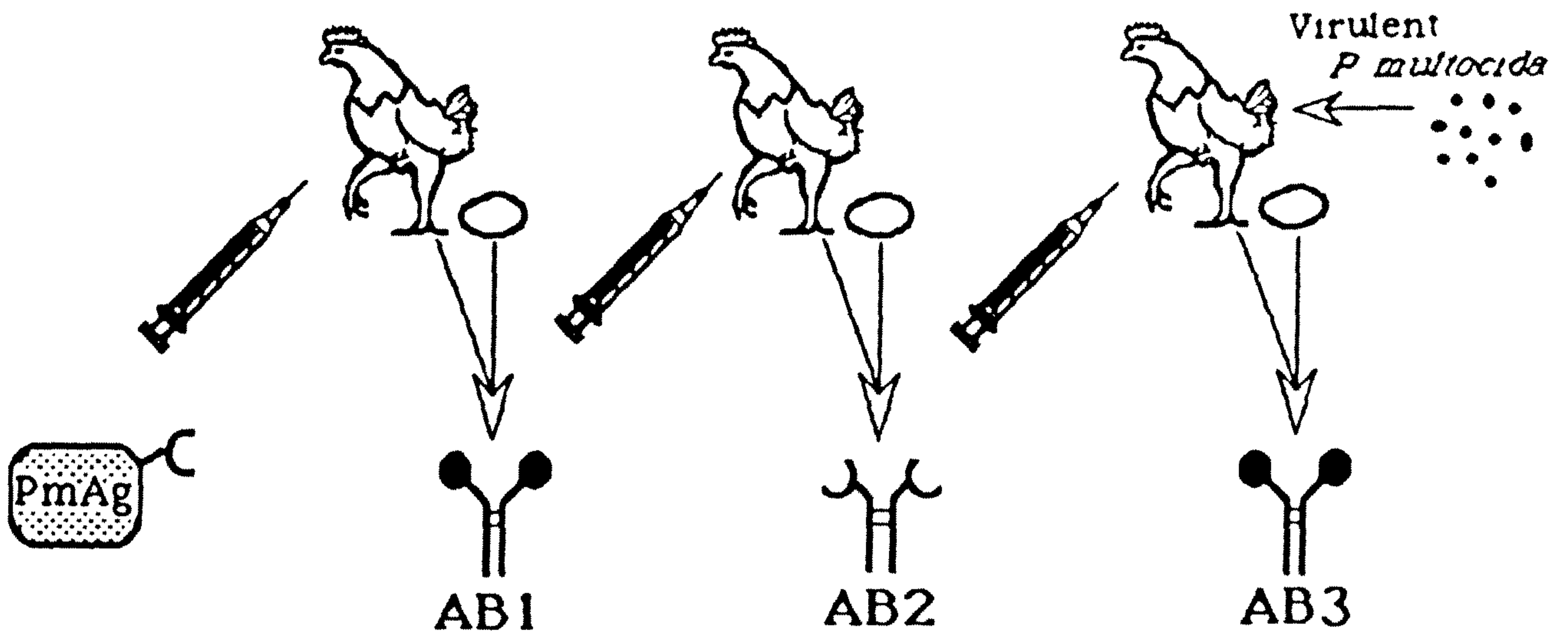
As an alternative to using LPS, a strategy of using anti-idiotypic antibodies as vaccine antigen (Hiernaux, 1988), which mimic LPS, was evaluated by Sutherland *et al.* (1993). Idiotypes are recognition sites located at or near the antigen binding site of an antibody molecule. Anti-idiotypic antibodies are antibodies against an idiotypic or another antibody molecule. Manipulation of the idiotypic network also has potential as an alternative means of vaccination as shown in Figure 3 (Zhang *et al.*, 1994). Although anti-idiotypic vaccines are unlikely to replace a majority of the conventional vaccines, they have some advantages such as no antigenic toxicity to the host, in some cases less cost to manipulate, easily produced, no antigenic variation and the avoidance of disease outbreak resulting from reversion of attenuated vaccines. Also, anti-idiotypic antibodies can mimic antigens that are difficult to synthesise or isolate in the laboratory. The induction of an active immune response by idiotypic antibodies has been shown by Sutherland *et al.* (1993) who found that polyclonal anti-idiotypic antibodies could be produced to mimic the linear *P. multocida* lipopolysaccharide molecule. The antibodies, when used as a vaccine antigen, induced antibodies that recognised LPS and imparted acquired protection when vaccinated mice were challenged with homologous organisms of *P. multocida* capsular type A isolated from bovine pneumonia.

Fig 3: A simplified schematic of the idiotypic network in poultry.

A chicken immunised with an antigen having a unique epitope produces specific antibodies, noted as AB1 idiotypic (anti-*P. multocida* antibody). This antibody can be isolated from serum or eggs. AB1 inoculated into a second chicken induces the synthesis of an AB2 antibody (anti-idiotypic) response. Isolation of AB2 is also possible from serum and eggs. Immunisation of a third chicken with affinity isolated anti-idiotypic results in production of AB3 or anti-anti-idiotypic antibody. The anti-anti-idiotypic antibody reacts with the original antigen even though the third chicken has never been exposed to the original antigen. (From Zhang *et al.*, 1994)

(PmAg: *P. multocida* antigen)

Fig 3:



1. 3. 7. 7. Live attenuated vaccines

Currently available parenteral inactivated vaccines are not very effective for prevention of diseases caused by enteric or respiratory bacteria. They do not induce a strong local immune response and, for invasive pathogens such as *Salmonella typhi*, they do not induce cell-mediated immunity. This led to much work on development of live attenuated vaccines for these types of pathogens.

Vaccination with live attenuated organisms is important because live attenuated vaccines mimic natural infections, without causing disease symptoms, and can leave an individual with strong, long-lasting immunity. Also, protection against some infections needs mucosal or cell-mediated immunity that live vaccines can induce better than killed vaccines. Finally, live vaccines are often cheap and easy to administer. One of the main problems with live vaccines in the past was that of reversion to virulence.

Two main strategies have been used in the past to obtain attenuated vaccines. A closely-related relatively avirulent micro-organism can be sought which induces protection against the pathogen in question, e.g. *Mycobacterium bovis* in BCG vaccine for tuberculosis or Vaccinia virus for smallpox. Alternatively, a pathogenic strain can be passaged many times *in vitro* until it loses pathogenicity, e.g., Sabin polio vaccine and the BCG vaccine strain. The latter type of vaccine can be problematic. The genetic lesion causing attenuation is often ill-defined and the strain could revert, causing vaccine-induced disease. Genetic manipulation techniques have led to the possibility of overcoming the problem of reversion, by introducing defined gene deletions. Recombinant DNA techniques, transposon mutagenesis and other modern methods are only now having an impact on the development of new bacterial vaccines. Efficient human and veterinary vaccination with easily prepared and administered vaccine strains could then ensue, resulting in decreased incidences of these insidious diseases world-wide. Experimentally, using animal models, the most

successful vaccines have been those based on live attenuated micro-organisms which set up a limited, clinically insignificant infection in the host that mimics infection by the virulent pathogen but arms the immune system to respond to further infection. Such vaccines include *Salmonella typhimurium aro* vaccine in calves (Ramos *et al.*, 1998), a streptomycin-dependent mutant of *Salmonella typhi* (Curtiss, 1990), and *aro* or *cya* *Salmonella typhi* strains in man (Tacket *et al.*, 1992), and *Pasteurella multocida* live streptomycin-dependent strains that act as immunogens in turkeys (Chengappa *et al.*, 1978; Briggs and Skeeles 1983), (Table 3).

Many vaccines in current use are not very effective at inducing long-lived immunity. More effective vaccines are needed but the mechanisms of pathogenicity and the important virulence factors involved are ill-defined. The antigens responsible for natural immunity are unknown. For this reason, there has been increasing interest in the use of "rational attenuation" to construct live attenuated bacterial strains that would not revert to virulence in the host. This has been well established for other bacterial pathogens in animal models (Table 3). The introduction of certain auxotrophic mutations in different genes, whose functions are essential for bacteria to survive and grow *in vivo* and thus cause disease, into the genome of virulent strains of different bacterial species, renders them avirulent for experimental models or natural hosts.

It is generally accepted that live vaccines confer better immunity than killed vaccines. De Alwis and Carter (1980) observed that susceptible buffalo calves exposed to natural HS infection and survived, developed considerably higher levels of antibody and immunity of longer duration than those vaccinated with oil-adjuvant vaccines. It was postulated that this immunity is caused by natural exposure to live virulent organisms in subinfective numbers.

Table 3: Individual genes in which mutation gives rise to attenuation in some pathogen species

Strain	Gene	Mutant phenotype	Reference
<i>Aeromonas hydrophila</i>	<i>aroA</i>	Requirement for aromatic A.A [♠] , PABA* and DHBA [♥]	28
<i>Bacillus subtilis</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	24
<i>Bordetella pertusis</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	33
<i>Erwinia amylovora</i>	<i>galE</i>	Renders cells reversibly rough	27
<i>Neisseria gonorrhoeae</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	3
<i>Neisseria meningitidis</i>	<i>galE</i>	Renders cells reversibly rough	22
<i>Pasteurella haemolytica</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	18
<i>Pasteurella multocida</i> A:3	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	17
<i>Pasteurella multocida</i>	<i>galE</i>	Renders cells reversibly rough	15
<i>Salmonella typhi</i>	<i>pab</i>	Requirement for PABA	2
<i>Salmonella dublin</i>	<i>pur</i>	Requirement for purines	26
<i>Salmonella typhimurium</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	9,16,29 25,34,23
<i>Salmonella typhi</i>	<i>aroA, C, D</i>	Requirement for aromatic A.A, PABA and DHBA	4,9,35,21
<i>Salmonella typhimurium</i>	<i>dap</i>	Requirement for diaminopimelic acid	5
<i>Salmonella typhi</i>	<i>purA</i>	Requirement for adenine	2
<i>Salmonella typhi</i>	<i>purHD</i>	Requirement for hypoxanthine and thiamine	10
<i>Salmonella typhimurium</i>	<i>purA</i>	Requirement for adenine	31
<i>Salmonella typhimurium</i>	<i>aroB</i>	Requirement for aromatic A.A, PABA and DHBA	14
<i>Salmonella typhimurium</i>	<i>galE</i>	Renders cells reversibly rough	12,19,20
<i>Salmonella typhi</i>	<i>galE</i>	" " " "	13
<i>Salmonella typhimurium</i>	<i>cya, crp</i>	Inefficient transport and use of carbohydrates and AA and inability to synthesise cell surface structure	7
<i>Salmonella typhimurium</i>	<i>phoP</i>	Regulates genes for acid phosphatase and virulence	11
<i>Salmonella enteritidis</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	6
<i>Salmonella typhimurium</i>	<i>ompR</i>	Outer membrane protein, modulates porin expression	8
<i>Shigella flexneri</i>	<i>aroA, D</i>	Requirement for aromatic A.A, PABA and DHBA	30,36
<i>Yersinia enterocolitica</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	1
<i>Yersinia pestis</i>	<i>aroA</i>	Requirement for aromatic A.A, PABA and DHBA	32

♠ Amino acid ♥ Dihydroxybenzoic acid *Para amino benzoic acid

References:

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|--------------------------------------|-------------------------------------|--------------------------------------|
| 1. Bowe <i>et al.</i> (1989) | 2. Brown and Stocker (1987) | 3. Chamberlain <i>et al.</i> (1993) |
| 4. Chatfield <i>et al.</i> (1992) | 5. Clark and Gyles (1987) | 6. Cooper <i>et al.</i> (1994) |
| 7. Curtiss and Kelly (1987) | 8. Dorman <i>et al.</i> (1989) | 9. Dougan <i>et al.</i> (1989) |
| 10. Edwards and Stocker (1988) | 11. Galan and Curtiss (1989) | 12. Germanier and Furer (1971) |
| 13. Germanier and Furer (1975) | 14. Gunel-Ozan <i>et al.</i> (1997) | 15. Henestrosa <i>et al.</i> (1997) |
| 16. Hoiseth and Stocker (1981) | 17. Homchampa <i>et al.</i> (1992) | 18. Homchampa <i>et al.</i> (1994) |
| 19. Hone <i>et al.</i> (1987) | 20. Hone <i>et al.</i> (1988) | 21. Hone <i>et al.</i> (1991) |
| 22. Jennings <i>et al.</i> (1993) | 23. Killer and Eisenstein (1985) | 24. Krispin and Allmansberger (1998) |
| 25. Lascelles <i>et al.</i> (1988) | 26. McFarland and Stocker (1987) | 27. Metzger <i>et al.</i> (1994) |
| 28. Moral <i>et al.</i> (1998) | 29. Mukkur <i>et al.</i> (1987) | 30. Noriega <i>et al.</i> (1994) |
| 31. O'Callaghan <i>et al.</i> (1988) | 32. Oyston <i>et al.</i> (1996) | 33. Roberts <i>et al.</i> (1990) |
| 34. Robertsson <i>et al.</i> (1983) | 35. Tacket <i>et al.</i> (1992) | 36. Verma and Lindberg (1991) |

Prolonged *in vitro* passage of *P. multocida* strains can lead to considerable attenuation, but this has the drawback of possible reversion to their original virulence especially on animal passage.

In the last three decades, different work has been done on chemically or physically altered live vaccines. Wei and Carter (1978) mutagenised a *P. multocida* type B strain with N-methyl-N-nitro-N-nitrosoguanidine (NMG) to obtain a genetically stable streptomycin-dependent mutant, from which a live vaccine was prepared that was highly immunogenic in mice and rabbits. De Alwis and Carter (1980) successfully immunised calves with the live streptomycin-dependent mutant by subcutaneous or intramuscular routes with one or two doses of vaccine. Also, streptomycin-dependent strains of *P. multocida* serogroups A:1 and A:3 produced after NMG and acriflavin (diaminoacridine salts) treatment were reported to be immunogenic in cattle, swine, sheep and rabbit, and protected against challenge with the wild-type strains (Chengappa *et al.*, 1979; Kucera *et al.*, 1981; Lu and Pakes, 1981). The molecular basis of the mutations was unknown.

A live vaccine of a serologically-related strain of *P. multocida* (serotype B:3, 4) to the HS-causing organism was isolated from deer and used in calves for protection against HS (Myint *et al.*, 1987). This vaccine, when inoculated subcutaneously showed some toxicity and was lethal in a few young buffalo calves but by the intranasal route it was safe and protection was reported against subcutaneous challenge (Myint and Carter 1989; 1990). It does have the advantages of apparent absence of anaphylactic shock, protection for up to a year, easy preparation, a relatively small dose and, when lyophilised, prolonged viability (Myint and Carter, 1990). This vaccine strain is rough and serologically different from typical HS strains that carry B:2 antigens. The difference in somatic antigens (type 3 and 4 carried by the vaccine strain and type 2 found in HS cultures) seems to be responsible for the reduction in

virulence, whereas the identical capsular type B helps in the development of protective immunity against HS (Myint, 1992). It has been used extensively by the intranasal route in several districts in Myanmar, and absence of outbreaks in these areas is reported, suggesting its efficacy against infection by natural routes (Carter *et al.*, 1991). This vaccine deserves a more comprehensive trial.

When using mutants which are not genetically defined, their stability is a matter for concern. Homchampa *et al.* (1992, 1994) constructed by recombinant DNA techniques *aroA* mutants of *P. multocida* serogroup A:1 and *P. haemolytica* biotype A:1 containing a Km^R cassette inserted into the *aroA* gene. These was done by using a suicide vector approach. They found that they were attenuated in a mouse model and protected the mice against wild-type challenge. Interestingly, the antibody levels in mice immunised with one or two doses of *P. multocida aroA* mutant did not show significant differentiation, suggesting a single dose may be sufficient for protection, but in compare, the antibody levels in mice which received two doses of *P. haemolytica aroA* mutant were approximately 50% higher than those which received single dose.

Homchampa *et al.* (1997) reported the cross protective immunity conferred by a marker-free *aroA* mutant of *P. multocida* serotype A:1. They found that the new marker-free *aroA* mutant strains were highly attenuated in the mouse model. Also, they showed that these mutant strains could confer protective immunity against homologous and heterologous (serotype A:3 strain) challenge with one or two doses of vaccine, but not against other biotypes.

1. 3. 8. The approaches used for production of attenuated vaccine strains with defined mutations

Many genetic manipulation experiments require the exchange of mutations either between strains, from plasmid into the chromosomal DNA or from the chromosome onto plasmids. Several widely used allelic exchange systems have been developed to facilitate these tasks.

1. 3. 8. 1. Use of plasmid incompatibility in selectable marker exchange

The properties of plasmid incompatibility, particularly that of IncP1 group, have been widely used as a means for marker exchange in a variety of Gram-negative bacteria (Ruvkun and Ausubel, 1981). This method can be used with any selectable marker, the fragment to be recombined contains a selectable marker such as kanamycin resistance (Km^R) and is cloned into a broad host range IncP1 plasmid [such as a tetracycline resistant (Tc^R) pLAFR derivative, Friedman *et al.*, 1982] and maintained in an *E. coli* host. This plasmid is introduced into the strain in which the mutation is to be recombined by electroporation or mating but, due to the large size of plasmid, mating is preferred. The pLAFR plasmids require helper functions for their mobilisation as provided by strain such as SM10 which contains a copy of plasmid RP4 integrated into the chromosome (Simon *et al.*, 1983). Recipients now carrying the pLAFR plasmid are then mated with a strain carrying a second transmissible IncP1 plasmid with a different selectable marker. Plasmid pPH1JI is a large self-transmissible plasmid which confers gentamycin resistance (Gm^R) and is often used for this "kickout" step. By selection for Gm^R and Km^R and appropriate counter selection against the plasmid pPH1JI donor cells, exconjugants arise that have lost the original IncP1 pLAFR plasmid; however, they retain the Km^R marker that is now integrated into genomic DNA via the homology surrounding the insertion (Fig. 4).

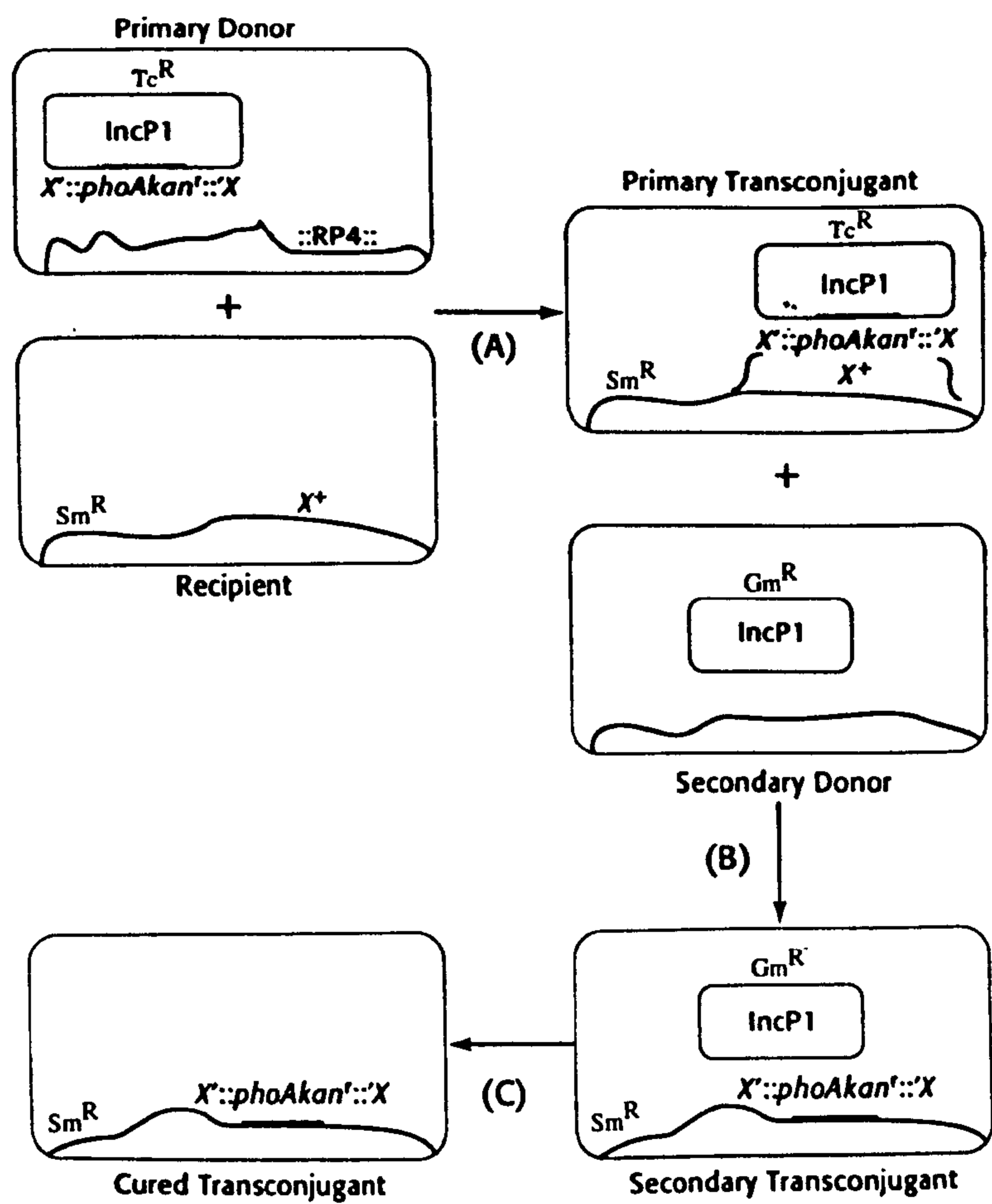
Generally, constructs that incorporate the antibiotic resistance marker within the genomic fragment of interest are first constructed on a small defined plasmid and then cloned into pLAFR for the allelic exchange into the appropriate recipient species.

1. 3. 8. 2. Use of conditional replicons ("suicide plasmids") as a means of constructing genetic duplications and null alleles

Fig 4: Allelic exchange of a *TnphoA* fusion using the IncP1 system.

The donor strain carries a pLAFR plasmid containing gene X that is disrupted with a *phoA* gene fusion marked by Km^R (*phoAKm^R*) as would be the situation for a mutation generated by *TnphoA* mutagenesis. The plasmid is present in a mobiliser strain such as SM10. This donor is mated with a recipient which carries a marker that will be used for counterselection against the donor, in this case Sm^R (A). Transconjugants from A are mated with a second IncP1 plasmid (e.g., pPH1JI) that confers Gm^R (B). Transconjugants from a second mating are selected on agar containing gentamycin and kanamycin for acquisition of the plasmid and retention of Km^R marker (C). Tc^S transconjugants are grown under conditions that promote curing of the Gm^R plasmid. (From Maloy, *et al.*, 1996)

Fig 4:



Plasmids that are conditional for their replication can be used to create defined duplications within a target genome. In this case, a DNA fragment is cloned into a plasmid and then introduced into a recipient strain in which the plasmid cannot replicate. Thus, selection for an antibiotic resistance marker carried by the plasmid, results in isolates that have integrated the plasmid into the genome via homology between the cloned DNA fragment and a corresponding region of the recipient chromosome.

Plasmid vectors used for this type of mutagenesis must have special properties desirable for such insertional mutagenesis, such as conditional replication to allow for selection of chromosomal integration, and must have a selectable marker and should be transferable to different bacterial species and finally must carry unique cloning sites.

1. 3. 8. 3. Use of suicide vectors for allelic exchange of non-selectable mutations

If the mutation or construction is initially induced on a plasmid, allelic exchange can be utilised to replace the wild type chromosomal copy of the gene with the altered gene. Such replacements are usually easily achieved only if the mutation is either the result of, or closely linked to, a selectable marker such as antibiotic resistance gene. If one of the duplicated sequences carries only a mutation, some of the resolved products will remove the wild-type sequence and leave the mutation in the chromosome (Fig. 5). The frequency with which the mutation becomes incorporated into the chromosome is influenced by the position of the mutation within the region of homology. In the absence of a method to select for loss of the integrated plasmid, a large number of colonies must be screened just to find those that had excised the plasmid sequences.

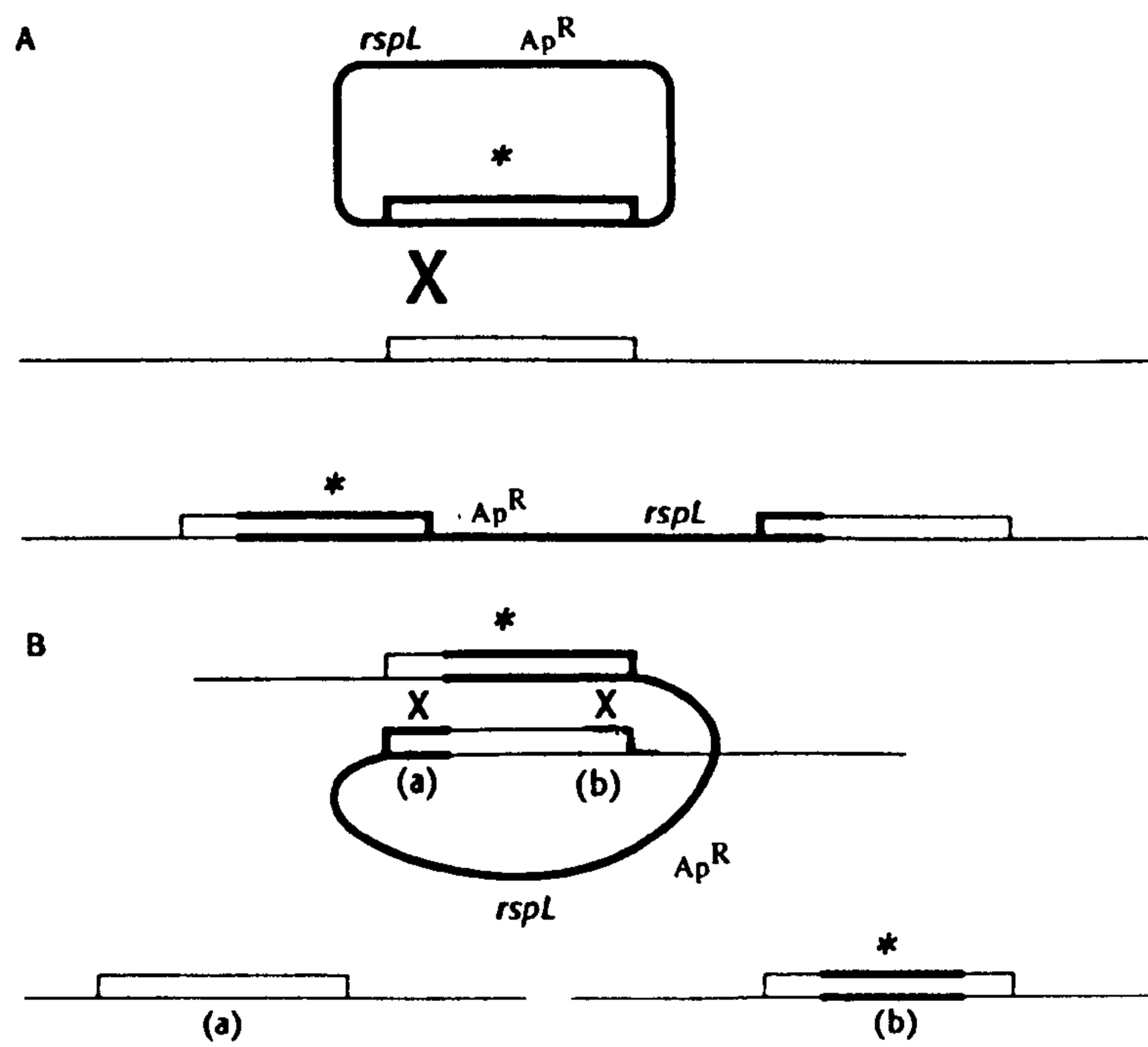
One marker that has been used to select for loss of an integrated plasmid that does not carry a selectable marker is the *sacB* gene from *Bacillus subtilis*. The *sacB* gene encodes the enzyme levansucrase, a 50 kDa enzyme secreted into the

Fig 5: Use of suicide vectors for allelic exchange of nonselectable mutations

(A). Chromosomal integration of a plasmid carrying a mutation (*) on a cloned fragment by homologous recombination generates a heterodiploid strain.

(B). Two possible excision events (a or b). Excision of the plasmid by homologous recombination on the opposite side of the mutation from which the integration occurred (excision b) results in allelic exchange of the mutation from the plasmid to the chromosome. The excised, non-replicating plasmid is lost upon cell division. (From Maloy, *et al.*, 1996)

Fig 5:



culture medium by *Bacillus subtilis* after induction by sucrose. Expression of this enzyme is toxic for Gram-negative bacteria such as *E. coli*, *Agrobacterium tumefaciens* and *Rhizobium meliloti*, (Blomfield *et al.*, 1991; Gay *et al.*, 1985) grown in the presence of 5% (w/v) sucrose, causing cell lysis within 1 h or inhibition of growth because of levansucrase secretion. Thus, only colonies that have removed *sacB* by recombining out the integrated plasmid sequences can survive in the presence of sucrose. In practice, some sucrose resistant colonies can arise from alterations in either the *sacB* gene or its expression, rather than by deletion of vector from chromosome. Thus, colonies arising from this selection are subsequently screened for concomitant loss of antibiotic resistance marker to ensure that the sucrose resistant phenotype is due to loss of the integrated plasmid. This approach has been used in construction of un-marked mutations in *Helicobacter pylori vacA* gene (Copass *et al.*, 1997) and construction of an *eae* deletion mutant of enteropathogenic *E. coli* (Donnenberg and Kaper, 1991). Also, *sacB* selection was used successfully by Akerley *et al.* (1995) and Tejada *et al.* (1996) in *Bordetella pertussis* and *Bordetella bronchiseptica*, respectively, for construction of $\Delta bvgAS$ and un-marked mutation in the *frlAB* or *frlA* (flagellar) gene.

Another strategy to select for loss of the integrated plasmid utilises the *E. coli rpsL* gene that encodes ribosomal protein S12. Integration of the plasmid carrying wild type *rpsL* into a streptomycin resistant strain carrying a chromosomal mutation in *rpsL* result is production of a streptomycin sensitive strain. This occurs because the mutation is recessive to the wild-type protein. Such a plasmid is pRTP1 a suicide vector for allelic exchange in *Bordetella pertussis* (Stibitz *et al.*, 1986; Stibitz, 1994).

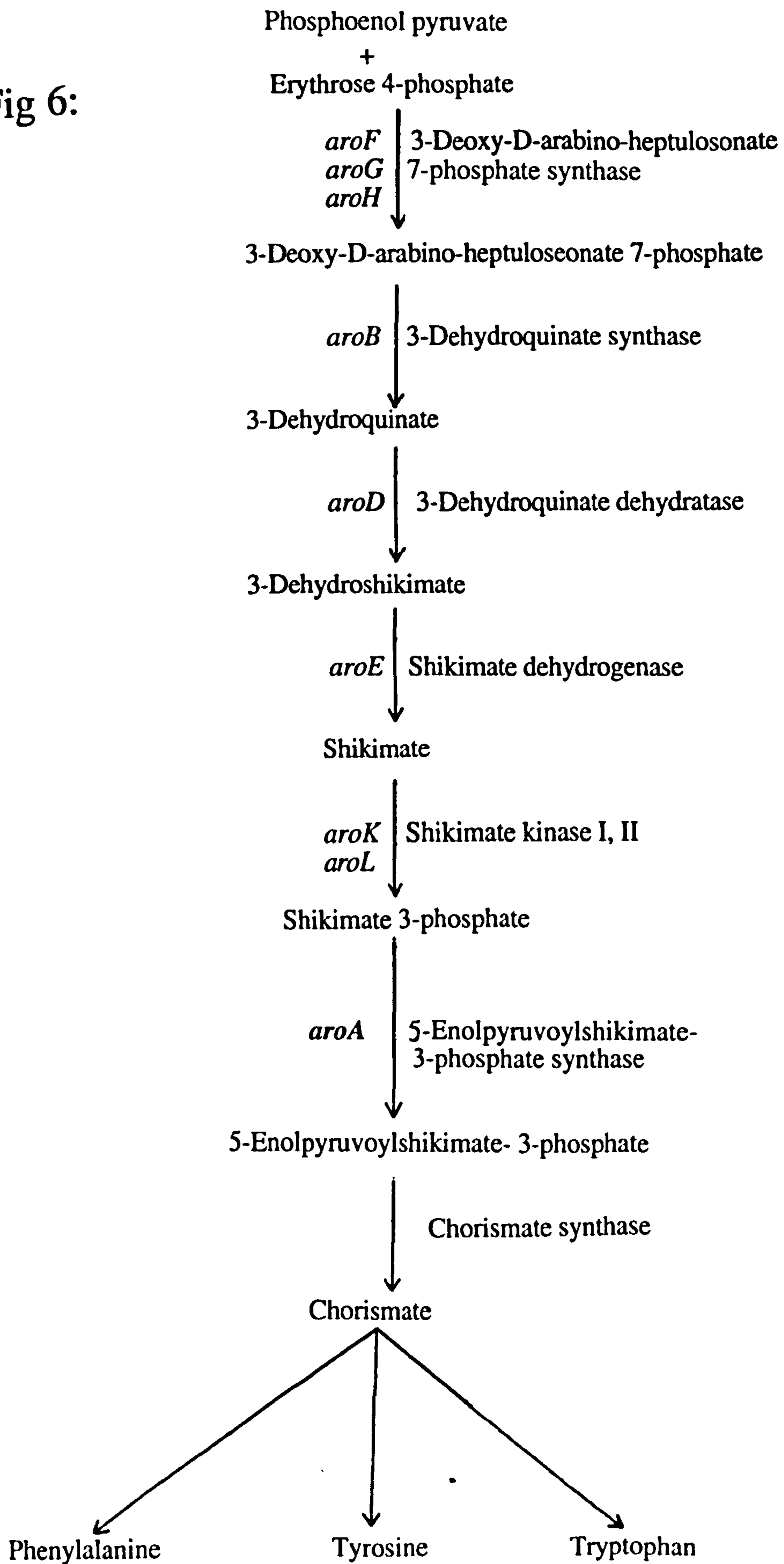
The inability of *colE1*-based plasmids to replicate in some bacterial species has also been exploited for allelic exchange in *P. multocida* (Nnalue and Stocker, 1989; Azad *et al.*, 1994). The high efficiency of allelic exchange,

especially as achieved with the selective marker, make it feasible to physically screen chromosomal integration of mutations for which there is no phenotypic screen. Such approaches are also useful for confirming mutations for which there is no phenotypic screen or selection. They generally involve analysis of an amplified product generated by polymerase chain reaction (PCR) using primers that flank the region of interest. The presence of insertion or deletion mutations that alter the size of particular DNA fragment can be determined directly by gel electrophoresis of the amplified products.

As mentioned, one approach to attenuating *P. multocida* has been to develop nutritional auxotrophs by interrupting the pathway for biosynthesis of aromatic metabolites which renders the strains dependent on *p*-aminobenzoic acid (PABA) and 2,3-dihydroxybenzoate, substrates not available to bacteria in mammalian tissues. Such mutants are unable to synthesis chorismic acid, an important precursor of aromatic compounds. Aromatic amino acids and other compounds containing an aromatic ring structure are bio-synthesised in bacteria, fungi and plants via the pre-chorismate or shikimate pathway, utilising erythrose-4-phosphate and phosphoenolpyruvate as precursor substrates. In bacteria, the seven enzymes in the pathway ultimately produce the compound chorismate (Fig. 6). Chorismate is the precursor of the aromatic amino acids (L-tyrosine, L-tryptophan and L-phenylalanine) as well as other aromatic ring-containing compounds such as quinones and folate. The five central steps of the pre-chorismate pathway are fused as a single pentafunctional enzyme called AROM in a number of lower eukaryotes (Hawkins *et al.*, 1993). In contrast, the corresponding enzymes of the pathway in the prokaryotes occur as monofunctional enzymes and their structural genes are scattered throughout the genome (Gunel-Ozcan *et al.*, 1997). As a consequence of this auxotrophy, the mutated bacteria should not have sustained proliferation within mammalian hosts, but they will reside and grow for long enough to stimulate protective immune responses. The introduction of certain auxotrophic mutations in genes

Fig 6: Genes, enzymes and metabolites comprising the shikimate (pre-chorismate) pathway in *Salmonella typhimurium*

Fig 6:



such as the *aroA* gene that produces 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase and catalyses the sixth step in shikimate pathway into the genomes of a variety of prokaryotes including *Salmonella* spp. (Hoiseh and Stocker, 1981; Mukkur *et al.*, 1987), *Bordetella pertussis* (Roberts *et al.*, 1990), *Bacillus subtilis* (Yazdi and Moir, 1990), *Pasteurella multocida* (Homchampa *et al.*, 1992), *Aeromonas hydrophila* (Moral *et al.*, 1998) and *Yersinia enterocolitica* (Bowe *et al.*, 1989) (Table 3) renders them avirulent for mice. Mice immunised with the appropriate dose of defined *aroA* mutants are generally well protected against a subsequent challenge with the virulent parental strain.

Mutation in the biosynthesis of surface components can create attenuated mutants of some bacterial species in experimental models. Dougan *et al.* (1989) reported that many rough variants of virulent *Salmonella* strains are less virulent than smooth parent strains. The smooth-to-rough phenotypic change involves loss of the ability to synthesise or assemble the O-specific side-chains of the surface lipopolysacchride (LPS) molecules. The LPS of Gram- negative bacteria is a major surface component present only in the outer leaflet of the outer membrane (Fig. 7). The LPS molecule consists of three covalently linked regions: the lipid A, which anchors the molecule in the outer membrane; the core, and the O-side chain. Galactose may be present in the O-antigen, and is also a constituent of the core portion of the LPS (Henestrosa *et al.*, 1997).

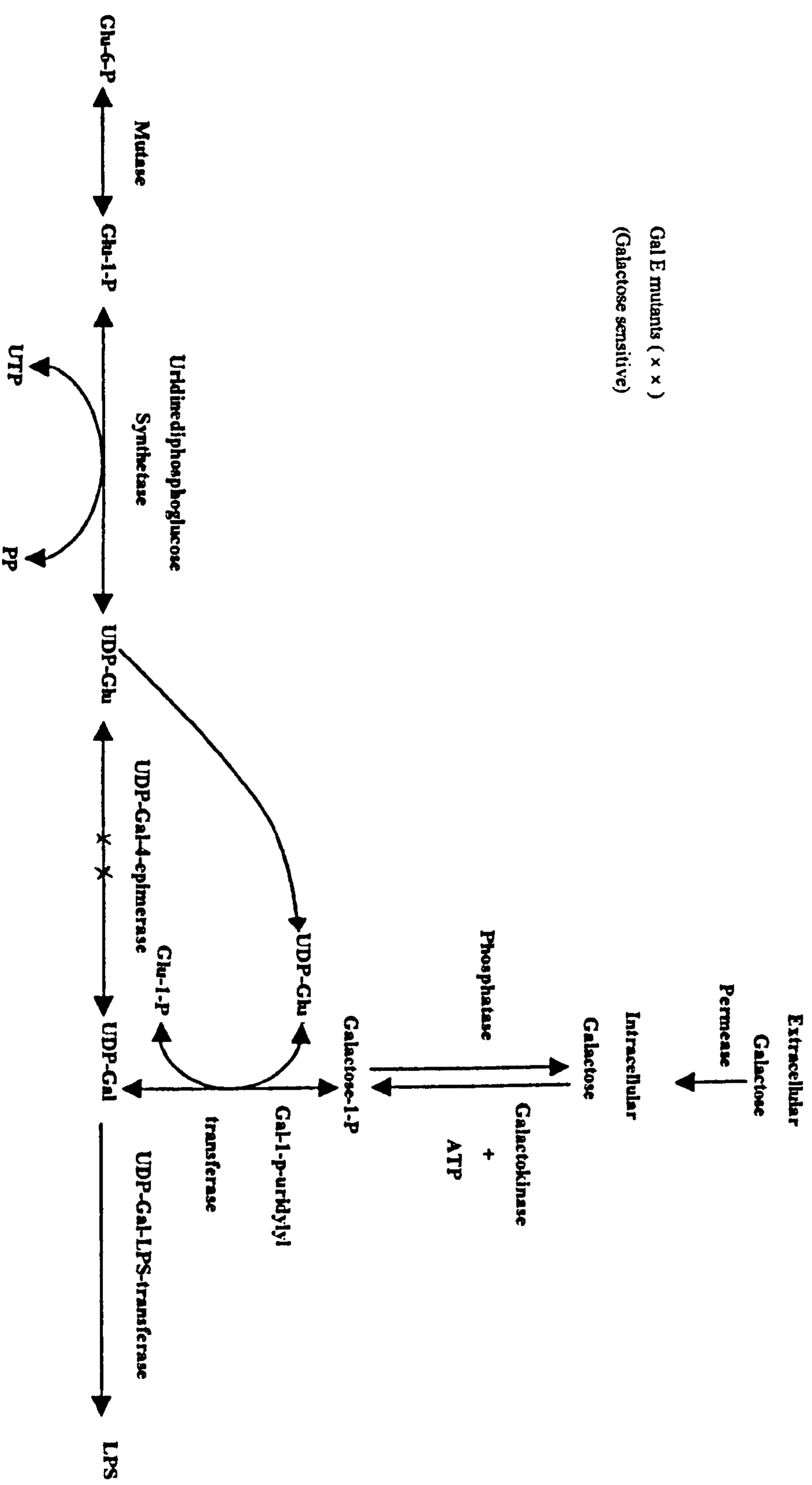
Enterobacteria utilize galactose by the Leloir pathway (Adhya, 1987). Metabolism of galactose requires the epimerization of uridine diphosphate (UDP)-galactose to UDP-glucose (Fig. 8). The *galE* product, UDP-Gal-4-epimerase, catalyses both this reaction and the reverse (Adhya, 1987). UDP-galactose is used as the donor for both core and O-polysaccharide biosynthesis. Thus *galE* mutants grown in glucose alone as a carbon source are unable to produce wild-type LPS structures on the cellular surface (Robertson *et al.*,

Fig 7: The Gram-negative bacterial envelope.

It contains 3 different parts; plasma membrane, periplasmic space and peptidoglycan and outer membrane. The LPS component of the outer membrane consist of three sections: lipid A, core and O-specific side chains. (From Prescott *et al.*, 1993)

Fig 8: Schematic representation of the Leloir pathway for galactose metabolism and incorporation of exogenous galactose into the LPS of *galE* mutants by *Salmonella typhi* illustrating the stage sensitive to galactose (× ×). Note that glucose-1-phosphate generated from galactose after the galactose-1-phosphate uridylyltransferase step is recycled to UDP-glucose by the uridinediphosphoglucose synthetase reaction. (Adapted from Walker, 1987)

Fig 8:



1993) and exhibit a rough colonial morphology due to incomplete LPS. If the growth medium is supplemented with galactose, cells begin to synthesise a smooth LPS with complete O-side chain, probably utilising galactokinase and galactose-1-phosphate-uridyl transferase (Dougan *et al.*, 1989). However, *galE* mutants accumulate a toxic phosphorylated derivative of galactose and autolyse. In this respect, the *galE* gene seems to play a role in the virulence of some bacteria, since it has been shown that *galE* mutants of *Salmonella typhi*, *Salmonella typhimurium*, *Haemophilus influenzae* and *Erwinia amylovora* are less virulent than their respective wild-type strains (Hone *et al.*, 1988; Dougan *et al.*, 1989; Maskell *et al.*, 1992; Metzger *et al.*, 1994). Dougan *et al.* (1989) found that a loss of the O-specific side chain but maintenance of a complete core in *Salmonella typhi* resulted in a two-log increase in LD₅₀ after intraperitoneal (i.p.) inoculation in BALB/c mice. Also, they found that a mutation in *rfaF* that affected core LPS structure increased the LD₅₀ an additional two-logs. Mutations that result in even shorter LPS structures are quite fragile, sensitive to detergents and have lost all virulence. Thus, LPS, on the basis of such rough mutants, has been implicated both as a virulence determinant and as a candidate protective antigen. With the exception of *galE* mutant, rough strains have not found use as *Salmonella* vaccines, although a spontaneously rough variant of *Salmonella dublin* has been sold for some time as a live cattle vaccine.

Nevertheless, the behaviour of the *galE* mutants is not the same in all bacterial species, since the pathogenic potential of strains of *Salmonella typhi* defective for this gene is not affected in humans (Hone *et al.*, 1988). Henestrosa *et al.* (1997) reported the importance of the *galE* gene on the virulence of *P. multocida* serogroup D and they showed that its *galE* mutant was slightly attenuated in a mouse model.

Another approach to attenuating *P. multocida* used here is by creating mutation in the *cya* (adenylate cyclase) gene. As was showed by Curtiss and Kelly (1987), *Salmonella typhimurium* deletion mutants lacking adenylate cyclase (AC) and cyclic AMP receptor protein are avirulent and immunogenic in mice. Cyclic AMP (cAMP) in *E. coli* is known to play a regulatory role in gene transcription via its receptor protein and catabolite gene activator protein. These are transcription regulators of many genes and operons concerned with the transport and breakdown of catabolites. Systems used for transporting carbon sources and several amino acid permeases are all under positive control by cAMP. Adenylate cyclase is the enzyme that converts ATP to cAMP. Aiba *et al.* (1984) cloned and sequenced the adenylate cyclase structural gene (*cya*) of *E. coli* and a model for the regulation adenylate cyclase activity has been proposed. On the basis of genetic experiments, it was suggested that the phosphorylated form of enzyme III-glucose, a component of phosphotransferase system, was an activator of adenylate cyclase (Mock *et al.*, 1991). When glucose transport takes place, the intracellular concentration of phosphorylated enzyme III-glucose decreases and correlates with a decrease in intracellular cAMP concentration.

Mock *et al.* (1991) cloned the *P. multocida* adenylate cyclase gene and expressed it in *E. coli* and they found that the *P. multocida* protein shares several regions of identity with *E. coli* and *Erwinia chrysanthemi* adenylate cyclases.

1. 3. 9. Immune and protective mechanisms

1. 3. 9. 1. Naturally acquired immunity

De Alwis and Carter (1980) described naturally acquired immunity to HS. In Sri Lanka, where high, moderate and low HS incidence areas can be identified, the naturally acquired immunity comes from sub-clinical infection. Occasionally, cattle have been found in Australia and USA with antibodies that

will protect mice against challenge with *P. multocida* 6:B (De Alwis, 1982). Antibodies against *P. multocida* capsular types B and E, which cause HS, were demonstrated in a high percentage of sera from domestic feeder calves that had not been vaccinated with any *Pasteurella* organisms. These antibodies were considered to be naturally acquired (Sawada *et al.*, 1985). Naturally acquired antibody has been demonstrated in sera from buffalo and Zebu cattle in HS enzootic areas of Asia and Africa (Bain, 1954; Peereau *et al.*, Cited by Verma and Jaiswal, 1998). De Alwis (1982) reported that when susceptible buffalo calves are exposed to natural infection with haemorrhagic septicaemia agent, some calves succumb to disease while the others develop varying degrees of immunity which in most instances is considerably higher than that induced by vaccination. Immunity has been attributed to protective antibodies that develop following non-fatal exposure and can persist for more than one year in some animals (Carter and De Alwis, 1989).

1. 3. 9. 2. Humoral and cell-mediated immune responses

There has been little work done on the surface components of *P. multocida* that contribute to interference with ingestion by phagocytosis. Mahesavaran and Theis (1979) studied phagocytosis of a non-capsulate *P. multocida* strain and an encapsulated type B strain by qualitatively measuring the uptake by bovine neutrophils of H³-thymidine-labelled bacteria in the presence of heat-stable and heat-labile opsonins. The non-capsulated strain was completely phagocytosed in the presence of normal and heat-inactivated bovine serum, which indicated that heat-labile factors in serum (complement) were not involved in opsonization. Encapsulated capsular type B strain was found to interfere with opsonization with normal serum. While testing the efficacy of alum-precipitated bacterin that provided protection to challenge, complement-fixation titres against whole cell bacteria, but not titres against saline capsular extract, were closely correlated with resistance (Sharma *et al.*, 1980). Cytophilic and opsonin

adhering antibodies were produced in rabbits immunised with oil-adjuvant vaccine and challenge infected with *P. multocida* (Maurya and Jaiswal, 1996).

The protective immune response to HS vaccination has long been attributed to humoral responses and can be transferred to native animals with serum from vaccinated animals (Maurya and Jaiswal, 1996). Outside the passive protective effect, very little is known about the kinetics of antibody response following oil-adjuvant vaccine or bacterin administration or even what constitutes a protective response, the functional classes of antibody involved in protection and the bacterial antigens responsible.

The majority of immune responses to vaccination in animals has been studied on the basis of data on morbidity/survival (Collins, 1973). The humoral response in a mouse protection test has been widely used as a relatively reliable assessment of immunity in cattle and buffalo. Vaccination of animals elicits a humoral immune response, and the presence of circulating antibody in cattle and buffalo correlates with immunity (Carter, 1967). Dhanda (Cited by Verma and Jaiswal, 1998). showed that levels of circulating antibodies were detectable from the first week up to 18 months post-vaccination with oil-adjuvant vaccine. It has been difficult to determine whether these protective antibodies operate by a bactericidal or an opsonizing mechanism (Wijewardana *et al.* 1990; Rimler and Jaiswal, 1998).

ELISA and immunoblotting techniques used to examine the humoral immune response to *P. multocida* in bovine sera from Indonesia and Malaysia showed elevated levels of antibody in vaccinated animals. The serum antibody response of buffalo immunised with three conventional HS vaccines, viz. broth bacterin (BB), alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV) and one experimental double emulsion vaccine (DEV), was determined by ELISA. Antibody levels were significantly higher in buffaloes immunised with the adjuvanted vaccines (APV, OAV, and DEV) than those immunised with BB

alone. There also appeared to be a relationship between ELISA titers and active protection in buffaloes (Verma and Jaiswal, 1998). A preliminary analysis of the antibody isotypes in the sera of buffalo immunised with the oil-adjuvanted vaccines showed the anti-*Pasteurella* activity to be associated predominantly with IgG₁ or IgG₂ isotypes. The principal response to OAV and bacterin vaccination was the IgG response with only a mild and transient IgM response, and the major protective role was due to the IgG antibody class. There appeared to be an association between pre-challenge antibody titers and protection in buffaloes following challenge. Buffaloes with initial high antibody levels were protected, whereas those buffaloes with low levels succumbed to challenge. No minimum antibody level necessary for protection has been attributed because of the difference in the types of methods of antigen preparation. Verma and Jaiswal (1997) measured the antibody response by indirect haemagglutination assay (IHA) and ELISA in cross-bred male calves immunised with multiple emulsion (ME) HS vaccine. In their study, an animal with a minimum pre-challenge IHA titre of 80 withstood the challenge infection with virulent *P. multocida*. However, because of the variation in the pre-challenge titre among the group of animals, no minimum level of pre-challenge antibody titre could be proposed.

There is no evidence of a role of a cell-mediated immune (CMI) response in HS (Bain *et al.*, 1982). However, the involvement of cell-mediated immunity cannot be discounted in protection (Wijewardana *et al.*, 1990). One of the reasons for not studying the role of cell-mediated immune response in cattle is that *P. multocida* has not been described as a facultative intracellular bacterium. As reported by Carter (1967), Hunter and Woebester (1980), Collins (1973) and Snipes *et al.* (1987) *P. multocida* appears to multiply extracellularly in the turkey and by inference, in other animals as well. The other reason is largely a humoral response in HS. However, avian serotype A strains of *P. multocida* have been reported to invade cultured mammalian cells

(Lee *et al*, 1994; Adler *et al*, 1996; Rabier *et al*, 1997), but other serotypes have not been examined. The leukocyte migration-inhibition test (LMIT) has been described as an indicator of the cell-mediated immune response (Timms, 1974). Verma and Jaiswal (1997) reported more than 20% leukocyte migration-inhibition in calves immunised with ME vaccine both at pre-challenge and post-challenge intervals, thus indicating the involvement of cell-mediated immune mechanisms.

1. 3. 10. Critical appraisal

Since the discovery of the attenuated *P. multocida* responsible for fowl cholera, and the first use of bacterin to prevent HS, more than a century has passed in which numerous efforts have been made to develop a suitable and cost-effective HS vaccine. Most success in HS vaccinology has been obtained with oil-adjuvant vaccines and recently, a live vaccine has been used in Myanmar (Table 2). Both of these vaccine types offer immunity for up to one year. Because the respiratory tract is the natural route of entry of *Pasteurella*, healthy animals carry the organisms that cause HS in their nasopharynx, and such animals are considered as seeds of an HS outbreak. Local secretory antibodies (specific IgA) are produced due to intranasal vaccination with live HS vaccine, which may be an advantage in preventing natural infection of HS. Another potential success in vaccinology has been the development of a multiple emulsion vaccine, which provided immunity for 52 weeks (Verma and Jaiswal, 1997), but this experimental vaccine is yet to go into field use.

It has been established from epidemiological studies of *P. multocida* that a high level of immunity is obtained following arrested infection (De Alwis, 1982; Verma and Jaiswal, 1998). This observation indicates the need to develop a safe, avirulent, stable variant strain that should produce the important immunogens *in vivo* when administered as a live vaccine. Alternatively, identification of the important immunogens responsible for the high level of

immunity attained on natural exposure needs to be done and development of artificial media to support *in vitro* expression of these immunogens or expression of recombinant proteins by DNA manipulation. Adjuvants have played a great role in the development of HS vaccine, and the duration of immunity depends on the type of adjuvant used. The use of adjuvants, including the new generation of adjuvants (Verma and Jaiswal, 1998) should be evaluated to give an easily injectable vaccine. But as mentioned in Table 2, the components used in vaccines, such as aluminium hydroxide gel, alum and mineral oil, can themselves elicit a humoral immune response.

Immune mechanisms in HS have been poorly understood. Investigation into the immune mechanisms, virulence determinants, comparison of live vaccines, killed vaccines and the carrier state are some important areas that have been suggested as future lines of research (Verma and Jaiswal, 1998).

1. 4. Objectives of research and experimental plan:

To construct defined attenuated strains, and to develop a general methodology that could be readily applied to a large number of vaccine strains of *P. multocida* B:2. The ultimate goals were:

1. Construction of live defined, attenuated (e.g. *aroA*, *cya*, *galE*) mutants of *P. multocida* strains that cause haemorrhagic septicaemia in cattle and buffalo
2. To test these attenuated strains in a mouse experimental model for their degree of attenuation and their protective properties.
3. Construction of marker-free deletion of the original mutants that showed promise as potential vaccine strains.
4. To do the above work in *P. multocida* strains of different geographical origin.

2. MATERIALS AND METHODS

2. 1. General bacteriological procedures

2. 1. 1. Sources of bacteria

All bacterial strains used in this study are given in Table 4. Strains were available from culture collections within the Division of Infection and Immunity, University of Glasgow or were available commercially.

2. 1. 2. Plasmids

Plasmids used in this study are given in Table 5.

2. 1. 3. Bacterial growth, storage and media

The compositions of the following media are given in the Appendices. Luria Bertani (LB) broth, Luria Bertani (LB) agar, Brain Heart Infusion (BHI) broth, Cyclodextrin liquid (CL) medium, BHI agar, Blood Agar (BA), Bordet-Gengou (BG) (Appendix 5. 16) medium, SOC, Terrific broth and Pasteurella Minimal Medium (PMM). All media were sterilised by autoclaving at 15 p.s.i. (121°C) for 15 min except where stated. Heat-labile ingredients such as antibiotics were sterilised by filtration through a sterile 0.22 µm filter (Gelman Sciences, USA). Glassware was sterilised by heating to 160°C for 2 hrs.

All strains were stored frozen in 50% (v/v) glycerol in BHI broth or LB at -80°C. *P. multocida* strains from the frozen glycerol stocks were subcultured routinely on Blood Agar (BA) or BHI agar medium with the supplementation of 5% (v/v) defibrinated sheep blood (B& E laboratories, Scotland).

2. 1. 4. Growth of *E. coli*

E. coli strains were subcultured on LB agar medium. All plates were incubated at 37°C overnight. Liquid cultures of the strains were grown in the

Table 4: Bacterial strains used in this study

Strain	Genotype	Source/ Remarks
<i>E. coli</i> DH5 α	<i>supE44 lacU169 (80lacZM15)</i> <i>hsd R17 recA1 endA1 gyrA96</i> <i>thi-1 relA1</i>	Infection and Immunity, University of Glasgow
<i>E. coli</i> XL1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR</i> <i>17 supE44relA1 lac[F' proAB</i> <i>lacZD M15 Tn10 (Tc^R)</i>	Stratagene
<i>E. coli</i> SM10 λ pir	<i>thi thr leu tonA lacY supE</i> <i>recA::RP4-2-Tc^R:: Mu, Km^R</i>	Infection and Immunity, University of Glasgow
<i>Bordetella</i> <i>pertussis</i> 18323	Wild type, standard strain for mouse protection test	Infection and Immunity, University of Glasgow
<i>E. coli</i> TOP10F'	F' { <i>lacIq, Tn10(Tc^R)</i> } <i>mcrA (mrr-</i> <i>hsdRMS-mcrBC) 80lacZ M15 lacX74</i> <i>deoR recA1araD139 (ara-leu)</i> <i>7639 galU galK rspL(Sm^R) endA1 nupG</i>	Invitrogen
<i>P. multocida</i> 85020	Wild type (B: 2)	Isolated from a case of HS, Sri Lanka
<i>P. multocida</i> Quetta	Vaccine strain (B: 2)	Pakistan
<i>P. multocida</i> JRMT1	<i>P. m. 85020 aroA::Km^R</i>	This work
<i>P. multocida</i> JRMT2	<i>P. m. Quetta aroA:: Km^R Sm^R</i> (JRMT11 derivative see below)	This work
<i>P. multocida</i> JRMT3	<i>P. m. Quetta cya::Km^R</i>	This work
<i>P. multocida</i> JRMT4	<i>P. m. 85020 cya::Ap^R</i>	This work
<i>P. multocida</i> JRMT5	<i>P. m. Quetta cya::Ap^R</i>	This work

Table 4: (continued)

<i>P. multocida</i> JRMT6	P.m. 85020 <i>cya::Ap^R /aroA::Km^R</i> (derivative of JRMT1)	This work
<i>P. multocida</i> JRMT7	P. m. Quetta <i>cya::Ap^R /aroA::Km^R Sm^R</i> (derivative of JRMT2)	This work
<i>P. multocida</i> JRMT8	P. m. 85020+ pJRMT29 (<i>Km^R-sacR -sacB</i>)	This work
<i>P. multocida</i> JRMT9	P. m. Quetta+ pJRMT29 (<i>Km^R-sacR-sacB</i>)	This work
<i>P. multocida</i> JRMT10	Spontaneous <i>Sm^R</i> derivative of <i>P. multocida</i> 85020 strain	This work
<i>P. multocida</i> JRMT11	Spontaneous <i>Sm^R</i> derivative of <i>P. multocida</i> Quetta strain	This work
<i>P. multocida</i> JRMT12	P. m. 85020 Δ <i>aroA</i> (142 bp)	This work
<i>P. multocida</i> JRMT13	P. m. Quetta Δ <i>aroA</i> (142 bp)	This work

Ap^R = Ampicillin resistance

Sm^R = Streptomycin resistance

Km^R = Kanamycin resistance

Table 5: The plasmids used in this study

Plasmid	Characteristics	Source/Reference	
pTAg	Ap ^R Km ^R cloning vector	4.8 kb	R&D Systems
pCR-Script	Cm ^R Cloning vehicle	3.4 kb	Stratagene
pCRII-TOPO	Ap ^R Km ^R PCR cloning vector	4.0 kb	Invitrogen
pUC19	Ap ^R Plasmid vector	2.7 kb	This lab
pACYC184	Tc ^R Ap ^R	4.24 kb	This lab
pUC4K	Ap ^R Km ^R Origin of the Km ^R cassette	3.91 kb	Pharmacia
pAKA16	Ap ^R cloning shuttle vector	5.3 kb	Azad <i>et al.</i> (1994)
pAKA19	Ap ^R cloning suicide vector	5.0 kb	Azad <i>et al.</i> (1994)
pEG18.3	Km ^R - <i>sacR</i> - <i>sacB</i>	11.8 kb	Cotter*
pJRMT1	Ap ^R Km ^R <i>aroA</i> ::pTAg (<i>aroA</i> from P.m. 85020)	5.02 kb	This work
pJRMT2	Ap ^R Km ^R <i>aroA</i> ::pTAg (<i>aroA</i> from P.m. Quetta)	5.02 kb	This work
pJRMT3	Ap ^R <i>aroA</i> ::pAKA19 (<i>aroA</i> from pJRMT1)	6.23 kb	This work
pJRMT4	Ap ^R <i>aroA</i> ::pAKA19 (<i>aroA</i> from pJRMT2)	6.23 kb	This work
pJRMT5	Ap ^R Km ^R Km::pJRMT3 (Km ^R from pUC4K)	7.47 kb	This work
pJRMT6	Ap ^R Km ^R Km::pJRMT4 (Km ^R from pUC4K)	7.47 kb	This work
pJRMT7	Cm ^R <i>cya</i> ::pCR-Script (<i>cya</i> from P.m. 85020)	5.9 kb	This work
pJRMT8	Cm ^R <i>cya</i> ::pCR-Script (<i>cya</i> from P.m. Quetta)	5.6 kb	This work
pJRMT9	Cm ^R Km ^R Km::pJRMT7 (Km ^R from pUC4K)	5.99 kb	This work
pJRMT10	Cm ^R Km ^R Km::pJRMT8 (Km ^R from pUC4K)	5.69 kb	This work
pJRMT11	Ap ^R Tc ^R Ap::pACYC184 (Ap ^R from pAKA16)	4.8 kb	This work
pJRMT12	Ap ^R Km ^R <i>cya</i> ::pAKA19 (<i>cya</i> from pJRMT9)	8.04 kb	This work
pJRMT13	Ap ^R Km ^R <i>galE</i> ::pCR-II Topo (<i>galE</i> from P.m. 85020)	5.02 kb	This work
pJRMT14	Ap ^R Km ^R <i>galE</i> ::pCR-II Topo (<i>galE</i> from P.m. Quetta)	5.02 kb	This work
pJRMT15	Ap ^R <i>galE</i> ::pUC19 (<i>galE</i> from pJRMT13)	3.72 kb	This work
pJRMT16	Ap ^R <i>galE</i> ::pUC19 (<i>galE</i> from pJRMT14)	3.72 kb	This work
pJRMT17	Ap ^R Km ^R Km::pJRMT15 (Km ^R from pUC4K)	4.96 kb	This work
pJRMT18	Ap ^R Km ^R Km::pJRMT16 (Km ^R from pUC4K)	4.96 kb	This work

Table 5: (continued)

pJRMT19	Ap ^R Km ^R <i>galE</i> ::pAKA19 (<i>galE</i> from pJRMT15)	7.27 kb	This work
pJRMT20	Ap ^R Km ^R <i>galE</i> ::pAKA19 (<i>galE</i> from pJRMT16)	7.27 kb	This work
pJRMT21	Ap ^R Cm ^R Ap::pJRMT7 (Ap ^R from pJRMT11)	6.52 kb	This work
pJRMT22	Ap ^R Cm ^R Ap::pJTMT8 (Ap ^R from pJRMT11)	6.22 kb	This work
pJRMT23	Ap ^R Km ^R Δ <i>aroA</i> ::pTAg (<i>aroA</i> from pJTMT1)	4.4 kb	This work
pJRMT24	Ap ^R Km ^R Δ <i>aroA</i> ::pTAg (<i>aroA</i> from pJTMT2)	4.4 kb	This work
pJRMT25	Ap ^R Km ^R Δ <i>aroA</i> ::pTAg (<i>aroA</i> from pJRMT1)	4.87 kb	This work
pJRMT26	Ap ^R Km ^R Δ <i>aroA</i> :: pTAg (<i>aroA</i> from pJRMT2)	4.87 kb	This work
pJRMT27	Ap ^R Δ <i>galE</i> ::pUC19 (<i>galE</i> from pJRMT15)	3.63 kb	This work
pJRMT28	Ap ^R Δ <i>galE</i> :: pUC19 (<i>galE</i> from pJRMT16)	3.63 kb	This work
pJRMT29	Ap ^R Km ^R <i>sacB</i> :: pAKA16 (<i>sacB</i> from pEG18.3)	9.1 kb	This work
pJRMT30	Ap ^R Km ^R <i>sacB</i> :: pAKA19 (<i>sacB</i> from pEG18.3)	8.8 kb	This work
pJRMT31	Ap ^R Δ <i>aroA</i> ::pAKA19 (Δ <i>aroA</i> from pJRMT25)	6.15 kb	This work
pJRMT32	Ap ^R Δ <i>aroA</i> ::pAKA19 (Δ <i>aroA</i> from pJRMT26)	6.15 kb	This work
pJRMT33	Ap ^R Km ^R <i>sacB</i> ::pJRMT31	9.95 kb	This work
pJRMT34	Ap ^R Km ^R <i>sacB</i> ::pJRMT2	9.95 kb	This work
pJRMT35	Ap ^R Km ^R Δ <i>galE</i> ::pCR-II Topo (<i>galE</i> from pJRMT27)	6.15 kb	This work
pJRMT36	Ap ^R Km ^R Δ <i>galE</i> :: pCR-II Topo (<i>galE</i> from pJRMT28)	6.15 kb	This work
pJRMT37	Ap ^R Δ <i>galE</i> ::pAKA19 (<i>galE</i> from pJRMT35)	5.9 kb	This work
pJRMT38	Ap ^R Δ <i>galE</i> ::pAKA19 (<i>galE</i> from pJRMT36)	5.9 kb	This work
pJRMT39	Ap ^R Km ^R <i>sacB</i> ::pJRMT37	9.7 kb	This work
pJRMT40	Ap ^R Km ^R <i>sacB</i> ::pJRMT38	9.7 kb	This work
pJRMT41	Ap ^R Δ <i>galE</i> ::pUC19 (<i>galE</i> from pJRMT15)	3.47 kb	This work
pJRMT42	Ap ^R Δ <i>galE</i> :: pUC19 (<i>galE</i> from pJRMT16)	3.47 kb	This work
pJRMT43	Cm ^R Δ <i>cya</i> ::pCR-Script (<i>cya</i> from P.m. 85020)	4.74 kb	This work
pJRMT44	Cm ^R Δ <i>cya</i> :: pCR-Script (<i>cya</i> from P.m. Quetta)	4.74 kb	This work
pJRMT45	Cm ^R Δ <i>cya</i> :: pCR-Script (<i>cya</i> from P.m. 85020)	5.67 kb	This work
pJRMT46	Cm ^R Δ <i>cya</i> :: pCR-Script (<i>cya</i> from P.m. Quetta)	5.37 kb	This work

*Gift from Dr. P. Cotter, Department of Microbiology, Immunology and Molecular Genetics, UCLA school of Medicine, University of California, U.S.A.

corresponding broth media in a Universal containers or dimpled conical flasks with shaking overnight on an orbital shaker at 150-200 rpm at 37°C. When required, antibiotic-resistant strains were grown on agar or in liquid media containing the appropriate antibiotic concentration.

2. 1. 5. Growth of *P. multocida*

P. multocida strains from the frozen glycerol stocks were subcultured routinely on BA or BHI agar medium with the supplementation of 5% (v/v) defibrinated sheep blood. Liquid cultures of the strains were grown in the BHI broth medium in Universal containers or dimpled conical flasks with shaking overnight on an orbital shaker at 150-200 rpm at 37°C. When required, antibiotic-resistant strains were grown on agar or in liquid media containing the appropriate antibiotic concentration.

2. 1. 6. Isolation of spontaneous streptomycin-resistant strains

P. multocida 85020 and Quetta strains were grown overnight (10^{12} cells ml⁻¹) in BHI broth with shaking at 37°C. A 100 µl portion of each culture was spread on BHI agar supplemented with 5% (v/v) sheep blood and streptomycin (100 µg ml⁻¹). The seeded plates were allowed to dry and then incubated at 37°C for 2-3 days. Streptomycin resistant colonies were picked, and their phenotype confirmed by subculture on agar medium containing the same concentration of streptomycin. All these strains were stored in 50% (v/v) glycerol at -80°C.

2. 2. Antibiotics

Antibiotics used in this study are: ampicillin (Ap), chloramphenicol (Cm), tetracycline (Tc), kanamycin (Km), streptomycin (Sm), gentamycin (Gm), polymyxin (Pm) and carbenicillin (Carb). All antibiotics were obtained from Sigma.

The sterile antibiotic solution was added to broth or agar media, after they had been autoclaved and then cooled to $\approx 55^{\circ}\text{C}$. The concentrations of the antibiotics used in selective media were ($\mu\text{g ml}^{-1}$): Ap (50), Cm (30), Tc (15), Km (50), Sm (100), Gm (350), Pm (350) and Carb (60).

2. 3. DNA extraction

2. 3. 1. Genomic DNA Extraction

Following growth of the bacteria in appropriate broth culture, the genomic DNA was extracted according to the method of Ausubel *et al.* (1990). Cells from 1.5 ml of an overnight liquid culture of *P. multocida* in 5 ml BHI broth were harvested by centrifugation at 13,000 rpm for 4 min in a Heraeus Medifuge and the bacterial pellet resuspended in 567 μl of TE buffer (Appendix 5. 5), by repeated pipetting. 30 μl of 10% SDS (w/v) and 3 μl of proteinase K (20 mg ml^{-1} in distilled water) were added and mixed thoroughly to give a final concentration of 100 $\mu\text{g ml}^{-1}$ proteinase K in 0.5% SDS. The cell mixture was incubated at 37°C for 1 h and 100 μl of 5 M NaCl and 80 μl of CTAB [10% CTAB (w/v) in 0.7 M NaCl] were added, mixed thoroughly and incubated at 65°C for 10 min in a water bath. After incubation, 750 μl of chloroform: isoamyl alcohol (24:1) (Sigma) was added and vortexed for 30 sec. The mixture was centrifuged at 13,000 rpm for 5 min and the aqueous phase was transferred into a clean microfuge tube to which was added 750 μl of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma). This was vortexed and centrifuged as above and the aqueous phase transferred to a clean tube. The DNA was precipitated by addition of 0.6 vol. of isopropanol (Sigma). The precipitated DNA was pelleted by centrifugation at 13,000 rpm for 5 min and washed with 1 ml of 70% (v/v) ethanol and re-centrifuged for 5 min at 13,000 rpm. The supernatant fluid was removed and the pellet dried briefly at room temperature and finally dissolved in 100 μl of TE buffer and stored at -20°C .

2. 3. 2. Plasmid DNA Extraction

Plasmid DNA was isolated according to the QIAprep miniprep procedure (Qiagen), based on alkaline lysis of bacterial cells followed by selective adsorption of plasmid DNA onto a silica gel membrane in high-salt buffer and subsequent elution in low-salt buffer. This procedure is a modification of the alkaline lysis method of Birnboim and Doly (1979). Bacteria are lysed under alkaline conditions, and the lysate is subsequently neutralised and adjusted to high-salt binding conditions in one step, and the lysate cleared by centrifugation. The plasmid DNA is adsorbed on to the silica-gel membrane of QIAprep columns and RNA, cellular proteins, and metabolites are not retained on the membrane upon washing but are found in the flow-through. Washing buffer and high-quality plasmid DNA is then eluted from the QIAprep column with distilled water to efficiently remove salts. The purified plasmid DNA is stored at -20°C .

2. 4. Restriction endonuclease digestion of 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 restriction enzymes used in this study are given in Table 6.

Restriction enzyme digestion was routinely performed in a $20\ \mu\text{l}$ volume containing approximately $2\ \mu\text{g}$ of DNA, 8 to 20 units of enzyme ($2\ \mu\text{l}$) and reaction buffer diluted to 1 fold. Sterile distilled water, reaction buffer (10 \times), 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 analysed by gel electrophoresis in 0.7 to 2% (w/v) agarose.

Table 6: The restriction endonucleases used in this study

<u>Restriction enzyme</u>	<u>Recognition sequence</u>	<u>Reaction buffer</u>	<u>Supplier name</u>
<i>AccI</i>	5'...GT↓ATAC...3'	NEBuffer 4	Biolab
<i>AflII</i>	5'...GT↓CGAC...3' 5'...C↓TTAAG...3'	NEBuffer 2+ BSA	Biolab
<i>ApaLI</i>	5'...G↓TGCAC...3'	0.5×U.Buffer	Stratagene
<i>AvaI</i>	5'...C↓pyCGpuG...3'	1×U.Buffer	Stratagene
<i>AvaII</i>	5'...G↓GTCC...3'	1×U.Buffer	Stratagene
<i>BamHI</i>	5'...G↓GATCC...3'	1×U.Buffer	Stratagene
<i>BglII</i>	5'...A↓GATCT...3'	Optimal buffer 4	Stratagene
<i>BglI</i>	5'.GCCNNNN↓NGGC.3'	NEBuffer 3	Biolab
<i>BsmI</i>	5'...GAATGCN↓...3'	NEBuffer 2	Biolab
<i>EarI</i>	5'...CTCTTC(N) ₁ ↓...3'	NEBuffer 1	Biolab
<i>EcoRI</i>	5'...G↓AATTC...3'	NEBuffer EcoRI	Biolab
<i>EcoRV</i>	5'...GAT↓ATC...3'	React 2 buffer	Gibco BRL
<i>HindIII</i>	5'...A↓AGCTT...3'	Buffer #3	Stratagene
<i>HpaI</i>	5'...GTT↓AAC...3'	NEBuffer 4	Biolab
<i>NsiI</i>	5'...ATGCA↓T...3'	NEBuffer NsiI	Biolab
<i>PstI</i>	5'...CTGCA↓G...3'	NEBuffer 3	Biolab
<i>PvuII</i>	5'...CAG↓CTG...3'	NEBuffer 2	Biolab
<i>SacI</i>	5'...GAGCT↓C...3'	Optimal buffer 4	Stratagene
<i>SacII</i>	5'...CCGC↓GG...3'	Optimal buffer 4	Stratagene
<i>ScaI</i>	5'...AGT↓ACT...3'	NEBuffer ScaI	Biolab
<i>SmaI</i>	5'...CCC↓GGG...3'	NEBuffer 4	Biolab
<i>XhoI</i>	5'...C↓TCGAG...3'	React 2	Gibco BRL

2. 5. Agarose gel electrophoresis

2. 5. 1. Sample preparation

The sample DNA (5-30 μ l) was mixed with 6 \times DNA loading buffer (Appendix 5. 9) in a ratio of 5:1 prior to loading into the wells. About 4 μ l of gel-loading buffer was mixed with each restriction digest and the mixture was centrifuged briefly to remove bubbles before loading onto the gel. Different types of DNA size markers were used, depending on the topological forms and the fragment sizes of DNA. For calculating the sizes of uncut plasmids, a supercoiled DNA marker (11 fragments) (Gibco BRL, Paisley, UK) was used. For sizing the digested or linearised DNA fragments, 1 Kb DNA marker (23 fragments) (Gibco BRL) was used.

2. 5. 2. Gel preparation

Agarose (type II-A medium EEO, Sigma) was suspended in 0.5 \times Tris-borate-EDTA (TBE) buffer (Appendix 5. 6) at a suitable concentration and heated until the agarose was completely dissolved. The solution was allowed to cool (hand-hot) and ethidium bromide (Bio-Rad, UK) was added to a final concentration of 0.5 μ g/ ml. A gel tray was prepared by taping the edges with adhesive tape and the gel cast to the desired thickness. Upon setting, the gel was immersed in 0.5 \times TBE buffer in a horizontal submarine electrophoresis tank (E-C Apparatus Corporation, USA).

2. 5. 3. Electrophoresis

A powerpack (model SL3655, Scotlab, UK) was used to provide a constant voltage corresponding to 1-5 volts/ cm^2 . Electrophoresis was carried out until the marker dyes migrated an appropriate distance.

2. 5. 4. Visualisation of DNA

An UV transilluminator (model TM-40, UVP Inc., California, USA) was used to visualise the ethidium bromide-stained DNA. Images were stored

electronically as appropriate using the Ultra Violet Products Gel Documentation System-Image Store 5000, version 7.2 (Ultra Violet Products, Cambridge, UK). Images were printed using a video graphic printer (model UP-860, Sony). Where appropriate, photographs were captured with Fotolook (version 2.07.2 Agfa, UK) using a flatbed scanner (model Studioscan IIsi, Agfa). Electronic images were edited using Adobe Photoshop 3.0 and images labelled with Microsoft Powerpoint 4.0.

2. 5. 5. Elution and purification of DNA from agarose gel

Before proceeding to the cloning protocol, DNA fragments were eluted and purified from agarose gel by the GenElute™ Agarose spin column (SUPELCO) or QIAEXII Gel Extraction kit (QIAGEN).

The DNA band of interest was excised from an ethidium bromide-stained agarose gel with a sharp scalpel under long wave UV light. For the GenElute agarose column procedure, 100 µl of TE buffer (Appendix 5. 5) were added to the column which was positioned in the top of a microfuge tube, centrifuged for 5 sec at maximum speed (13,000 rpm) in a Heraeus microcentrifuge. The agarose gel slice containing DNA was then placed in the washed spin column, which was put on top of a clean tube and centrifuged for 10 min at maximum speed (13,000 rpm) in the microcentrifuge. The DNA was collected in a volume of approximately 30-50 µl TE buffer.

For QIAEXII Gel Extraction kit, the gel slice was put in a pre-weighed microfuge tube. About 3 volumes of buffer QX1 and Qiaex resin (10-15 µl) were added and incubated at 55°C in a water bath for 10 min with shaking every 2 min until the agarose was dissolved. Following centrifugation for 1 min at 13,000 rpm the supernate was removed. The pellet was then washed once with 500 µl of Buffer QX1 and twice with 500 µl of buffer PE, with centrifugation as before. The resin/ DNA pellet was air-dried, 10-25 µl of sterile distilled water was added and, after incubation for 5 min at 50°C, the

resin was removed by centrifugation as before and the eluted DNA in a volume of approximately 20 μ l distilled water and retained for immediate use or stored at -20°C .

2. 6. Estimation of DNA concentration

The concentration of DNA was estimated by measuring the absorbance at 260_{nm} in a 1 cm quartz cuvette in a spectrophotometer (Shimadzu recording spectrophotometer UV-240) and by applying the equation:

$$\text{Concentration of DNA (ng/ } \mu\text{l)} = \text{AB } 260_{\text{nm}} \times 50 \times \text{dilution factor}$$

Alternatively, the approximate concentration of DNA fragments was determined visually on agarose gel, by comparison of their staining intensities with those of known quantities of marker DNA of a similar size.

2. 7. Concentration of DNA

To precipitate and concentrate the DNA, 3 volumes of ethanol 95% (v/v) and 0.1 volume of 3 M sodium acetate (pH 5.3) was added to the sample, mixed and the resultant mixture stored at either -20°C overnight or at -80°C for 30 min depending on the time available. The sample was then centrifuged at 15,000 rpm (Biofuge, Rotor: HFA 14.2) for 15 min and the supernate discarded. The DNA pellet was washed with cold ethanol 70% (v/v) and dried at room temperature for 10-15 min. The DNA was resuspended in an appropriate volume of sterile distilled water and if necessary heated to 65°C for 30 min to aid dissolving.

2. 8. Polymerase chain reaction (PCR)

2. 8. 1. PCR primers

During primer design, care was taken to avoid potential internal secondary structure and where possible a GC clamp was engineered at the 3' end of the primer. Additionally, for PCR, primers were checked for overlap, potential for

secondary structure and for compatible T_m values. T_m values were calculated according to the equation:

$$T_m (^{\circ}\text{C}) = 4(\text{G}+\text{C}) + 2(\text{A}+\text{T}) - 5^{\circ}\text{C}$$

Primers (50 nmol, desalted and deprotected) were obtained from Gibco BRL (Paisley, UK) and resuspended in sterile distilled water to give final concentrations of 50 pmol/ μl for PCR. Table 7 shows the primers used in this study.

2. 8. 2. Components of PCR

Except where otherwise stated, the reaction mixture (25 μl) contained (final concentration) 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.0 mM MgCl_2 , 0.2 mM of each deoxynucleoside triphosphate (dNTPs) (Life Technologies Paisley, Ltd. UK), 100 pmol each primer, 1.0 U of *Taq* DNA polymerase (Life Technologies Ltd, Paisley, UK) and 2.5 μl of template DNA preparation. The PCR assays were performed in 0.5 ml microfuge tubes (Sarsted Ltd., Leicester, UK).

2. 8. 3. Conditions for PCR

Amplification was done in a Hybaid thermal cycler (model: Touchdown, Hybaid Ltd, Middlesex, UK) using 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, extension at 72°C for 6 min with a final extension at 72°C for 6 min. The products of the PCR were stored at -20°C or used immediately.

2. 8. 4. Analysis of amplicons

The amplified products (8-10 μl) were mixed with 2 μl of 6-fold loading buffer (Appendix 5. 9) and electrophoresed in a 1% (w/v) agarose gel. The 1 Kb DNA ladder was used as DNA molecular weight markers.

Table 7: PCR primers used in this study

Primer name	Primer sequence	Primer position	Reference
AroA1 (forward)	5'TT ACT CTC AAT CCC ATC AGC 3'	nt. 315-335	<i>aroA</i> gene of <i>P. multocida</i> Genbank accessio No. Z14100
AroA2 (reverse)	5'A CAA TGC GAT TAA AGC AAA G 3'	nt. 1495- 1514	<i>aroA</i> gene of <i>P. multocida</i> Genbank accession No. Z14100
Cya4 (reverse)	5'GCA TGG TAA GGT GTC AGC TA 3'	nt. 2655-2674	<i>cya</i> gene of <i>P. multocida</i> Genbank accession No. M68901
Cya5 (forward)	5'TTC AAT TAC GAT CTC TTT AGT GCC 3'	nt. 173-196	<i>cya</i> gene of <i>P. multocida</i> Genbank accession No. M68901
Cya6 (reverse)	5'AGG TTC GTG AGG TAT CGG GTA AGG CGC 3'	nt. 2348-2375	<i>cya</i> gene of <i>P. multocida</i> Genbank accession No. M68901
GalE1 (forward)	5'AGG TTC GTG AGG TAT CGG GTA AGG CGC 3'	nt. 379-406	<i>galE</i> gene of <i>P. multocida</i> Genbank accession No. U76617
GalE2 (reverse)	5'ATC TCG ATA GCC TTT AGG 3'	nt. 1378-1396	<i>galE</i> gene of <i>P. multocida</i> Genbank accession No. U76617

2. 9. CLONING

2. 9. 1. PCR product cloning

2. 9. 1. 1. Cloning into pCR-Script™

Cloning was performed using the pCR-Script™ cloning kit (Stratagene, Cambridge, UK) according to manufacturer's instructions. This procedure involves blunt cloning of the "polished" PCR product into a pre-digested *SrfI* vector site. The ligation occurs in the presence of *SrfI*, which reduces the frequency of vector self-ligations. PCR products were electrophoresed in agarose gel, purified by gel extraction kit (QiaexII) and then "polished" to generate blunt ends (section 2. 9. 4). The polished PCR product was then added to the ligation reaction containing the following:

pCR-Script™ Cm SK(+) cloning vector (10 ng/ µl)	1.0 µl
pCR-Script 10 reaction buffer	1.0 µl
ATP (10 mM)	0.5 µl
Polished PCR product	2-4 µl
<i>SrfI</i> (5 U/ µl)	1.0 µl
T4 DNA ligase (4 U/ µl)	1.0 µl

The ligation mixture was mixed gently and incubated at room temperature for 1 h, then incubated at 65 °C for 10 min, transformed into *E. coli* (section 2. 10. 2) and a blue-white screen was performed (section 2. 10. 3).

2. 9. 1. 2. Cloning into pCR®2.1-TOPO cloning vector

PCR products were routinely cloned using the pCR®2.1-TOPO vector included in the TOPO-TA cloning kit (Invitrogen, The Netherlands). Amplicons of interest were excised following agarose gel analysis and DNA extracted using the QiaexII kit. The DNA was resuspended to a concentration of approximately 50 ng/ µl. Ligation of the PCR product into pCR®2.1-TOPO was performed according to manufacturer's instructions. Briefly, 4 µl of

cleaned PCR product was mixed gently with 1 µl of TOPO mix (containing topoisomerase, prepared pCR®2.1-TOPO and ligation buffer). After incubation for 5 min at room temperature, the mixture was put onto ice. Immediately, one vial of TOP10F' One Shot competent cells were thawed gently on ice and 2 µl mercaptoethanol (0.5 M) was added. To the cells, 2 µl of the ligation mix was added with gentle mixing. After incubation on ice for 30 min, the cells were then heat-shocked at 42°C for 30 sec in a water bath and returned to ice for 2 min. SOC medium (Appendix 5. 7) 250 µl was added to the vial and the cells shaken at 37°C for 2 hrs before 100 µl aliquots were spread on selective agar medium.

2. 9. 2. Standard cloning protocol

2. 9. 2. 1. DNA preparation

Vector and insert DNA were subjected to restriction enzyme digestion to obtain complementary cohesive overhangs and the resultant fragments were gel-purified. DNA concentrations were estimated according to section 2. 6 and ratios determined as follows:

$$\frac{100 \text{ ng vector} \times ? \text{ kb insert}}{? \text{ kb vector}} = ? \text{ ng insert DNA to every 100 ng vector for a 1:1 ratio}$$

Insert: Vector ratios of 1: 1 and 3: 1 were commonly used.

2. 9. 2. 2. Ligation strategies

Ligation reactions were performed in a total volume of 10 µl using 3 units of DNA ligase (Promega, USA) and Promega ligase buffer. After incubation at 16°C for 18 h the reactions were transferred to 4°C for 2 hrs. The ligase was inactivated by heating to 70°C for 10 min and the products stored at -20°C until use.

2. 9. 2. 3. Ligation Express Kit

The ligation was performed according to the manufacturer's instructions (Clontech). Compatible vector and insert termini were prepared and ratios calculated as described previously (section 2. 9. 2. 1). Approximately 300 ng of total DNA was used per reaction (maximum volume of DNA solution was 7 μ l). To the DNA was added 1.5 μ l of plasmid ligation buffer, 1.2 μ l of ATP (10 mM) and 1.0 μ l of T4 DNA ligase (100 U). After thorough mixing the reaction was incubated for 30 min at 16°C. To this, 1 μ l of glycogen (20 μ g), 85 μ l of distilled water and 250 μ l of ethanol 100% was added. The DNA was precipitated using a dry ice/ ethanol bath for 30-60 min and the DNA recovered by centrifuging at 15,000 rpm in a Biofuge (Rotor HFA 14.2). After careful removal of the supernate, the pellet was briefly air-dried and a wash in ethanol 70% (v/v) was performed to remove salts. The DNA was resuspended in 5 μ l of distilled water and stored at -20°C.

2. 9. 3. Dephosphorylation of linearized DNA

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) (Promega), according to the protocol of Sambrook *et al.* (1989).

Plasmid DNA (2-4 μ g) was digested to completion with the appropriate restriction enzyme in a 40 μ l volume. Then 2.5 μ l (1 U/ μ l) of CIP and 4.5 μ l CIP buffer were added to the digestion mix and incubated at 37°C for 30-60 min. The CIP was heat inactivated at 75°C for 15 min and the dephosphorylated DNA fragments were purified by agarose gel electrophoresis in TBE buffer (Appendia 5. 6), followed by GenElute agarose Spin Column preparation (Suppelco, UK) (section 2. 5. 5).

2. 9. 4. "Filling in" or "Trimming" reaction for blunt end ligation

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 μ l volume and fragments were separated by agarose gel electrophoresis. DNA fragments of interest were purified by the GenElute agarose spin column (Suppelco, UK) (section 2. 5. 5) and taken up in 30-50 μ l distilled water in a fresh microfuge tube to which REact 4 was added as a substitute for DNA polymerase buffer. dNTPs mix (a mixture of 25 mM dATP, dCTP, dGTP and dTTP; obtained from Life Technologies Ltd. UK) was added to the tube at a final concentration of 1mM (filling reaction) or 2 mM (trimming reaction). To this was added 1-2 μ l of Klenow fragment of *E. coli* DNA polymerase I (Biolab UK) for the "filling in" reaction or bacteriophage T4 DNA polymerase (Biolab UK) for the "trimming" reaction. The reactions were incubated for 15 min at room temperature (filling in) or for the same period at 12° C (trimming). DNA polymerase enzymes were inactivated by heating the reaction mixture to 75° C for 10 min before proceeding with the ligation or cloning protocols.

2. 10. DNA transformation

In order to study the molecular basis of pathogenesis, an efficient means of transforming bacteria is necessary. The efficient introduction of DNA into the bacteria is a phenomenon of great practical importance in molecular biology. Methods of chemical treatment yielding high efficiency transformation with some strains of *E. coli* have been described; but the preparation of such highly competent cells is affected by many factors and the level of competence obtained can vary considerably from batch to batch (Hanahan, 1983).

Rendering prokaryotic and eukaryotic cells permeable to nucleic acid by exposure to electrical fields is now a commonly used technique and is referred to as electroporation (Dower *et al.*, 1988). A possible mechanism suggested to explain this technique is the opening of a new compartment to the interior of the cell to passive diffusion of DNA and the bulk of flow of medium, containing the DNA, into the cells. Both *E. coli* and *P. multocida* strains have been transformed to reasonable efficiencies with this technique (Dower *et al.*, 1988; Jablonski *et al.*, 1992a)

2. 10. 1. Preparation of electroporation-competent cells

An overnight culture of *E. coli* or *P. multocida* was diluted 1 in 100 in pre-warmed 500 ml of LB or BHI broth respectively in a 2 litre dimpled flask. The flask was shaken vigorously at 37°C until an OD_{540nm} of 0.5-0.7 was obtained. The flask was then chilled on ice for 20 min and cells harvested at 10,000 rpm for 15 min in a Sorvall Superspeed (rotor GS-3) at 4°C. The resultant bacterial pellet was resuspended in 500 ml of cold sterile distilled water and centrifuged as described previously. The cell pellet was then resuspended in 250 ml of cold sterile distilled water, centrifuged as before and resuspended in 100 ml of cold sterile 10% (v/v) glycerol, centrifuged as before and the cell pellet was resuspended in 10 ml of cold sterile 10% (v/v) glycerol. Following a final centrifugation, the cells were resuspended in 1.5 ml of cold sterile 10% (v/v) glycerol and 100 µl aliquots were snap frozen in liquid nitrogen. Cells remained cold (on ice) at all times during the procedure and were stored at -80°C.

2. 10. 2. Electroporation procedure

The *E. coli* and *P. multocida* strains were transformed by electroporation procedures described by Dower *et al.* (1988) and Jablonski *et al.* (1992a). Prior to electroporation, a 2 mm electroporation cuvette (Flowgen, UK) and the safety chamber were chilled at -20°C. To the cold cuvette, 1-5 µl of DNA

(ligation mixture or plasmid preparation) and 40 μ l of competent cells (cells from -80°C were thawed on ice) were added. The mixture was shaken to the bottom of the cuvette and a Bio-Rad Gene Pulser (model 1652078, Bio-Rad laboratories, UK) set at 2.0 KV (field strength 10 KV/ cm^2) for *E. coli* and 2.5 KV (field strength 12.5 KV/ cm^2) for *P. multocida* strains and 25 μ FD was connected to a Bio-Rad pulse controller (model 1652098) set to 200 Ω resistance for *E. coli* and 400 Ω resistance for *P. multocida* strains. The cells were pulsed once to give a time constant of 4-5 msec for *E. coli* and 9-10 msec for *P. multocida* strains. Immediately following electroporation, one ml of pre-warmed SOC medium for *E. coli* or BHI broth for *P. multocida* strains was added and the cells incubated at 37°C , *E. coli* cells for one h and *P. multocida* cells for 3-4 hrs, without shaking, to allow expression of plasmid-encoded antibiotic resistance. Aliquots of 100 μ l transformed cells were then spread onto selective plates containing appropriate antibiotic(s) and the plates were incubated at 37°C to obtain transformant colonies.

2. 10. 3. Blue-white screening of recombinants

Following incubation of the transformed cells in SOC medium, 100 μ l aliquots were spread onto pre-warmed LB agar plates containing appropriate antibiotics, 2 mg/ml of isopropyl-D-thiogalactopyranoside (IPTG) (Sigma) and 40 mg/ml of 5-bromo-4-chloro-3-indolyl-D-galactoside (X-GAL) (Sigma). The remainder of the SOC suspension was centrifuged and the cell pellet resuspended in 150 μ l of SOC and spread onto other plates to increase the cell concentration. The plates were then incubated at 37°C overnight. Resultant white colonies (potentially containing insert) were cultured and plasmid DNA prepared according to section 2. 3. 2. The plasmids were analysed by restriction enzyme digestion (section 2. 4) for the presence of an insert.

2. 11. Conjugation (plate mating)

This was carried out by the method of Bradley *et al.* (1980). Each strain was grown overnight at 37°C on a shaker in 5 ml BHI for *P. multocida* recipient strain or LB broth for *E. coli* donor strain containing the appropriate antibiotic. An aliquot (0.5 ml) of overnight culture was inoculated into 10 ml pre-warmed BHI or LB broth and incubation continued at 37°C with shaking, *E. coli* for 2 hrs and *P. multocida* for 4 hrs, until the culture reached an optical density OD_{540nm} of ≈0.5-0.6 (≈5 × 10⁷ cells ml⁻¹). The cells were harvested by centrifugation at 5000 rpm for 5 min and washed with the same media and resuspended in 200 µl of media. Equal volumes (50-100 µl) of the cultures of donor and recipient strains were mixed gently in a microfuge tube by repeating pipetting and put on top of a sterile filter paper (cellulose nitrate membrane filters, Whatman) on BA plates which were allowed to dry at room temperature, then incubated at 37°C face up overnight. Growth from the mating on top of the filter paper was resuspended in 5 ml of BHI broth, and 100 µl aliquots of neat sample and 10× dilutions were spread onto BHI agar transconjugant- selection plates containing the required selective antibiotics and were incubated at 37°C for 48 hrs.

2. 12. Southern blot hybridisation

In this study, PCR amplimers of the *P. multocida aroA* gene, *cya* gene and *galE* gene, plasmid pAKA19, a Km^R cassette and an Ap^R cassette were used as probes for hybridisation to digested genomic DNA of *P. multocida* strains or PCR products. The transfer and hybridisation of DNA fragments were essentially carried out according to Southern (1975), as described by Sambrook *et al.* (1989) or by Ausubel *et al.* (1990).

2. 12. 1. Assembly of the blot

DNA fragments generated by restriction enzyme digestion were separated by gel electrophoresis, stained with 0.5 µg ml⁻¹ ethidium bromide, and

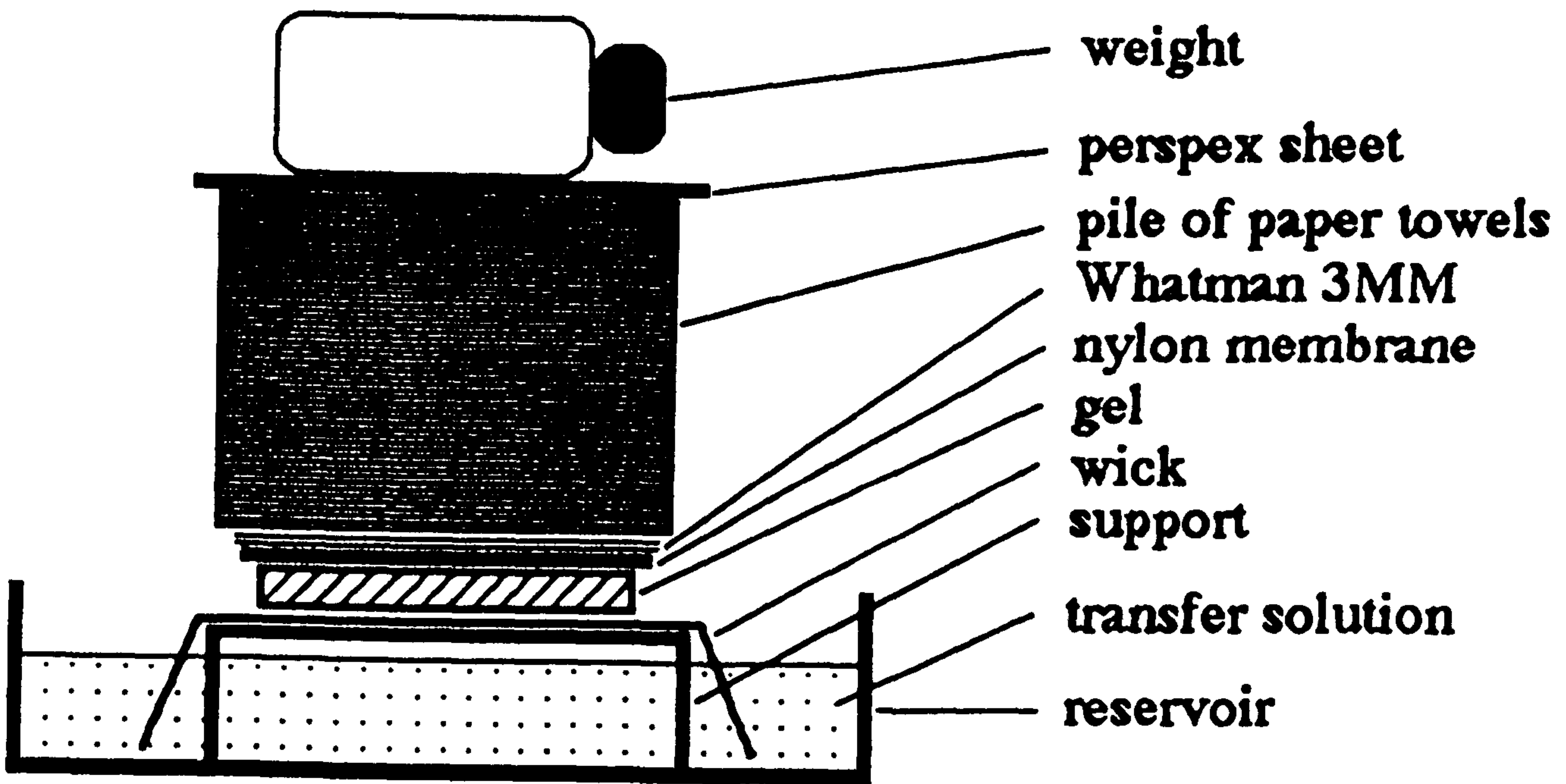
photographed under UV light. One corner of the gel was cut off for orientation and the DNA denatured in the gel by soaking the gel twice for 15 min in denaturation solution (Appendix 5. 12) with constant, gentle agitation. After that the gel was rinsed twice in distilled water and placed twice for 15 min in neutralisation solution (Appendix 5. 13) with gentle agitation.

A DNA transfer pyramid was assembled (Fig. 9) by filling a reservoir with transfer solution (20× SSC) (Appendix 5. 11) and the gel support was put in place. A wick, made from Whatman 3 MM paper, was prepared so that each end dipped into the transfer solution and the middle lay flat on the support. Air bubbles between the wick and support were removed by rolling a pipette over the surface of the paper. The gel was removed from the neutralisation solution and placed over the Whatman 3 MM wick, with sample wells face down and any air bubbles between the wick and the gel were smoothed away as before. A sheet of nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech, UK) was cut to the exact size of the gel, placed on top of gel, again avoiding trapped air bubbles beneath the membrane, and with scissors the corner of the membrane that corresponded to the cut corner of the gel was snipped off, the edges of the membrane surrounded with Nesco film membrane, to stop "short circuits" (in which transfer solution flows directly from the reservoir to the dry paper towels above the membrane, rather than passing through the gel) and then, three pieces of pre-wetted 3 MM filter paper, cut to exactly the same size as the nylon membrane, were placed on top of the membrane and air bubbles were smoothed out. Finally, a pile of paper towels (5-8 cm high) was placed on top. A glass plate was put on top of the pile and the layers weighed down with a weight (0.7-1 kg e.g. a bottle). For convenience, the assembled blot was usually left undisturbed overnight. After blotting overnight, the nylon membrane was wrapped between two sheets of plastic and both sides of the wet membrane, without prior washing, exposed to a source of UV radiation and the DNA crosslinked to the membrane using a UV crosslinker

Fig 9 : Uni-directional capillary Southern blotting

(From Darling and Brickell, 1994)

Fig 9:



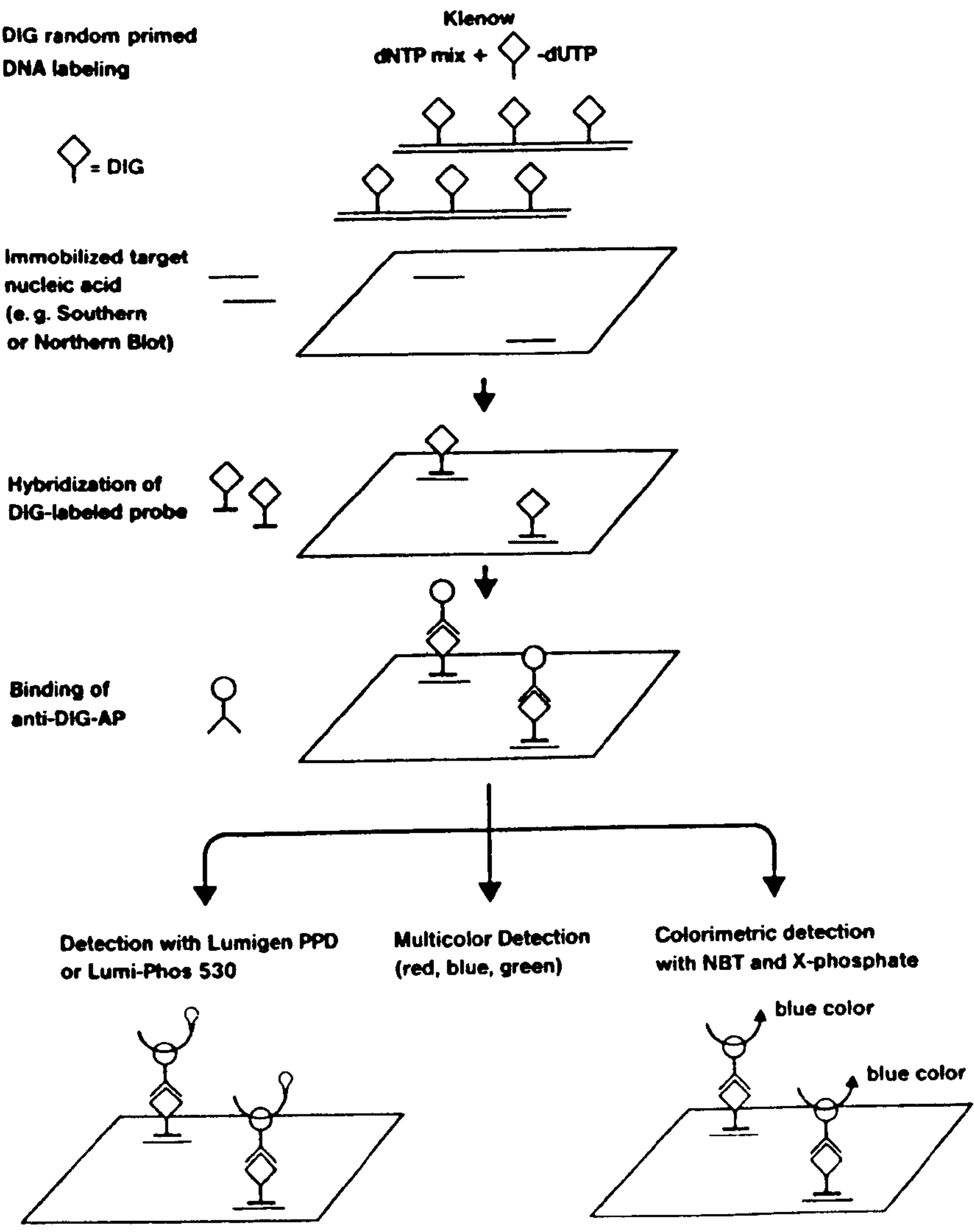
(Spectrolinker XL-1000, Spectroline, New York, USA). A proportion of the thymine residues in the DNA will cross-link to amine groups on the surface of the membrane (Darling and Brickell, 1994) and fix the DNA to the membrane. The membrane was then prehybridised for a minimum of 4 hrs in a rolling hybridisation oven (model HB-1D, Techne, Cambridge, UK) with at least 20 ml of prehybridising solution (Appendix 5. 11). The temperature of the oven was set within the range 50°C and 68°C depending on the desired hybridisation stringency. This temperature was maintained for the subsequent hybridisation and washing steps (see below).

2. 12. 2. Preparation of digoxigenin-labelled probes

Template DNA was excised from an agarose gel with a sterile scalpel. Extraction was done by the method that explained in section 2.3 and the concentration of DNA estimated as described in Section 2. 6. The random priming method was utilised as described in The DIG System User's Guide for Filter Hybridisation (Boehringer Mannheim, Germany). The nonradioactive DIG system uses digoxigenin (DIG), a steroid hapten, to label DNA, RNA or oligonucleotides for hybridisation and subsequent colour or luminescence detection. For DNA labelling, digoxigenin is coupled to dUTP via an alkaline-labile ester-bond. The use of the alkaline-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridisation experiments with a second DIG-labelled probe (Fig. 10). Briefly, approximately 1 µg of template DNA was diluted in distilled water to a total volume of 15 µl. The DNA was denatured in a boiling water bath for 10 min followed by immediate chilling on ice. Hexanucleotide mixture (10×) (Boehringer Mannheim, Germany) and dNTP labelling mixture (10×), 2 µl of each, were added to the tube on ice. To the reaction, 1 µl of labelling-grade Klenow DNA polymerase enzyme was added to a final concentration of 100 U/ml. After brief centrifugation, the reaction was incubated overnight at 37°C, then terminated by adding 2 µl of EDTA (200 mM, pH 8). The contents of the labelling tube were then stored at

Fig 10: The labelling and detection alternatives offered by the DIG system
(Boehringer Mannheim)

Fig 10:



-20°C. Denaturation of the probe was performed for 10 min in a boiling water bath followed by immediate chilling on ice. The denatured probe was diluted into 10 ml of pre-hybridisation solution.

2. 12. 3. Probe hybridisation

The prehybridising solution was discarded and the denatured probe immediately added to the roller tube. Hybridisation was performed for a minimum of 8 hrs at 68°C. The membrane was washed at the hybridisation temperature for 2 × 15 min in 100 ml of 2× washing solution (Appendix 5. 11) followed by 2 × 15 min washes in 100 ml 0.5× washing solution (Appendix 5. 11) at 68°C.

2. 12. 4. Chemiluminescent detection

Detection was performed according to the protocol described in The DIG System User's Guide for Filter Hybridisation (Boehringer Mannheim, Germany). The washed membrane was equilibrated for 1-3 min in washing buffer (Appendix 5. 11). Antibody blocking solution was then incubated with the membrane for 30-60 min. The antibody (anti-DIG conjugated to alkaline phosphatase) was diluted to 1 in 10,000 in the blocking solution and the membrane was incubated for 30 min in at least 20 ml of antibody solution. The membrane was washed for 2 × 15 min in washing buffer and then equilibrated in detection buffer (Appendix 5. 11) for 3 min. The alkaline phosphatase substrate used (CSPD) was diluted to 1 in 100 in detection buffer and this was dripped onto the membrane prior to sealing within a plastic sheet. After 5 min, the excess substrate was removed using a damp paper towel on the top plastic surface. The plastic was heat sealed and then incubated at 37°C for 15 min, to allow the alkaline phosphatase chemiluminescent reaction to occur at a steady rate. The membrane was then exposed in the sealed plastic to photosensitive film (Kodak, X-ray film, UK) for up to 24 hrs, although 15-30 min was usually sufficient. After exposure, the autoradiograph was immersed in

developing solution (LX24, Kodak, UK) with agitation for 2-3 min. The film was then rinsed in water and fixed, with agitation, in Unifix solution (Kodak, UK) for 15 min. Finally the film was washed with tap water for 3-5 min and air dried.

For re-probing the blot, the membrane was thoroughly rinsed in distilled water, incubated twice for 20 min in 0.2 M NaOH, 0.1% (w/v) SDS at 37°C with gently agitation, rinsed in 2× SSC and then re-hybridized overnight with another DNA probe.

2. 13. Virulence and protection tests

2. 13. 1. Inoculum preparation

A stock culture of *P. multocida* maintained at -80°C in 50% glycerol was plated onto BA plates and incubated overnight at 37°C, then a single colony was inoculated into 5 ml BHI broth and incubated overnight at 37°C with shaking. One ml of the culture was inoculated into 20 ml of pre-warmed BHI broth and the OD at 540_{nm} measured at intervals of 2 hrs for 12 hrs to obtain the growth curves for each strain (Fig. 11).

Generally at OD_{540nm} ≈ 1 (late exponential phase, Fig. 11), 10× serial dilutions of cells in phosphate-buffered saline (PBS) as a suspension medium were prepared and 1, 2 and 3 hrs after dilution a 100 µl portion of diluted culture in PBS was plated onto BA for checking the survival time of bacteria in PBS.

For animal inoculation, when the OD_{540nm} of cultures reached ≈ 1.0 (10⁹ C.F.U./ ml), 10× serial dilutions in PBS were prepared and from dilution 10⁻⁵, 10⁻⁶, 10⁻⁷ and 10⁻⁸ and 100 µl aliquots were spread onto BA plates for viable counting.

Fig 11: Growth curve of 4 different strains of *P. multocida* in BHI broth

10 ml-volumes of BHI broth were inoculated with overnight cultures of the different bacterial strains and incubated at 37°C with shaking. The OD_{540 nm} was measured at intervals

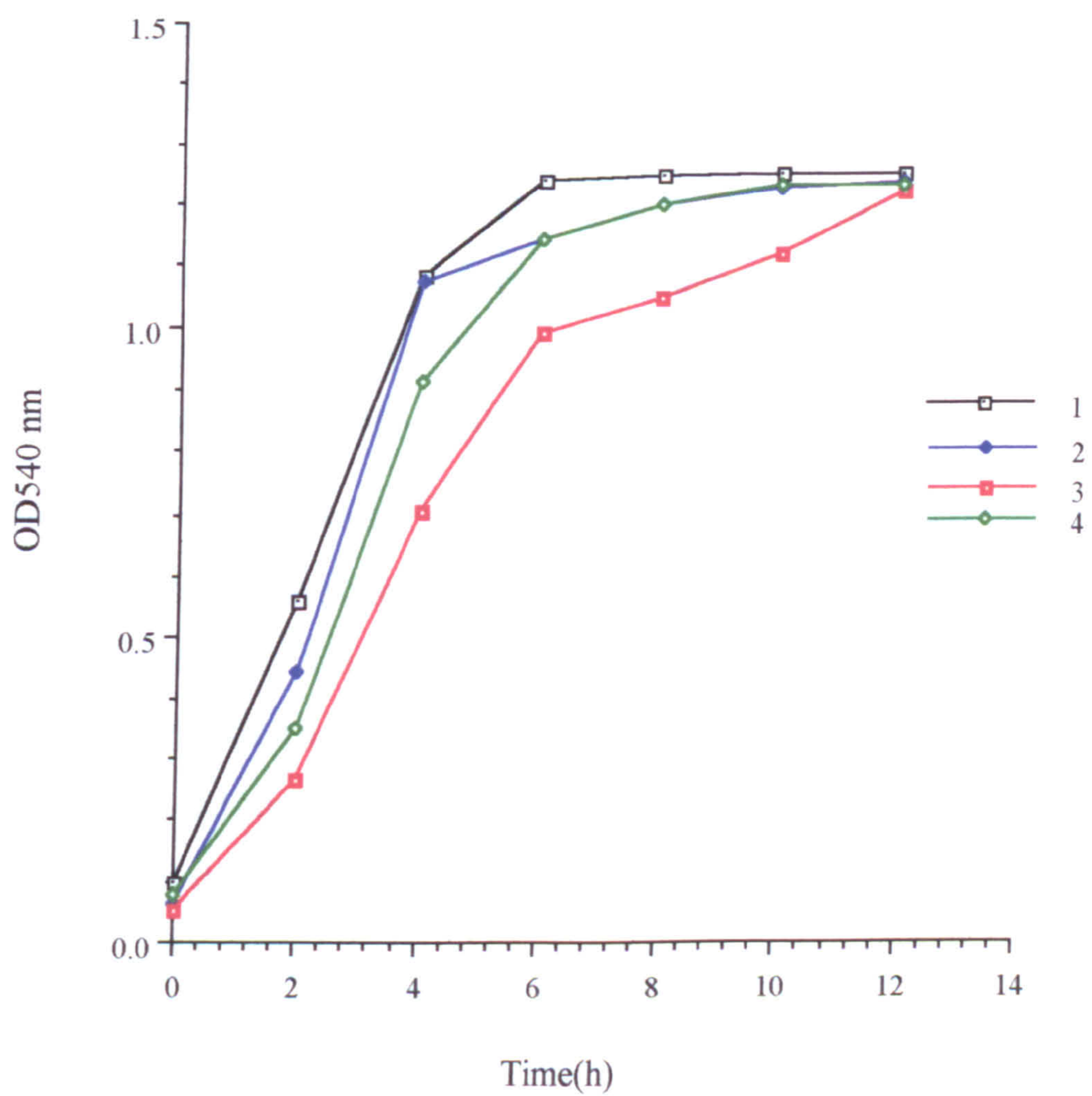
1: *P. multocida* 85020 (wild type)

2: *P. multocida* JRMT1 (*aroA*⁻)

3: *P. multocida* Quetta (wild type)

4: *P. multocida* JRMT2 (*aroA*⁻)

Fig 11:



2. 13. 2. Selection of animal models

Animals vary in their susceptibility to *P. multocida* infection and this may in turn be influenced by different factors; such as the number of organisms and route of the inoculation. Although virulence of *P. multocida* may vary considerably for different animal species, the mouse appears to be the animal most susceptible to lethal infection. Virulence of different serogroups is roughly comparable in mice (Rimler and Rhoades, 1989). HS working parties set up by the UN Food and Agricultural Organisation have recommended the use of the mouse for testing HS vaccines. The mouse provides a good model of infection as it manifests a septicaemia similar to HS in natural hosts (Bain *et al.*, 1982; De Alwis *et al.* 1990).

Groups of 3-6 female 4-6 week old BALB/c (inbred) mice (Harlan Olac, Shaws farm, Black-thorn, Bicester, UK) were used in this study. The mice were injected intraperitoneally (i.p.) with 0.5 ml of 10× serial dilutions in PBS of exponential phase cultures ($OD_{540nm} \approx 1$) in BHI broth.

For intranasal (i.n.) inoculation the method described by Rushton (1978) was used. Mice responded satisfactorily to halothane administered in the same way and there was time for handling of mice before they made a rapid recovery. When the level of narcosis with halothane was correct, mice were recumbent, insensitive and breathing evenly and shallowly. First the mice were anaesthetised with halothane, and then in deep narcosis, 50 µl of appropriate bacterial dilution was applied to the nares and allowed to be inhaled. Subcutaneous (s.c.) vaccination consisted of injecting 200 µl of appropriate bacterial dilutions into the nape of the neck of the mouse. Mice vaccinated s.c. were also anaesthetised with halothane. Before and after injection, the mice were weighed and checked daily. For collecting blood samples and taking the internal organs, the mice were sacrificed in the CO₂ cabinet and then disinfected with chlorhexidine skin disinfectant (Adams Health Care, UK). The

appropriate samples were prepared aseptically and collected in pre-weighed Universal bottles and the weight of each organ was calculated. The organs were then homogenised in 10 ml-volumes of sterile PBS using a sterile homogeniser (Silverson machines,UK). The samples, 0.1 ml of neat and 10× serial dilutions for each organ were plated onto BA and incubated overnight at 37°C.

It should be noted that no statistical analysis was performed with these tests. This was because only small numbers of mice were used as clear differences were apparent between parent and mutant strains.

2.14. Adenylate cyclase assay

2. 14. 1. Preparation of cell free extract

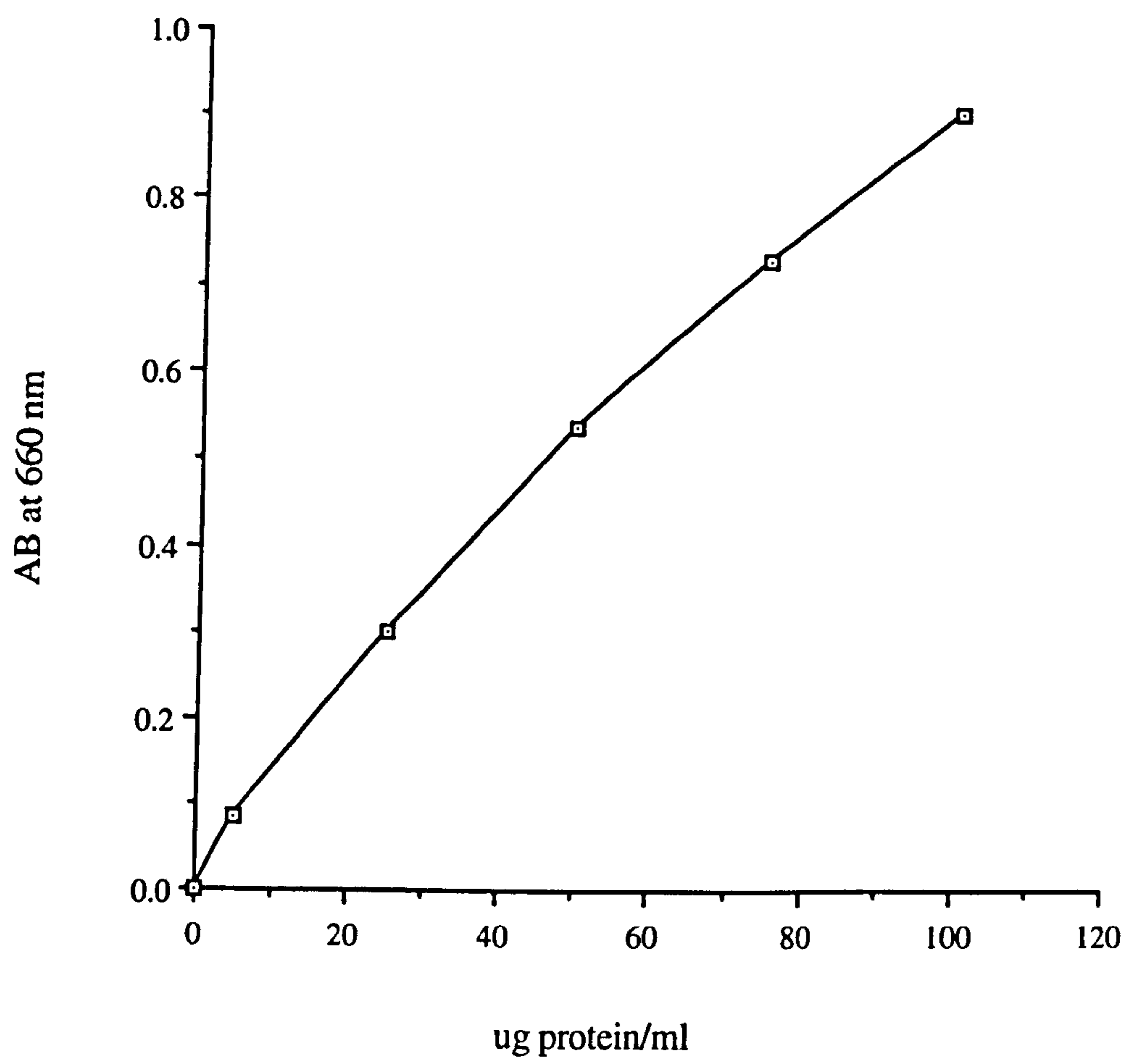
P. multocida strains were grown in BHI broth to late exponential phase ($OD_{540nm} \approx 1.5$) and *Bordetella pertussis* was grown in CL (Imaizumi *et al.*, 1983) (Appendix 5. 17) medium and served as a positive control. The cultures were centrifuged at 10,000 rpm for 15 min at 4°C and the cell pellet resuspended in 10 ml 25 mM HEPES (pH 7.5) (Sigma). The cells were sonicated 3 times for 20 seconds with 40 seconds cooling intervals. The ruptured cells were centrifuged at 33,000 rpm for 90 min at 4°C (Sorvall, Combi) and the supernate stored in 0.5 ml aliquots at -70°C.

2. 14. 2. Quantification of proteins using the Lowry procedure

This assay relies on the formation of a protein-copper complex (Biuret reaction) and the reduction of the phosphomolybdate-phosphotungstate reagent (Folin-ciocalteu phenol reagent, Sigma) by the tyrosine and tryptophan residues of the protein. This method is applicable when the protein concentration per assay ranges between 20 and 200 µg. The method also needs a standard curve (Fig. 12), which is generally established with bovine serum albumin (BSA) (Sigma).

Fig 12: Protein estimation standard curve developed by the Lowry method
(Section 2. 14. 2)

Fig 12:



Protein standards containing 25 to 100 μg protein per ml were used. Dilutions of the sample of unknown protein concentrations were prepared in triplicate, in a total volumes of 200 μl . A blank tube with no protein sample was also prepared. One ml of reagent C (Appendix 5. 14) was added to each tube and the contents vortexed and allowed to stand at room temperature for 10 min. Solution D (Appendix 5. 14) (100 μl) was added rapidly to each tube, with immediate mixing. All solutions was allowed to stand for 30 min at room temperature before their absorbance at 660 nm was recorded.

2. 14. 3. Adenylate cyclase assay

The enzyme activity of adenylate cyclase was measured in an assay which involved two steps, incubation of the sample with ATP, the substrate from which the adenylate cyclase generates cyclic 3', 5' adenosine mono phosphate (cAMP), and then measurement of the cAMP generated by a competitive binding assay. This was based on the method described by Hewlett and Wolff (1976). Test tubes containing 80 μl of 180 mM Tricine [N-tris-(hydroxymethyl)-methyl glycine] pH 8.0 and 40 μl of unknown sample were incubated for 4 min in a 30°C water bath. The reaction was started by addition of 120 μl 2 mM ATP in 20 mM MgCl_2 . The reaction was terminated after 120 min by addition of 200 μl cAMP assay buffer (Appendix 5.14) and immersion in a boiling water bath for 10 min. Samples were stored sealed at -20°C and assayed for cAMP in triplicate at a later date.

2. 14. 4. cAMP enzyme immunoassay (EIA) kit (Amersham Life Science)

This procedure combines the use of a peroxidase-labelled cAMP conjugate, a specific antiserum which can be immobilised on to pre-coated microtitre plates, and a stabilised substrate solution. The assay is based on the competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP-specific antibody. With fixed amount of antibody and peroxidase-labelled cAMP, the

amount of peroxidase-labelled ligand bound by the antibody will be inversely proportional to the concentration of added unlabelled ligand. The peroxidase ligand that is bound to the antibody is immobilised on to polystyrene microtitre wells pre-coated with second antibody, as demonstrated in Figure 13. Thus any unbound ligand can be removed from the wells by a simple washing procedure.

The amount of peroxidase-labelled cAMP bound to the antibody is determined by addition of a tetramethylbenzidine (TMB)/ hydrogen peroxidase substrate. The reaction is stopped by addition of an acid solution, and the resultant colour read at 450_{nm} in a microtitre plate spectrophotometer (Dynatech Laboratories).

cAMP may be measured in the range 12.5-3200 fmol per well (4-1052 pg/well). According to the manufacture instructions, a working standard curve was prepared (Fig. 14) and samples assayed by non-acetylation procedure (Table 8).

2. 15. 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase

assay procedure:

5-enolpyruvylshikimate 3-phosphate (EPSP) synthase is assayed in either the forward or reverse direction by using appropriate coupling enzymes. For the forward assay, formation of EPSP is coupled to the chorismate synthase reaction while for the reverse reaction the release of phosphoenolpyruvate is coupled to the pyruvate kinase and lactate dehydrogenase reactions.

EPSP synthase was assayed by method of Lewendon and Coggins (1987). EPSP is prepared enzymatically from shikimate 3-phosphate and phosphoenolpyruvate using purified *E. coli* EPSP synthase. Shikimate 3-phosphate is isolated from an *aroA*⁻ strain of *Klebsiella pneumoniae* (ATCC 25597) as described by Coggins *et al.* (1987). Chorismate synthase, for use as a coupling enzyme, is partially purified from *Neurospora crassa* (Coggins *et al.*, 1987)

Fig 13: The simplified cAMP assay by competition between unlabelled cAMP and fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP-specific antibody (Amersham)

Fig 13:

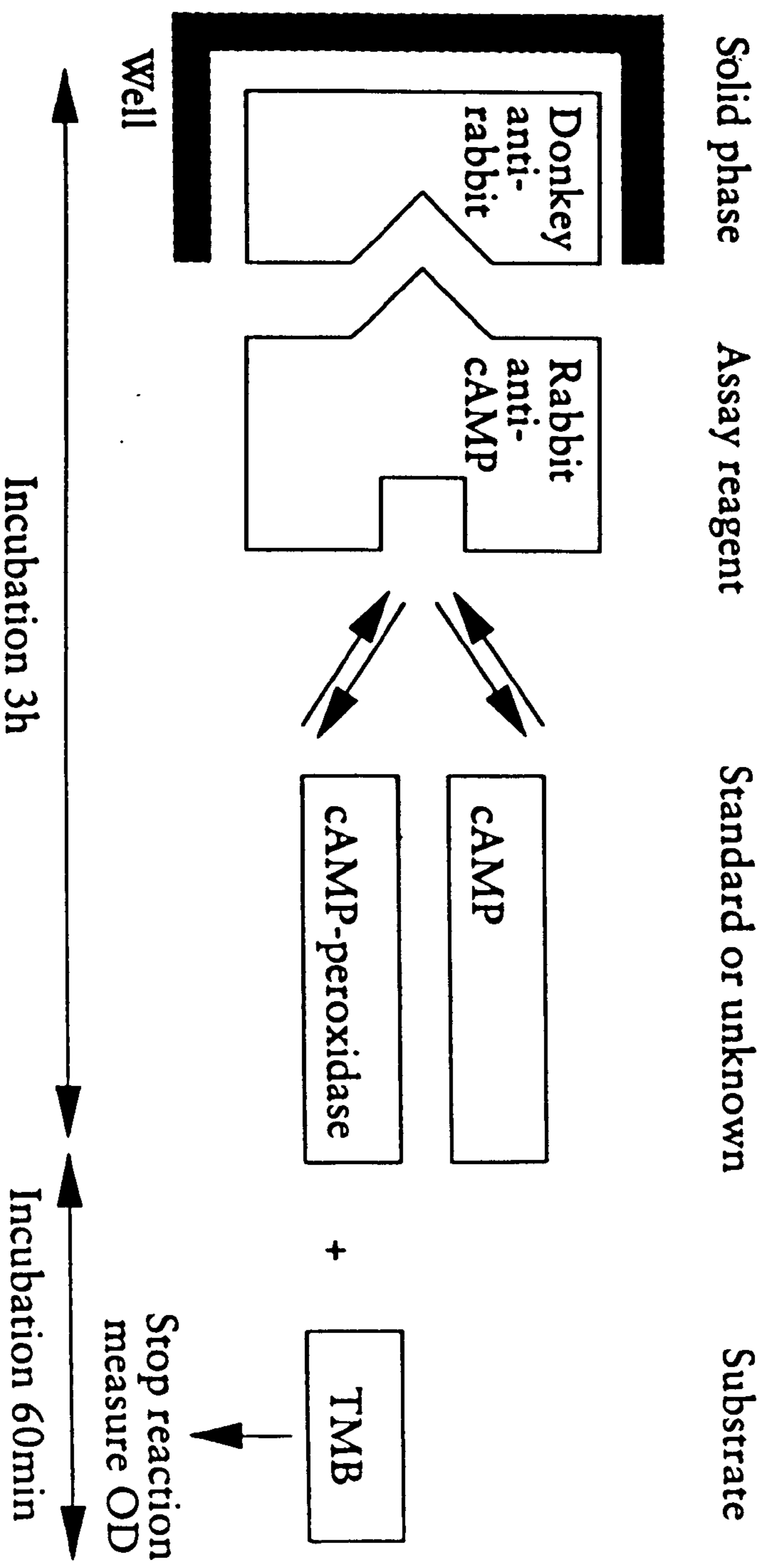


Fig 14: Standard curve for competitive EIA reaction of cAMP assay

(Non-acetylation protocol)

Points represent the log₁₀ cAMP concentration against (B/B₀) for each set of replicated wells. For calculation of standard curve data, the average absorbance (AB) at 450nm was calculated for each set of replicate wells. The percent bound for each standard or test sample was calculated from the following relationship:

B: AB_{450nm} of standard or test sample

B₀: Zero standard (no-enzyme blank)

NSP: Non-specific binding (no-antiserum blank)

$$\%B/B_0 = \frac{\text{Standard or sample AB} - \text{NSB AB}}{B_0 \text{ AB} - \text{NSB AB}} \times 100$$

Fig 14:

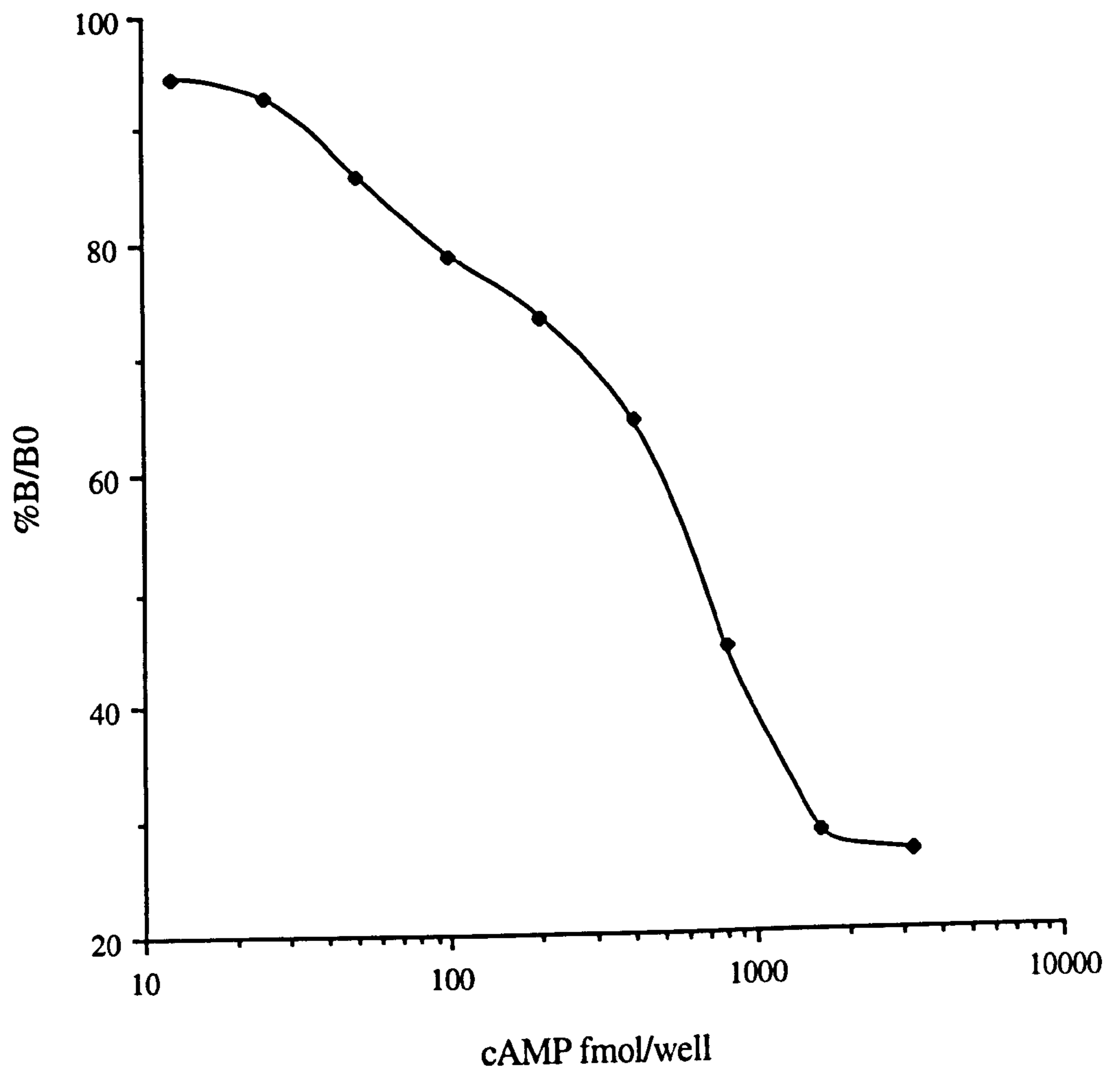


Table 8: Enzyme immunoassay protocol for cAMP; non-acetylation assay

(all volumes are in microlitres)

	NSB	Zero standard blank (B ₀)	Standards	Test samples
Assay buffer	200	100	----	----
Standard	----	----	100	----
Test sample	----	----	----	100
Antiserum	----	100	100	100
Covered plate, incubated at 3-5 °C for 2 hrs				
Peroxidase conjugate	50	50	50	50
Covered plate, incubated at 3-5 °C for 60 min Aspirated, washed all wells four times with 400 µl washing buffer				
Substrate	150	150	150	150
Covered plate, incubated at room temperature for exactly 60 min while shaking				
1.0 M sulphuric acid*	100	100	100	100

* = Reaction can be read at 630 nm before acidification but halting the reaction prior to end point determination is recommended.

and the coupling enzymes pyruvate kinase and lactate dehydrogenase are obtained from Boehringer (Mannheim, Germany). (EPSP, shikimate 3-phosphate and chorismate synthase were kindly supplied by Professor J. R. Coggins, Glasgow University).

For routine purposes and in particular for monitoring the enzyme purification, EPSP synthase is assayed in the reverse direction. In this procedure the following reagents were mixed together and NADH oxidation is monitored continuously at 340_{nm} in a 1 ml cuvette at 25°C: cell free extract 395 µl, EPSP synthase assay buffer (Appendix 5. 14) 500 µl, 2 mM NADH 50 µl, pyruvate kinase/lactate dehydrogenase 5 µl, EPSP (0.7 mg/ ml) 50 µl.

$$\frac{(\text{Change in AB} / \text{min} / \text{ml})}{6.2} = \text{U} / \text{ml}$$

2. 16. Phagocytosis assay

The murine macrophage-like cell line RAW267.7 was maintained in RPMI 1640 medium (containing calf serum [10% v/v] and 10 mM glutamine) at 37°C in the absence of antibiotics. Approximately 10⁵ cells ml⁻¹ were seeded in tissue culture plates or flasks and incubated for 48 to 72 hrs until cell numbers had increased to 5 × 10⁵ cells ml⁻¹. Cell numbers and viability were confirmed by trypan blue exclusion counts. Infections were carried out with suspensions of *P. multocida* strains at concentrations of 2-3 × 10⁸ C.F.U. ml⁻¹ which were prepared in RPMI by resuspension of exponential cultures grown in BHI broth for 4 hrs. After incubation for 2 hrs at 37°C, the cells were washed twice with PBS, and extracellular bacteria were killed by addition of RPMI supplemented with polymyxin B sulphate (Sigma U.K) and gentamycin to a final concentration of 350 µg/ ml of each and incubation continued for 2 hrs. This concentration of polymyxin and gentamycin killed >99.99% of 5 × 10⁹ C.F.U. *P. multocida* organisms over the 2 hrs period, as confirmed by plate counting.

The cells were then washed three times with PBS and resuspended in RPMI and, at chosen intervals, digitonin (Sigma, UK) was added to final concentration of 100 $\mu\text{g}/\text{ml}$ and left for 30 min at 37° C to lyse the eukaryotic cells. Surviving bacteria were estimated by plating aliquots on BA.

3. RESULTS

3. 1. Construction of defined mutations in *P. multocida* strains

3. 1. 1. Cloning of the *aroA* gene

Forward and reverse primers were designed according to the *aroA* sequence of *P. multocida* strain PBA100 serotype A:1 a chicken isolate, published by Homchampa *et al.* (1992) (Genbank accession number Z14100). The *aroA* genes of *P. multocida* 85020 and Quetta strains were amplified by PCR using primers AroA1 and AroA2 (Table 7) as 1.2 kb amplicon (nt. 315-1514). The PCR products were electrophoresed in a 0.8% (w/v) agarose gel and the DNA bands cut out from the gel and purified by Agarose Spin Columns.

A ligation mixture containing the *aroA* gene fragment and the pTAg cloning vector was incubated at 16°C overnight. The ligation mixture was heat inactivated and used to transform *E. coli* XL1 Blue competent cells. Then the bacteria were spread onto LB-X-Gal-IPTG agar containing Ap, Km (vector antibiotic markers) and Tc (competent cells are resistant to Tc) and incubated overnight at 37°C. White colonies were picked and inoculated into 5 ml of LB broth containing appropriate antibiotics. Recombinant plasmids were analysed and confirmed by restriction digestion and PCR. Digestion with *Bam*HI and *Xho*I which cut in the MCS of pTAg produced fragments of ≈1.2 kb and ≈4.0 kb equivalent to the *aroA* insert and cut vector respectively (Fig. 15A, lanes 1 and 2). PCR analysis using AroA primers 1 and 2 (Table 7) produced a ≈1.2 kb fragment from both genomic and plasmid DNA (Fig. 15 B). The ≈4.0 kb band in lanes 2 and 4 are probably plasmid DNA and the very low molecular weight band is probably primer dimers. The plasmids were designated

Fig 15: Analysis of plasmids pJRMT1 and pJRMT2 (size as kb)

A: Restriction analysis

Lane M: 1 kb DNA marker

Lane 1: plasmid pJRMT1 digested with *Bam*HI/*Xho*I

Lane 2: plasmid pJRMT2 digested with *Bam*HI/*Xho*I

Lane 3: plasmid pJRMT1 undigested

Lane 4: plasmid pJRMT2 undigested

Lane M: supercoiled DNA marker

B: PCR analysis of plasmid pJRMT1 and pJRMT2 with primers AroA1 and AroA2

Lane 1: *P. multocida* 85020 genomic DNA

Lane 2: plasmid pJRMT1 DNA

Lane 3: *P. multocida* Quetta genomic DNA

Lane 4: plasmid pJRMT2

Lane M: 1 kb DNA marker

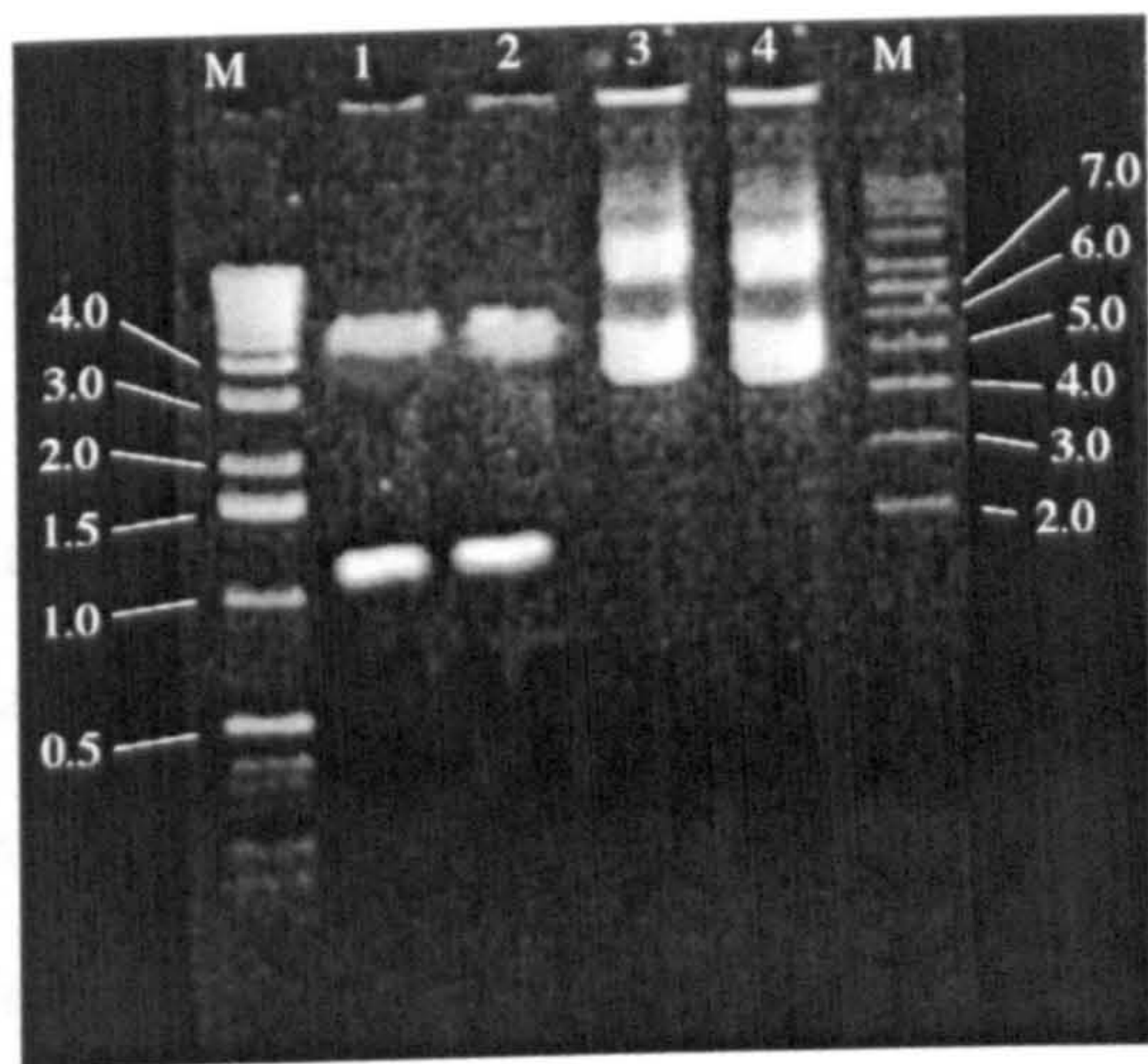
pIPMT1 level from the *P. maritimus* strain (Fig. 16).

3.1.2.5 Fig 15:

Plasmid pIPMT1 was used to transform *P. maritimus* strains *P. maritimus* (1680) and *P. maritimus* (1681).

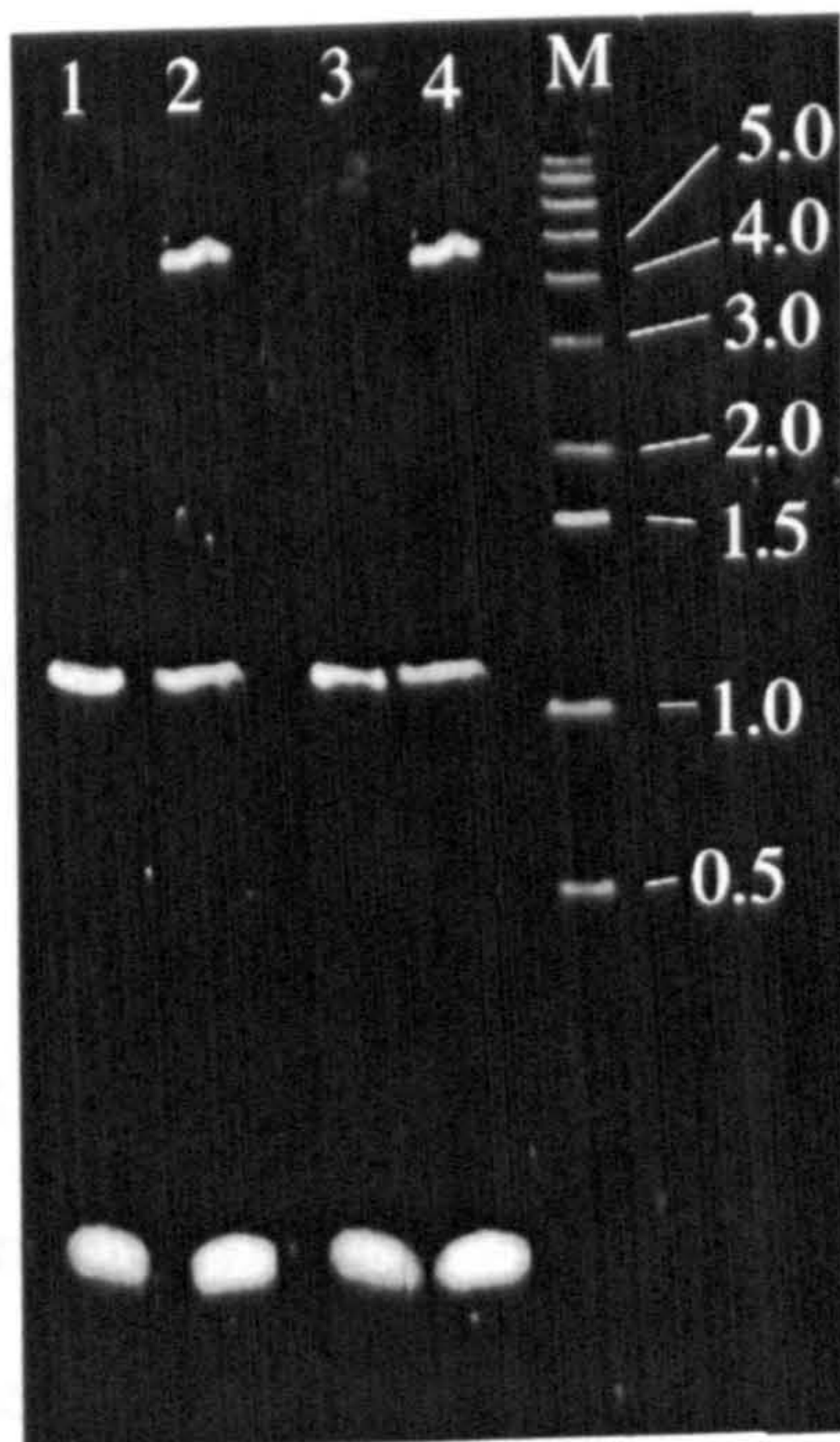
A.

was obtained from *P. maritimus* (1680) and *P. maritimus* (1681) using the same protocol as described above. The plasmid was then digested with *Pvu*I and the resulting fragments were separated by agarose gel electrophoresis. The DNA was stained with ethidium bromide and visualized under UV light.



B.

Pasteurella sp. (1682) was transformed with the plasmid pIPMT1 and the resulting transformants were cultured in a minimal medium. The plasmid was then digested with *Pvu*I and the resulting fragments were separated by agarose gel electrophoresis. The DNA was stained with ethidium bromide and visualized under UV light. The plasmid was then digested with *Pvu*I and the resulting fragments were separated by agarose gel electrophoresis. The DNA was stained with ethidium bromide and visualized under UV light.



pJRMT1 (*aroA* insert from 85020) and pJRMT2 (*aroA* insert from Quetta) (Fig. 16).

3. 1. 2. Subcloning of *aroA* gene into suicide vector pAKA19

Plasmid pAKA19 is a suicide derivative of pAKA16 which was developed for gene transfer to *P. multocida* and *P. (Mannheimia) haemolytica*. pAKA16 was obtained by insertion of the *lacZ* peptide-encoding region and a multiple cloning site from pIC20H into a plasmid which was originally isolated from *P. haemolytica* serotype A1 (Azad *et al.*, 1994). The vector encodes ampicillin resistance, and contains 14 unique restriction sites and the property of phenotypic identification of recombinant clones in *E. coli* by insertional inactivation of β -galactosidase activity. It can be transferred by conjugation to *P. multocida* and *P. haemolytica*. pAKA19 was constructed by replacement of the origin of DNA replication (*ori p*) of pAKA16 with a *ColE1* type *ori* from plasmid pBR322. This derivative will replicate in *E. coli*, but not in either *P. multocida* or *P. haemolytica*, and is suitable for use as a suicide vector for these *Pasteurella* species (Azad *et al.*, 1994). For *E. coli* hosts containing low copy number plasmids like pAKA16, pAKA19 and pEG18.3, Terrific broth (Tartof and Hobbs, Cited by Azad, *et al.*, 1992) containing 1% (w/v) yeast nitrogen base (Difco) was used. Cells were inoculated into 5 ml of volumes Terrific broth and incubated at 37°C for 2-3 hrs until the OD_{540nm} reached $\approx 0.3-0.4$, then chloramphenicol 30 or 170 $\mu\text{g}/\text{ml}$ was added and incubation at 37°C continued for another 16-18 hrs. The amplification of *ColE1*-type plasmids in *E. coli* by inhibition of protein synthesis with Cm (Clewell, 1972) or amino acid starvation (Schroeter *et al.*, 1988) is well established. In our work, pAKA16 was amplified very well in presence of 170 $\mu\text{g}/\text{ml}$ Cm, whereas plasmids pAKA19 and pEG18.3 amplified better in Terrific broth plus 1% (w/v) YNB without Cm (Fig. 17).

Fig 16: The map of plasmids pJRMT1 and pJRMT2

The *aroA* genes of *P. multocida* strains 85020 and Quetta were amplified by PCR and cloned into the pTA_g cloning vector.

Fig 16:

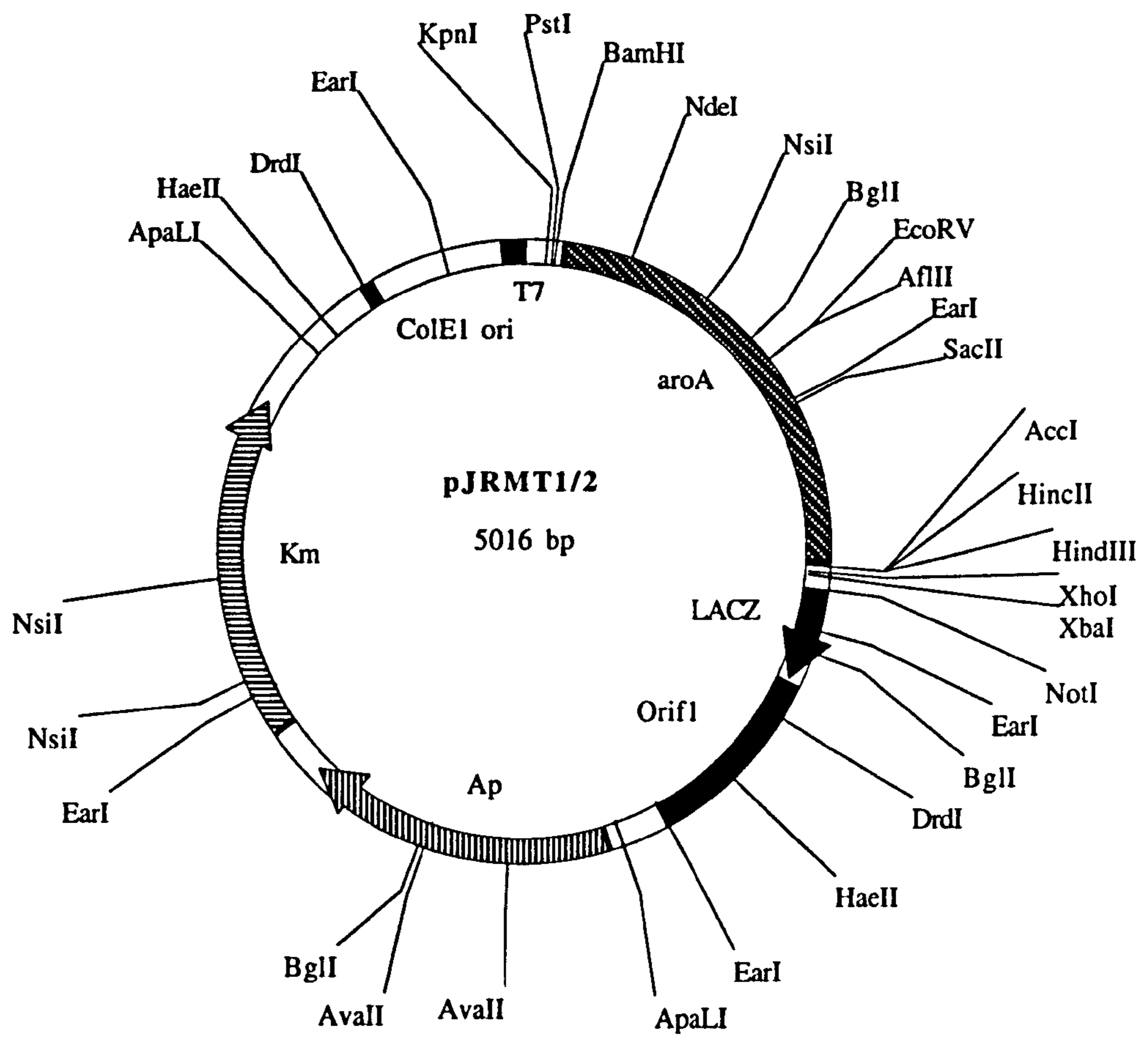


Fig 17: Plasmid DNA concentration from *E. coli* DH5 α containing low copy number plasmids in different culture media (size as kb).

A: Plasmid pAKA16 from *E. coli* DH5 α grown in:

Lane 1: LB broth

Lane 2: Terrific broth

Lane 3: Terrific broth+1% yeast nitrogen base

Lane 4: Terrific broth+1% yeast nitrogen base+170 μ g/ml chloramphenicol

Lane 5: Terrific broth+1% yeast nitrogen base+30 μ g/ml chloramphenicol

Plasmid pAKA19 from *E. coli* DH5 α grown in:

Lane 6: LB broth

Lane 7: Terrific broth

Lane 8: Terrific broth+1% (w/v) yeast nitrogen base

Lane 9: Terrific broth+1% (w/v) yeast nitrogen base+170 μ g/ml
chloramphenicol

Lane 10: Terrific broth+1% (w/v) yeast nitrogen base+30 μ g/ml
chloramphenicol

Lane M: supercoiled DNA marker

B: Plasmid pEG18.3 from *E. coli* DH5 α grown in:

Lane 1: LB broth

Lane 2: Terrific broth

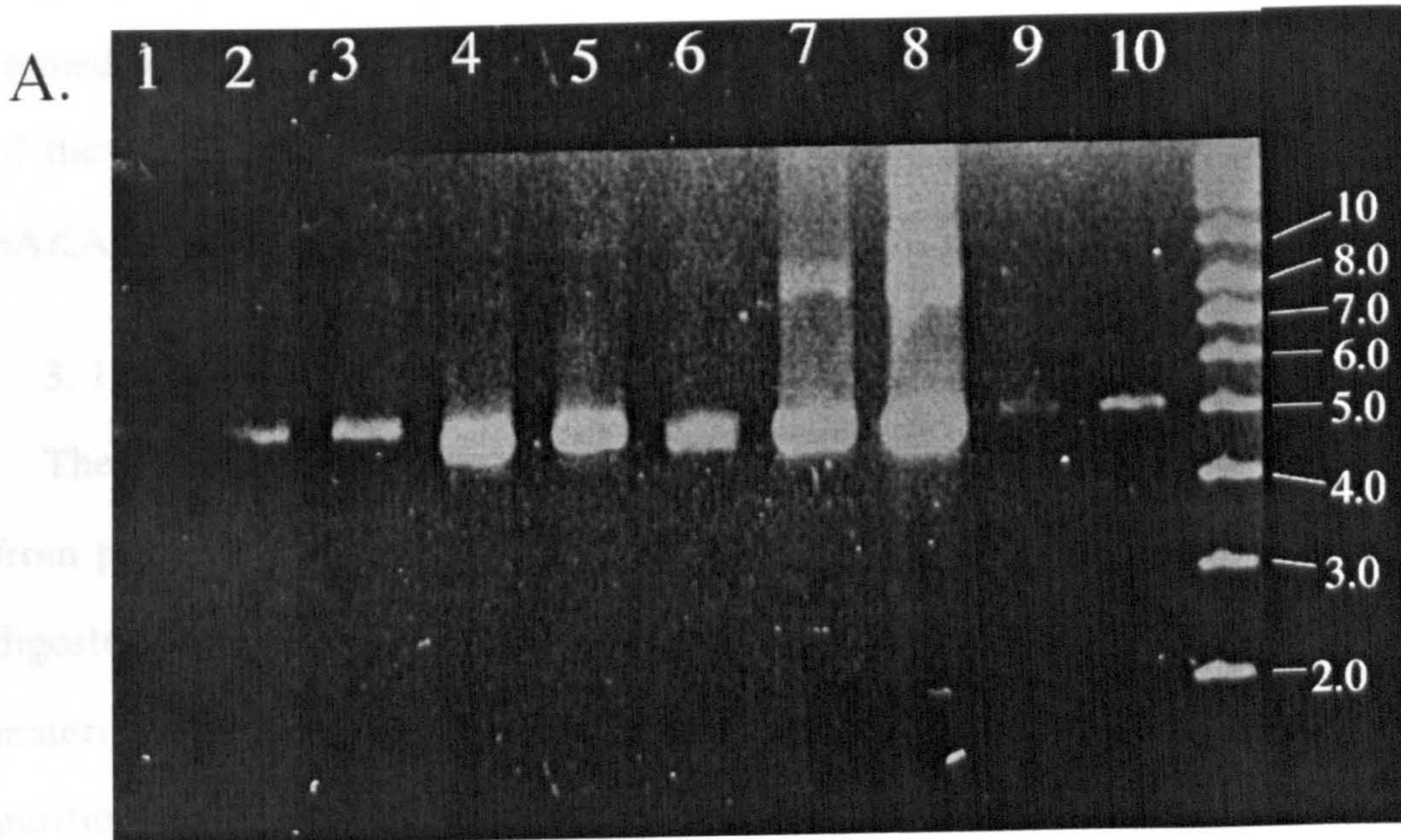
Lane 3: Terrific broth+1% (w/v) yeast nitrogen base

Lane 4: Terrific broth+1% (w/v) yeast nitrogen base+170 μ g/ml
chloramphenicol

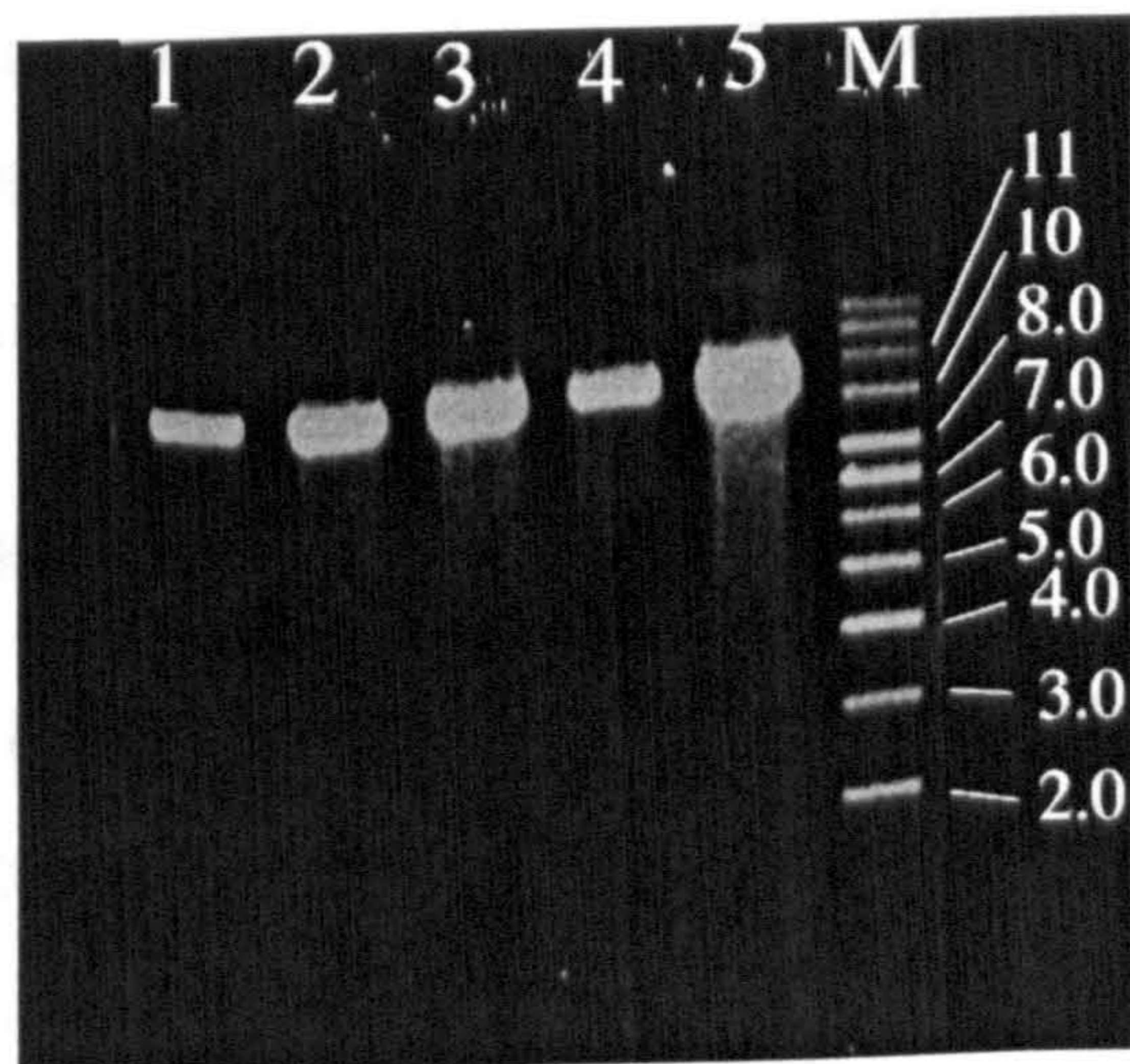
Lane 5: Terrific broth+1% (w/v) yeast nitrogen base+30 μ g/ml
chloramphenicol

Lane M: supercoiled DNA marker

Fig 17:



B.



The *aroA* fragments were cut out from plasmids pJRMT1 and pJRMT2 with *Bam*HI and *Xho*I and plasmid pAKA19 was digested with *Bam*HI and *Xho*I and dephosphorylated. The fragments were ligated and transformed into *E. coli* DH5 α . White colonies were selected from LB-X-Gal-IPTG plates containing ampicillin. Recombinant plasmids were confirmed by restriction analysis and termed pJRMT3 and pJRMT4 respectively (Fig. 20 A). *Bam*HI/*Xho*I digests of these plasmids produced the expected \approx 1.2 kb *aroA* insert and \approx 5.0 kb pAKA19 vector (Fig. 18).

3. 1. 3. Insertion of a kanamycin resistance cassette into the *aroA* gene

The Km^R cassette was cut out as a 1.24 kb fragment (from nt. 421-1661) from plasmid pUC4K with *Pst*I and plasmids pJRMT3 and pJRMT4 were digested with *Nsi*I (at nt. 718) (Fig. 20A) and dephosphorylated. Digested material was run in a 0.8% (w/v) agarose gel and the relevant fragments purified and ligated. The ligation mixture was transformed into *E. coli* DH5 α competent cells by electroporation and colonies resistant to both Ap and Km were selected on LB agar containing these antibiotics. Recombinant plasmids were confirmed by restriction analysis and designated as pJRMT5 and pJRMT6 respectively (Fig. 20B). *Bam*HI/*Xho*I digestion showed a \approx 2.4 kb *aroA*::Km^R insert and the \approx 5.0 kb pAKA19 vector (Fig. 19).

3. 1. 4. Allelic exchange

The suicide plasmids pJRMT5 and pJRMT6 were used with *P. multocida* strains for allelic exchange, and introduced into strain 85020 by electroporation and into the Sm^R Quetta strain JRMT11 (Table 4) by conjugation. For the latter procedure, the plasmids were transformed to *E. coli* SM10 λ pir by electroporation. 100 μ l aliquots of bacteria were spread onto BHI agar supplemented with 5% (v/v) sheep blood containing kanamycin only for strain 85020 and kanamycin with streptomycin for strain Quetta Sm^R in order to select Km^R and Km^R-Sm^R colonies respectively. For each strain picked up 50

Fig 18: Restriction analysis of plasmids pJRMT3 and pJRMT4 (the *aroA* genes cloned into pAKA19 vector, size as kb)

Left:

Lane 1: plasmid pJRMT3 digested with *Bam*HI/*Xho*I (bands of ≈ 1.2 and ≈ 5.0 kb)

Lane 2: plasmid pJRMT4 digested with *Bam*HI/*Xho*I (bands of ≈ 1.2 and ≈ 5.0 kb)

Lane M: 1 kb marker

Right:

Lane M: supercoiled DNA marker

Lane 1: plasmid pJRMT3 undigested

Lane 2: plasmid pJRMT4 undigested

Fig 19: Restriction analysis of plasmids pJRMT5 and pJRMT6 (size as kb)

Left:

Lane 1: plasmid pJRMT5 digested with *Bam*HI/*Xho*I (bands of ≈ 2.4 and ≈ 5.0 kb)

Lane 2: plasmid pJRMT6 digested with *Bam*HI/*Xho*I (bands of ≈ 2.4 and ≈ 5.0 kb)

Lane 3: 1 kb DNA marker

Right:

Lane 1: plasmid pJRMT5 undigested

Lane 2: plasmid pJRMT6 undigested

Lane M: supercoiled DNA marker

Fig 18:

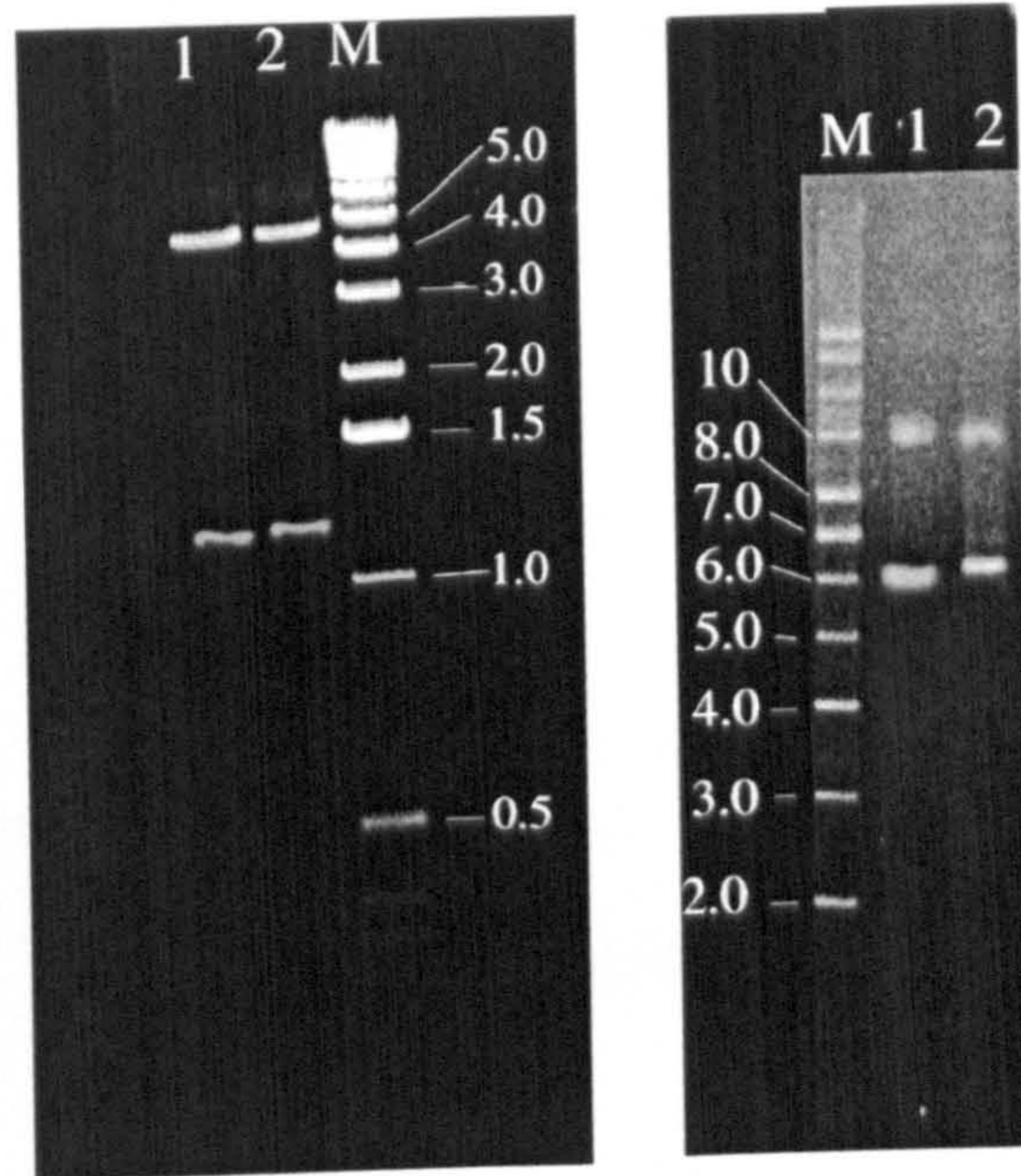


Fig 19:

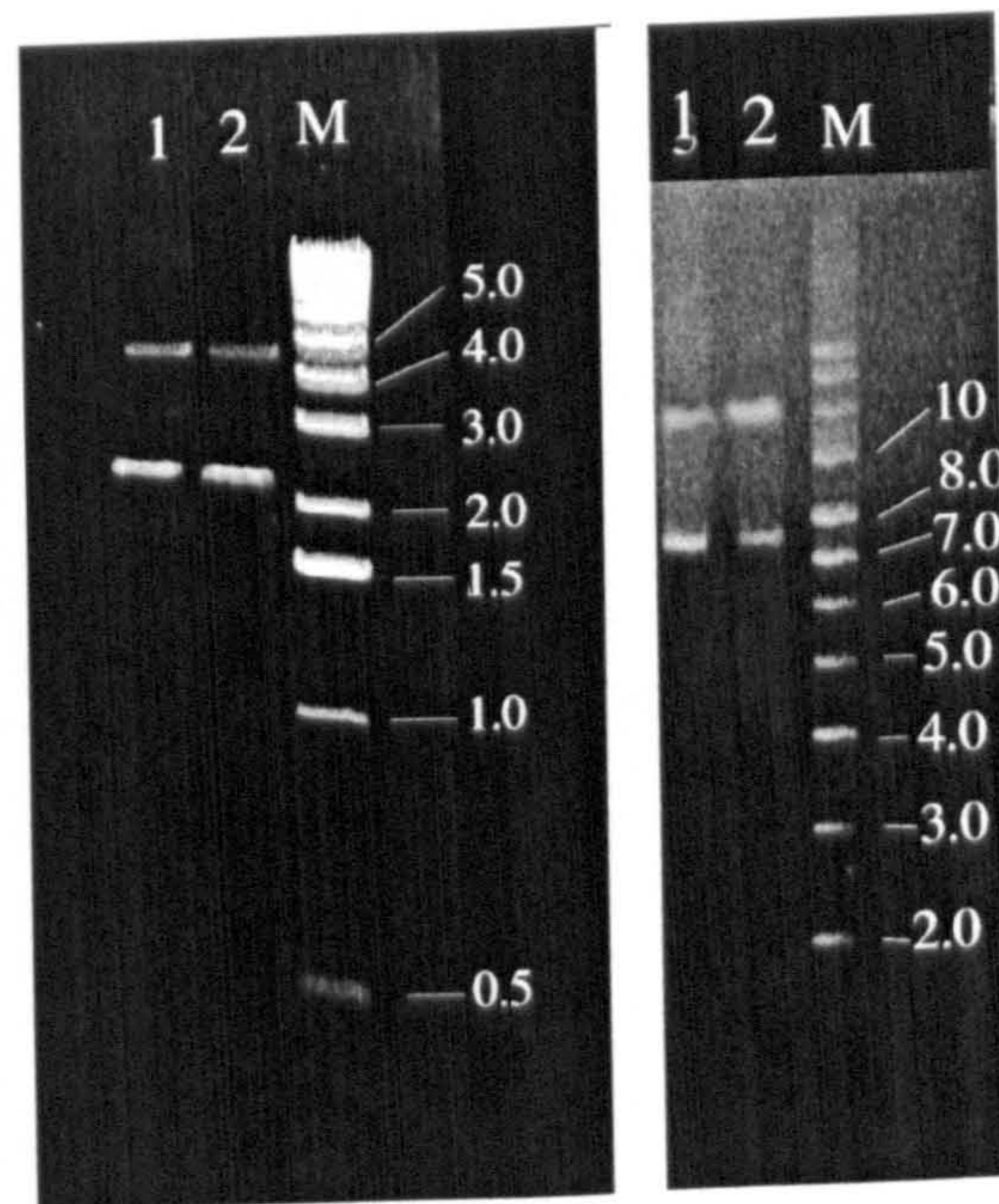


Fig 20:

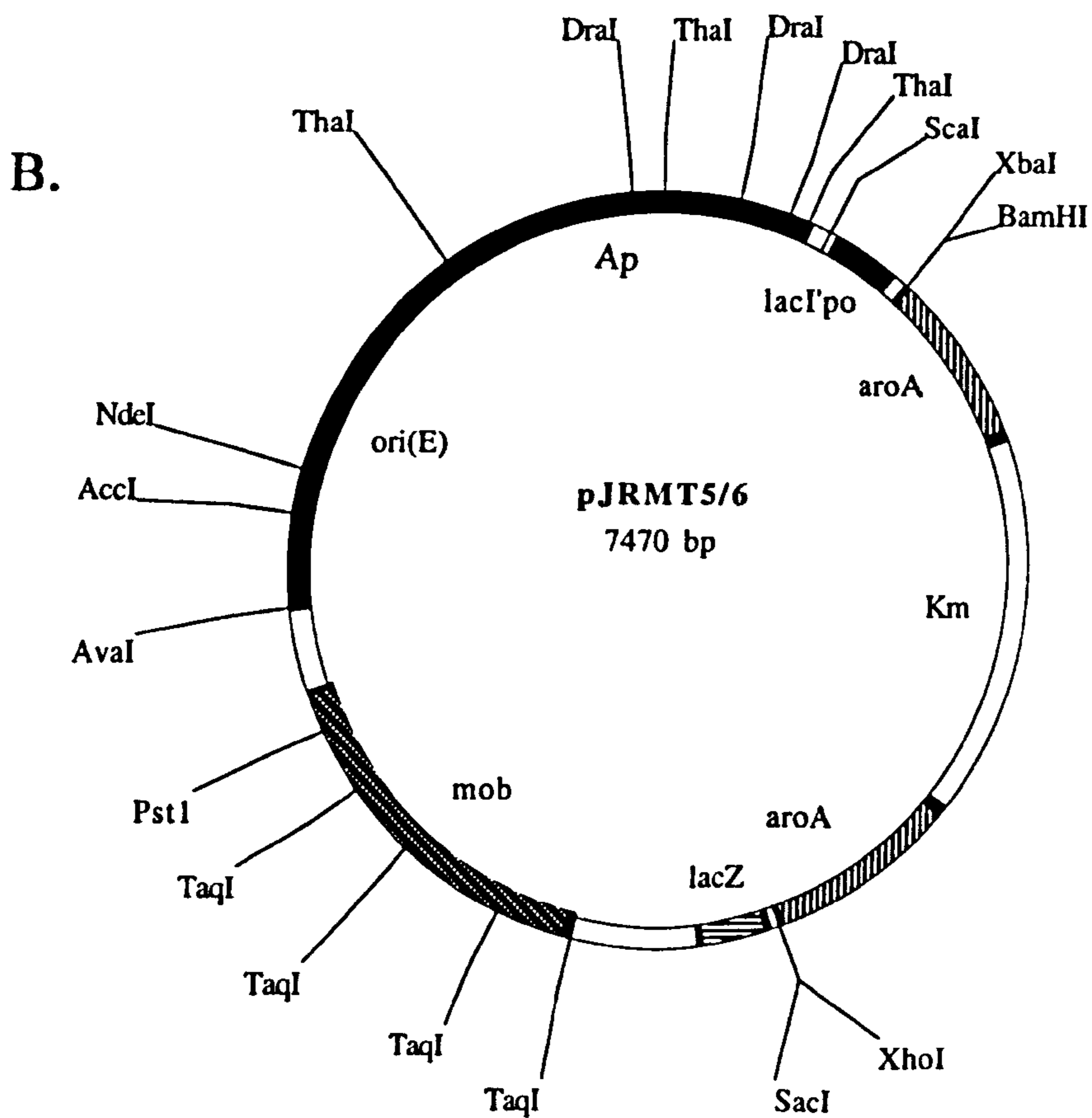
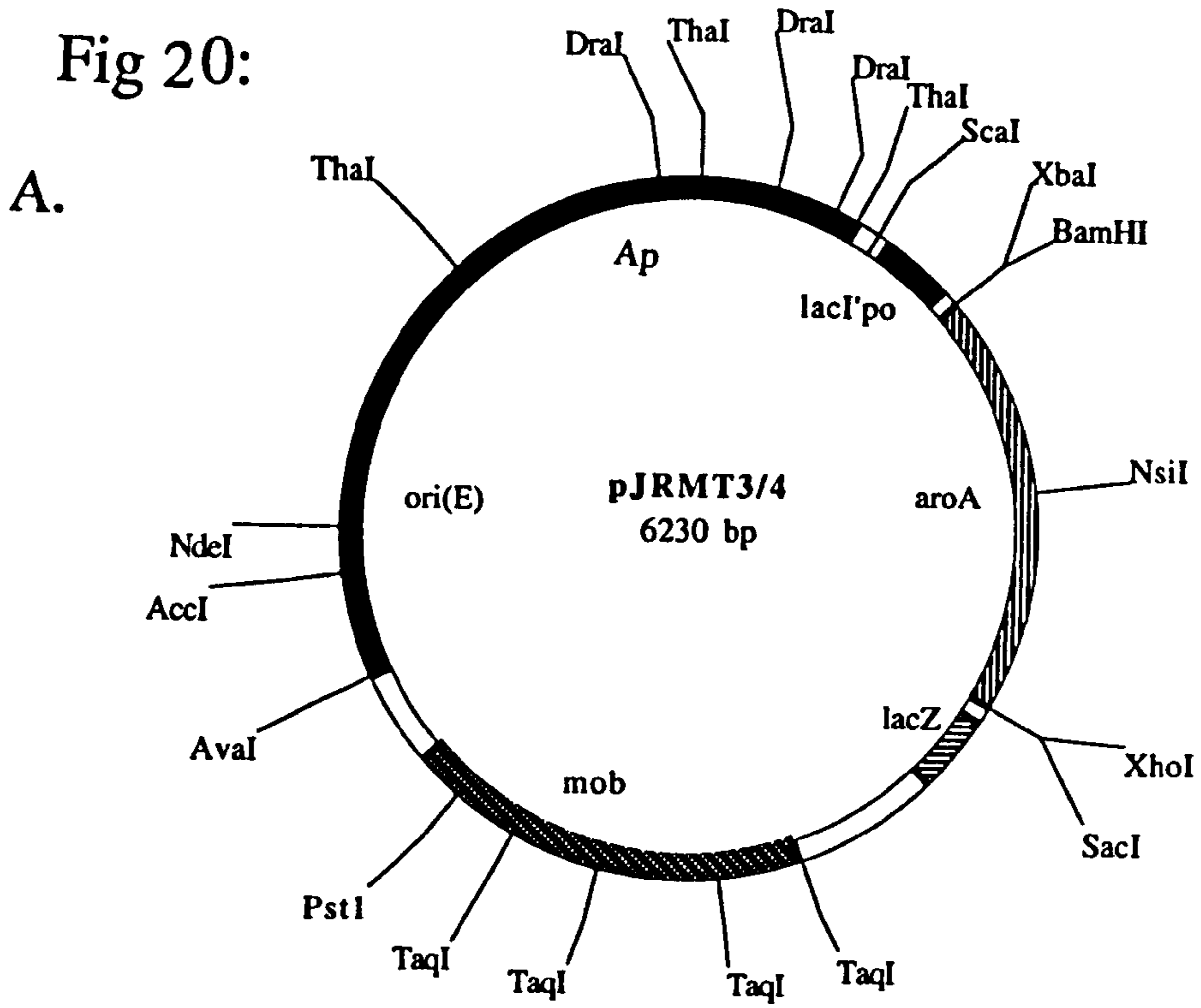
A: The map of plasmids pJRMT3 and pJRMT4

The *aroA* gene was inserted as a *Bam*HI/*Xho*I fragment into pAKA19 which had been digested with *Bam*HI/*Xho*I and dephosphorylated.

B: The map of plasmids pJRMT5 and pJRMT6

The kanamycin resistance cassette from plasmid pUC4K was inserted into plasmids pJRMT3 and pJRMT4.

Fig 20:



single colonies from different selective plates and subcultured on BHI agar supplemented with 5% (v/v) sheep blood containing appropriate antibiotics for five days. then 20 single clones of each strain checked by PCR. From these clones just 8 or 9 clones were showed correct predicted pattern that selected and one of them for each *P. multocida* strains have been chosen as *aroA* mutant strain for further works.

3. 1. 5. Confirmation of the *P. multocida aroA* mutant strains

Potential mutant derivatives were tested by PCR, growth in Pasteurella Minimal Medium (Jabloneski *et al.*, 1992b), assay for the enzyme EPSP synthase and Southern blot hybridisation.

3. 1. 5. 1. PCR

The *aroA* primers AroA1 and AroA2 amplified a larger ≈ 2.4 kb amplimer(*aroA* gene+ kanamycin resistance cassette) from *P. multocida* JRMT1, derived from *P. multocida* 85020, and JRMT2, derived from *P. multocida* Quetta Sm^R, than from the parent strains which produced the expected ≈ 1.2 kb amplimer (Fig .21).

3. 1. 5. 2. Growth in Pasteurella Minimal Medium (PMM)

Bacteria were grown on BHI agar supplemented with 5% (v/v) sheep blood at 37°C overnight. Several colonies were inoculated into PMM with and without aromix supplementation and incubated at 37°C with shaking (250 rpm). The OD_{540nm} values were measured after 24 and 48 hrs (Fig. 22). Both mutant strains failed to grow in PMM unless supplemented with aromix.

3. 1. 5. 3. Southern blot hybridisation

The PCR products and genomic DNA, digested with *EcoRV*, of the wild type and the mutant strains were electrophoresed in a 0.7% (w/v) agarose gel and a blot processed for hybridisation with the *aroA* probe (*aroA* gene of *P. multocida* 85020 amplified by PCR and dig-labelled) and with the kanamycin

Fig 21: PCR analysis of *aroA* in wild-type and mutated *P. multocida* strains
(size as kb)

The AroA primers were used with genomic DNA preparations as template

Lane 1: *P. multocida* 85020

Lane 2: *P. multocida* JRMT1

Lane 3: *P. multocida* Quetta

Lane 4: *P. multocida* JRMT2

Lane M: 1 kb DNA marker

Fig 21:

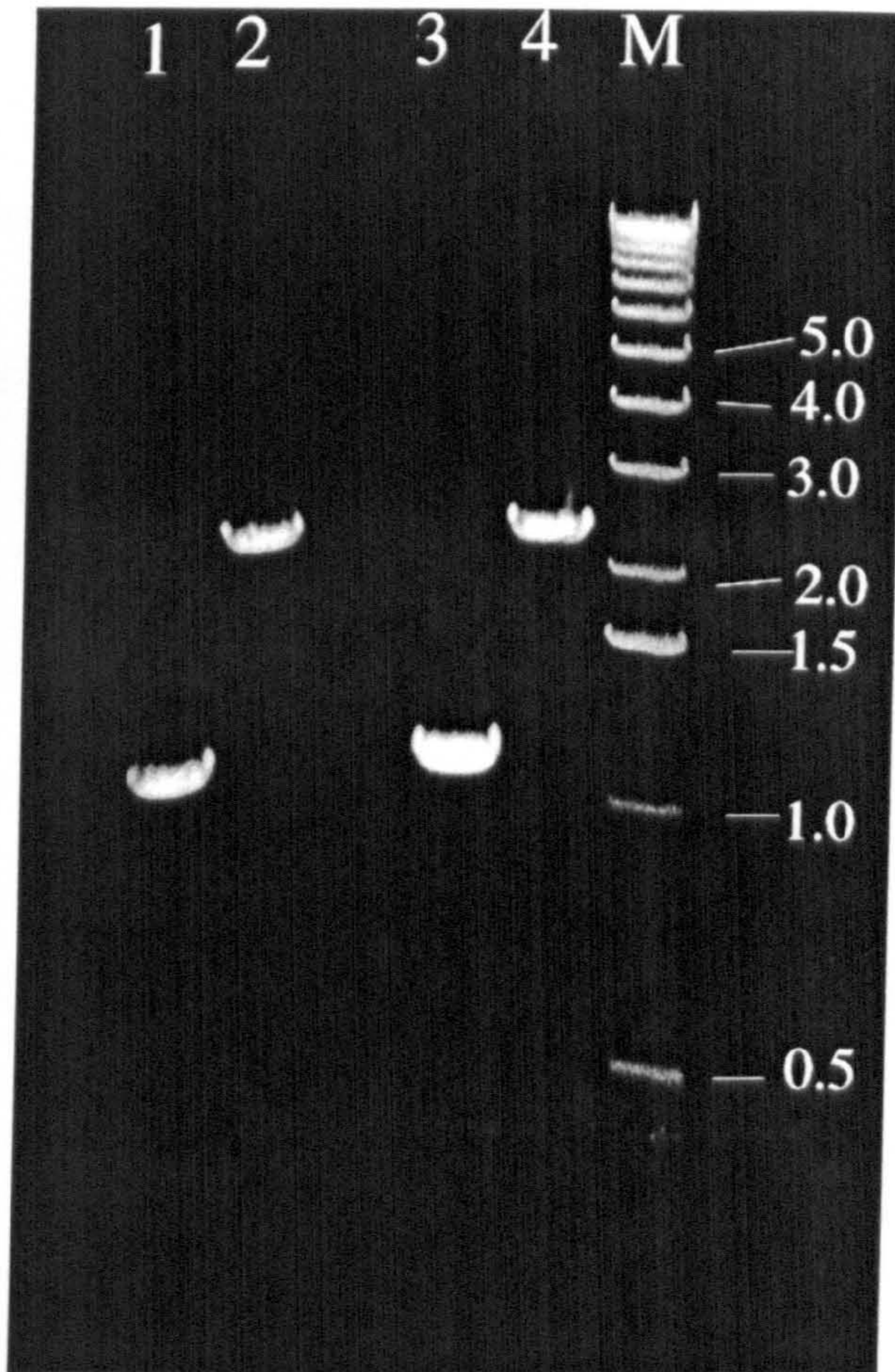
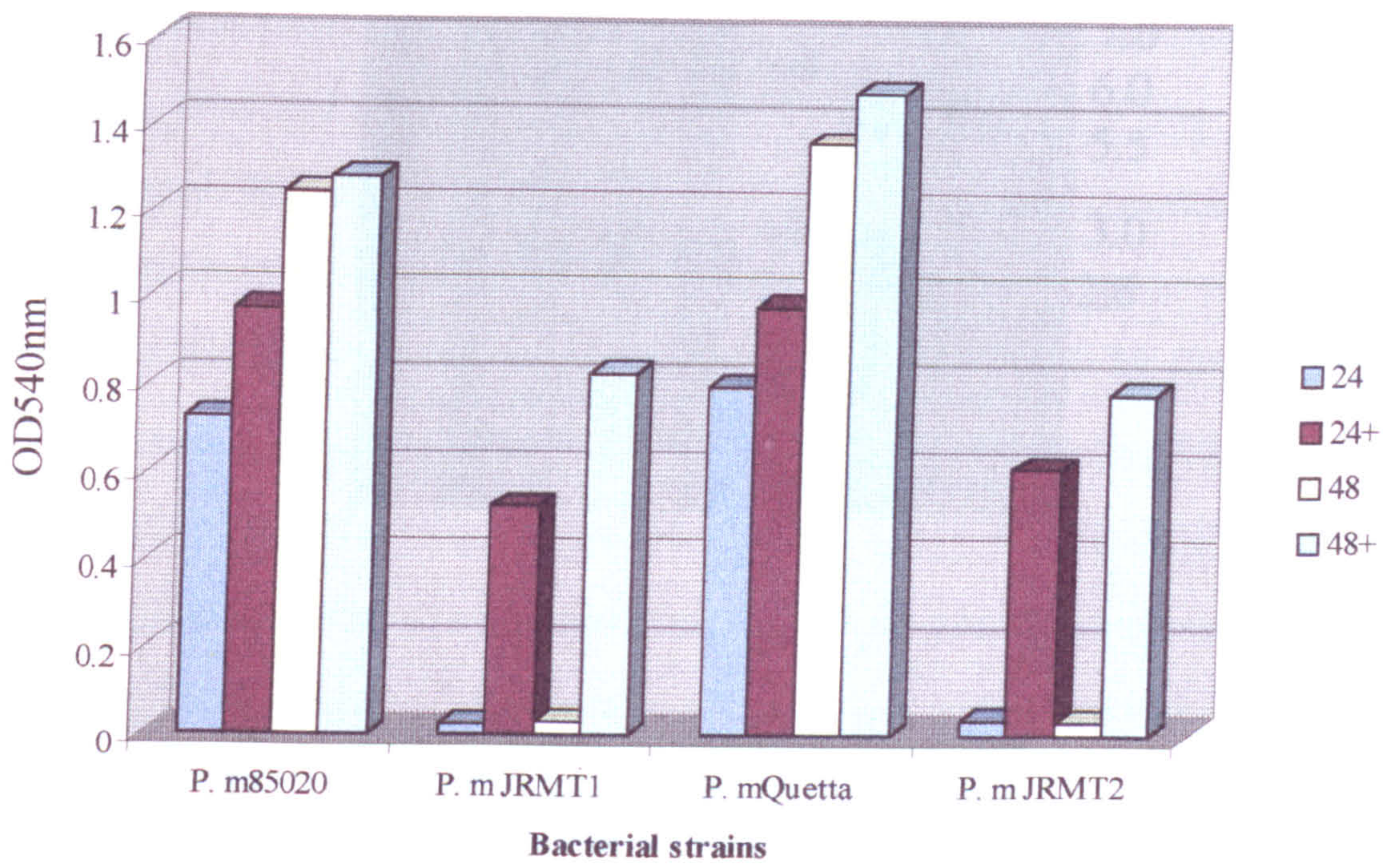


Fig 22: Growth of *P. multocida* strains in Pasteurella Minimal Medium

P. multocida strains 85020, JRMT1, Quetta and JRMT2 were inoculated into PMM with (indicated by +) or without aromix supplementation and the OD_{540nm} was measured after shaking for 24 and 48 hrs at 37°C.

Fig 22:



resistance cassette (*Pst*I fragment of plasmid pUC4K). With the PCR products, the *aroA* probe hybridised to ≈ 1.2 kb band for the wild type and the ≈ 2.4 kb band for the mutant strains as expected (Fig. 23A, left). The Km^{R} probe hybridised to only the ≈ 2.4 kb band obtained from PCR of the mutant strains (Fig. 23A, right). In genomic DNA digested with *EcoRV*, the wild-type strains showed two bands of ≈ 1.8 and ≈ 1.6 kb. This would confirm the presence of an *EcoRV* site in the *aroA* gene (nt. 931). No hybridisation was detected with the Km^{R} probe. For the mutant strains, two bands of ≈ 1.8 and ≈ 2.8 kb were visible with the *aroA* probe (Fig. 23B, left) and the Km^{R} probe hybridised only with the bigger band (Fig. 23B, right). This confirmed insertion of the Km^{R} cassette into the section of *aroA* represented by the 1.6 kb *EcoRV* fragment.

3. 1. 5. 4. EPSP synthase assay

For the EPSP synthase assay, a cell free extract was prepared and the protein concentration measured by Lowry procedure. Total protein concentrations for *P. multocida* 85020 and Quetta wild-type strains was 12.8 and 14.7 mg/ ml respectively and for *aroA* mutant JRMT1 and JRMT2 strains was 9.6 and 9.9 mg/ ml respectively. The values for EPSP synthase enzyme activity obtained for *P. multocida* 85020 and Quetta wild-type strains was 0.024 and 0.021 U/ ml respectively, whereas no activity was detected for *aroA* mutant JRMT1 and JRMT2 strains.

3. 1. 6. Cloning of *cya* gene

Primers were designed according to the sequence of the *cya* gene of *P. multocida* CNP1 (NCTC 10332) (Mock *et al.*, 1991; Genbank accession number M68901). The *cya* gene amplified from *P. multocida* strain 85020 was 2503 bp (from nt. 171-2674) using the primers Cya4 and Cya5 (Table 7). Primer Cya4 was not used successfully with strain Quetta and a supplementary primer Cya6 had to be designed (Table 7). The *cya* gene amplified from *P.*

Fig 23: Southern blot hybridisation *P. multocida* stains 85020, Quetta and their *aroA* mutants, JRMT1 and JRMT2 (size as kb)

A: PCR products obtained with AroA primers from genomic DNA of *P. multocida* strains

Left: Hybridisation with *aroA* probe

Right: Hybridisation with Km^R probe

Lane 1: *P. multocida* 85020 (wild-type)

Lane 2: *P. multocida* JRMT1 (*aroA* mutant)

Lane 3: *P. multocida* Quetta (wild-type)

Lane 4: *P. multocida* JRMT2 (*aroA* mutant)

B: The genomic DNA of *P. multocida* strains, digested with *EcoRV*

Left: Hybridisation with *aroA* probe

Right: Hybridisation with Km^R probe

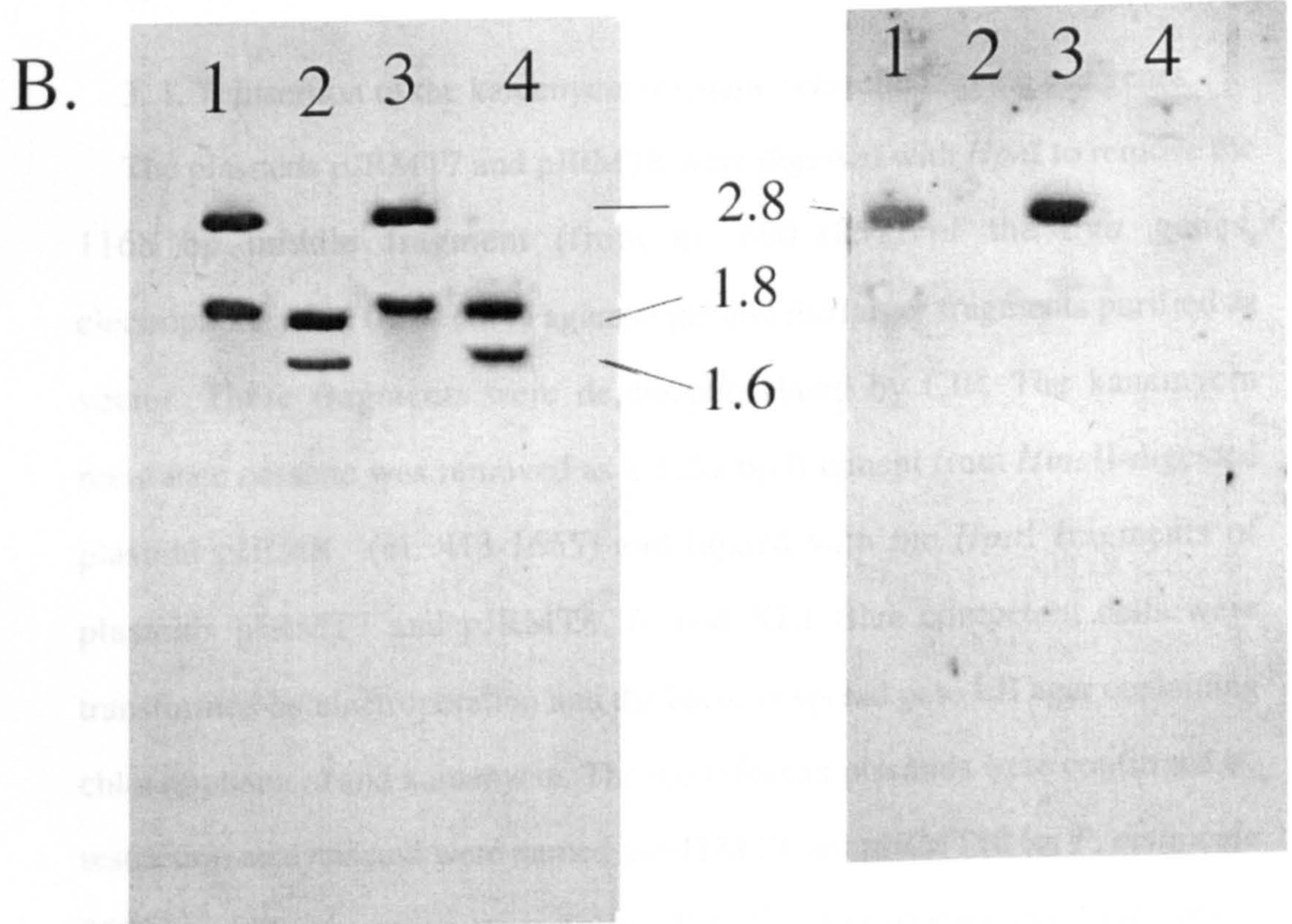
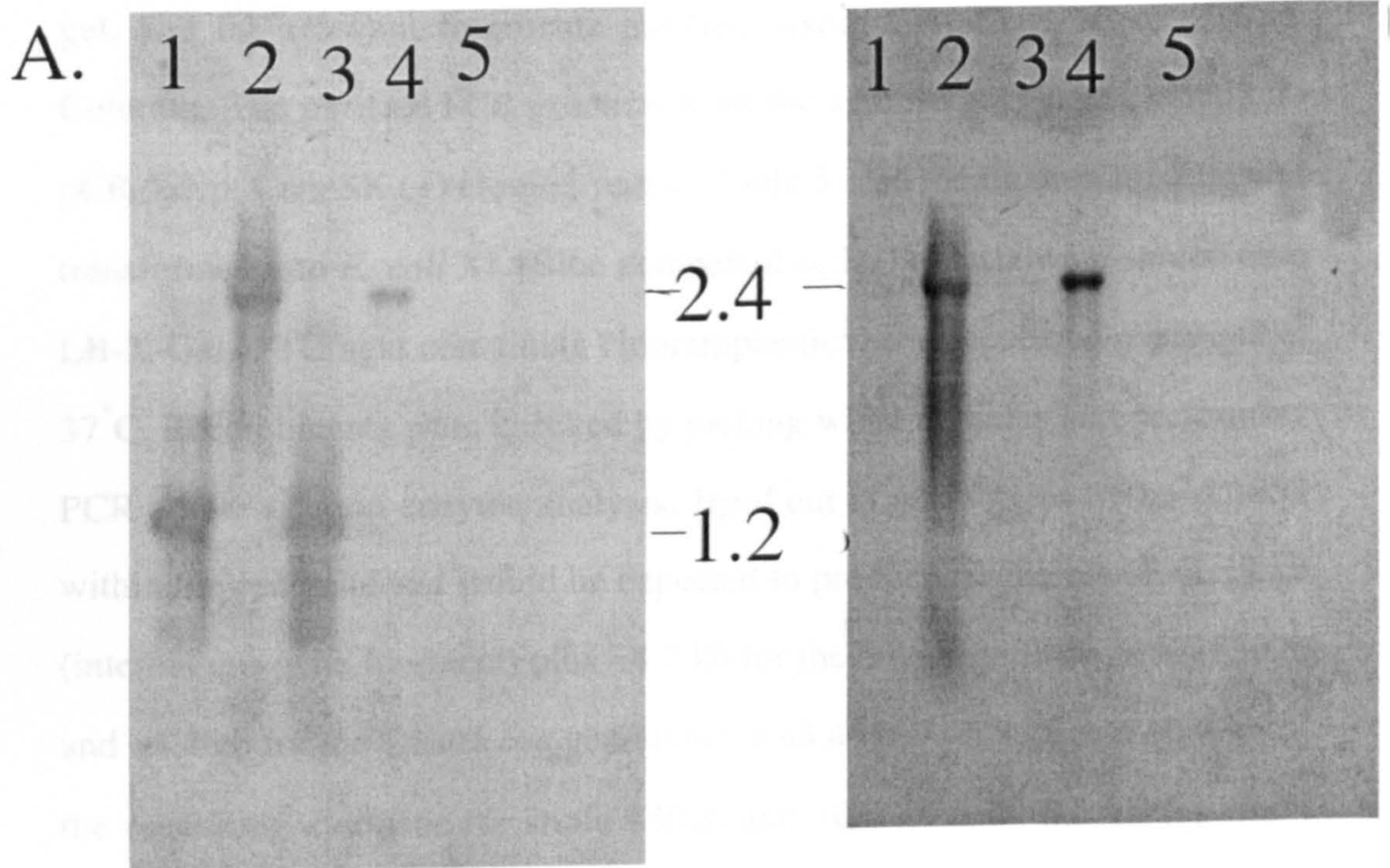
Lane 1: *P. multocida* JRMT1 (*aroA* mutant)

Lane 2: *P. multocida* 85020 (wild-type)

Lane 3: *P. multocida* JRMT2 (*aroA* mutant)

Lane 4: *P. multocida* Quetta (wild-type)

Fig 23:



multocida strain Quetta was 2203 bp (from nt. 171-2372) using primers Cya5 and Cya6 (Table 7).

The above PCR products were electrophoresed on a 0.8% (w/v) agarose gel, and the relevant fragments purified, using Gen Elute Agarose Spin Columns. The purified PCR products from the agarose gels were cloned into pCR-Script Cam SK (+) cloning vector (Table 5) and the ligation mixture was transformed into *E. coli* XL1Blue competent cells. Bacteria were spread onto LB-X-Gal-IPTG agar containing chloramphenicol and incubated overnight at 37°C. Recombinants were checked by picking white colonies and performing PCR and restriction enzyme analysis. *HpaI* cut at nucleotides 690 and 1857 within the *cya* gene and would be expected to produce fragments of ≈1.17 kb (internal *cya* gene fragment) plus ≈4.7 kb for the *cya* gene from strain 85020 and ≈4.4 kb for the Quetta *cya* gene (vector ≈3.4 kb + ≈0.5 kb and ≈0.8 kb of the remaining *cya* gene for strain 85020 and ≈0.5 kb and ≈0.5 kb for strain Quetta). The new plasmids were designated pJRMT7 for *P. multocida* 85020 and pJRMT8 for *P. multocida* Quetta (Fig. 24 & 25).

3. 1. 7. Insertion of the kanamycin resistance cassette into the *cya* genes

The plasmids pJRMT7 and pJRMT8 were digested with *HpaI* to remove the 1168 bp middle fragment (from nt. 690-1857) of the *cya* genes, electrophoresed in 0.7% (w/v) agarose gel and the larger fragments purified as vector. These fragments were dephosphorylated by CIP. The kanamycin resistance cassette was removed as a 1252 bp fragment from *HincII*-digested plasmid pUC4K (nt. 413-1665) and ligated with the *HpaI* fragments of plasmids pJRMT7 and pJRMT8. *E. coli* XL1 Blue competent cells were transformed by electroporation and the bacteria spread onto LB agar containing chloramphenicol and kanamycin. The recombinant plasmids were confirmed by restriction analysis and were named as pJTMT9 and pJRMT10 for *P. multocida* 85020 and Quetta strains respectively (Fig. 27). As expected, *HpaI* did not cut

Fig 24:

A: Restriction analysis of plasmids pJRMT7 and pJRMT8 (*cya* genes cloned into pCR-Script, size as kb)

Lanes 1-4: plasmid pJRMT7 digested with *Hpa*I (different clones)

Lanes 5-7: plasmid pJRMT8 digested with *Hpa*I (different clones)

Lane M: 1 kb marker

B: PCR analysis of plasmids pJRMT7 and pJRMT8 with *Cya* primers

Lane M: 1kb DNA marker

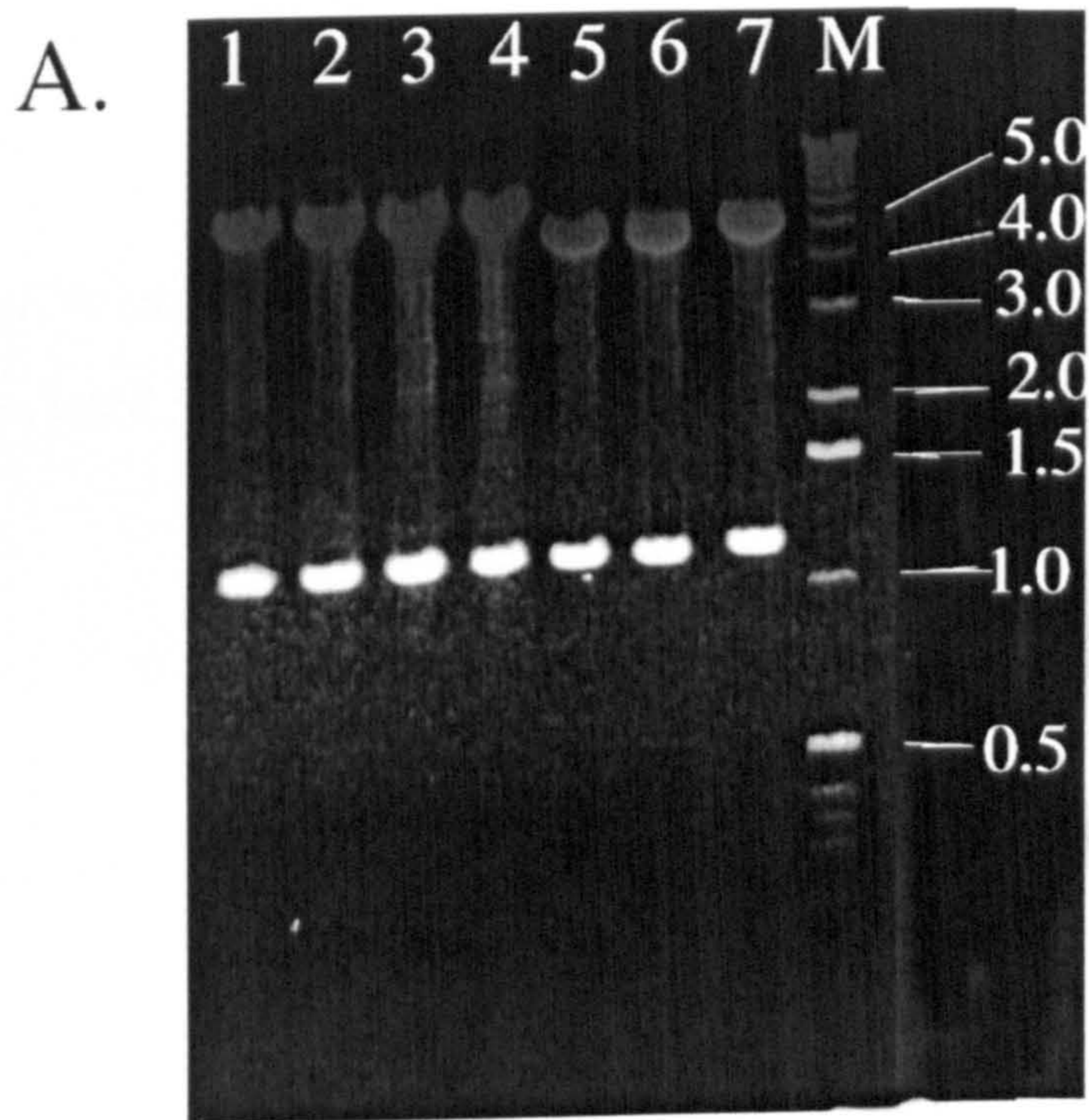
Lane 1: plasmid pJRMT7 (plasmid DNA as template) with primers *Cya*4 and *Cya*5

Lane 2: plasmid pJRMT8 with primers *Cya*5 and *Cya*6

Lane 3: *P. multocida* 85020 genomic DNA with primers *Cya*4 and *Cya*5

Lane 4: *P. multocida* Quetta genomic DNA with primers *Cya*5 and *Cya*6

Fig 24:



B.

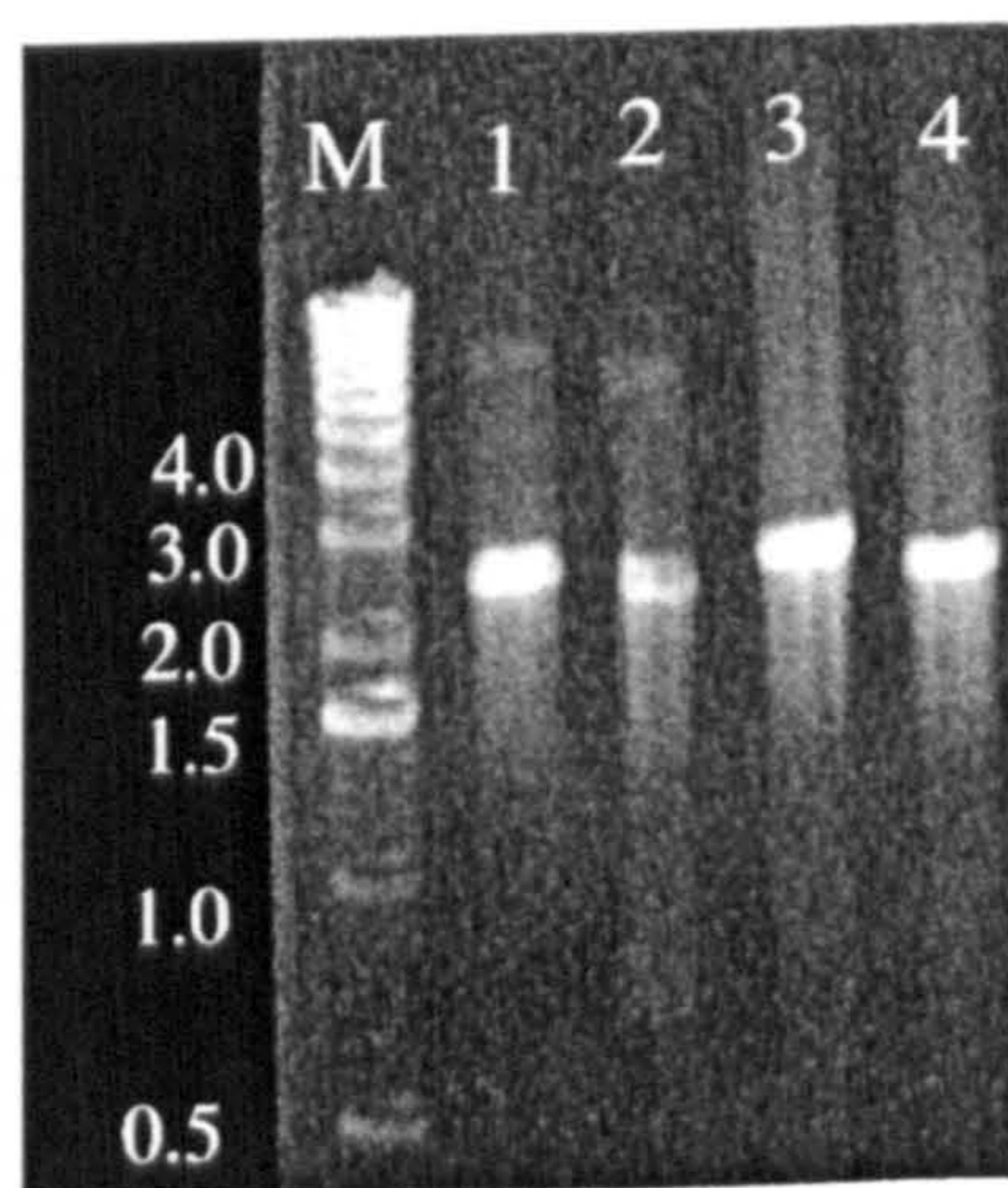
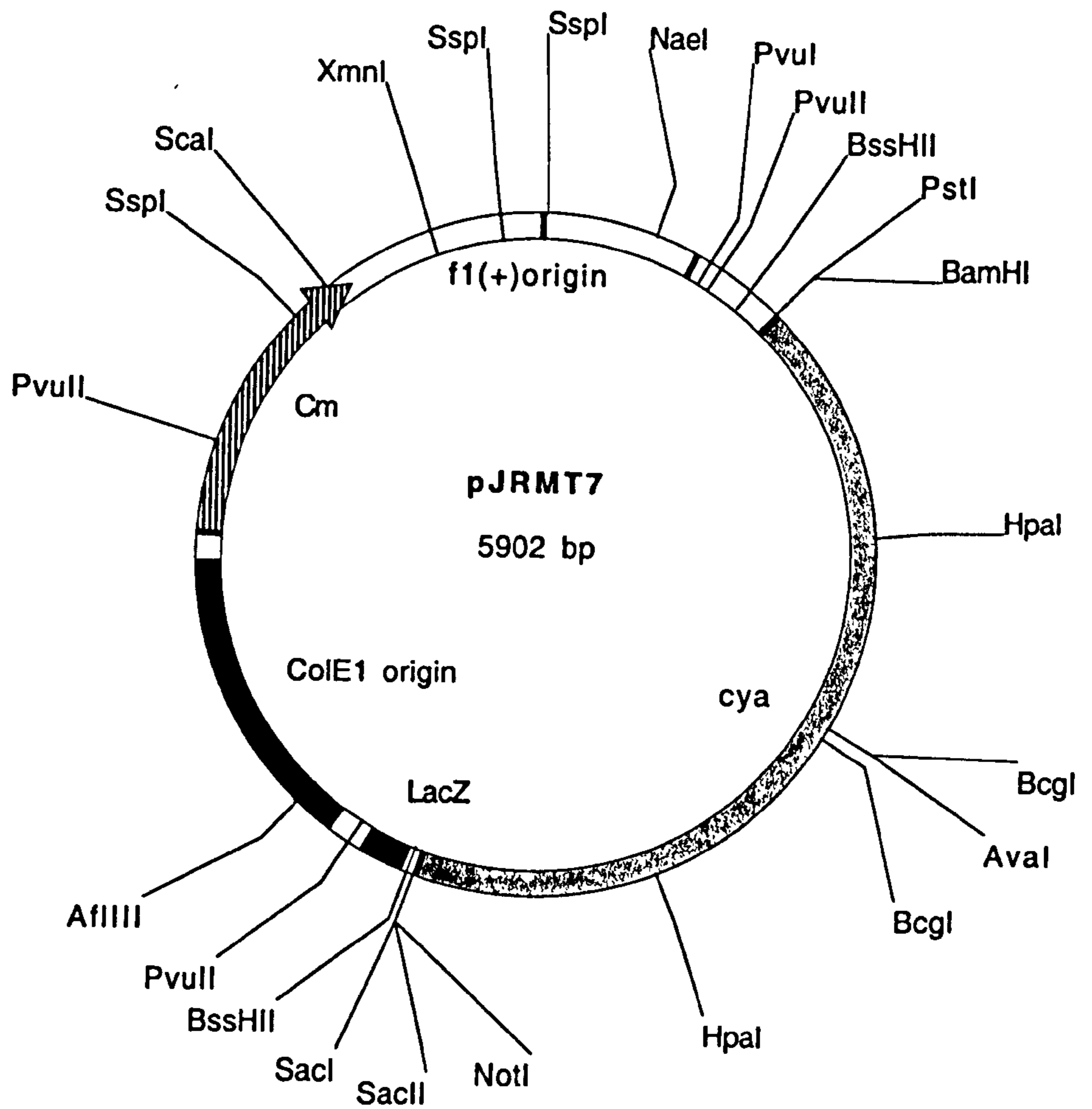


Fig 25: Map of plasmids pJRMT7 and pJRMT8

The *cya* genes of *P. multocida* strains were amplified by PCR (as 2.5 and 2.2 kb fragments for 85020 and Quetta strains respectively) and cloned into pCR-Script cloning vector

Fig 25:

A.



B.

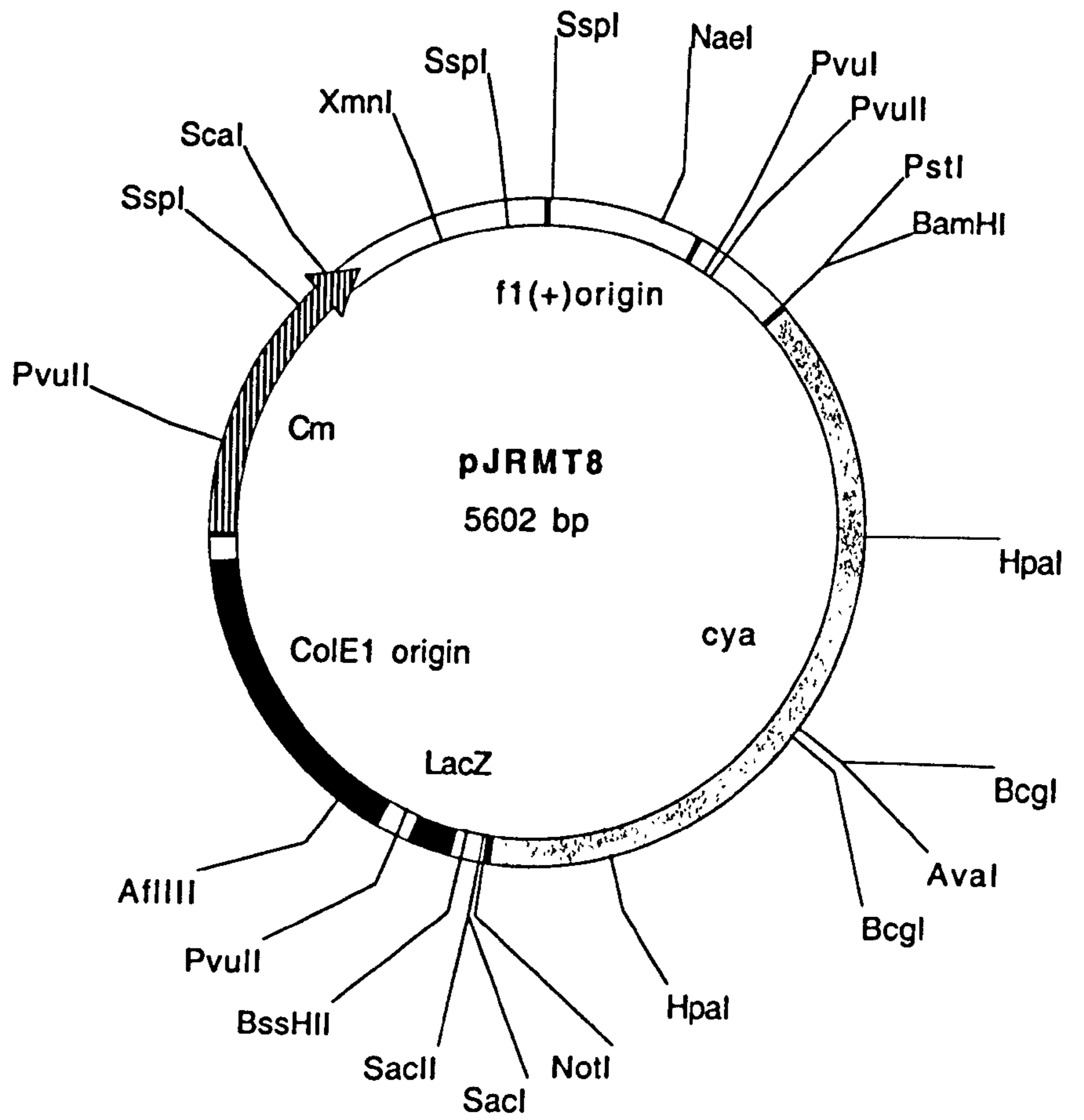


Fig 26: Restriction analysis of plasmids pJRMT9 and pJRMT10 (size as kb)

Lane 1: plasmid pJRMT9 digested with *HpaI* (un-affected)

Lane 2: plasmid pJRMT10 digested with *HpaI* (un-affected)

Lane 3: plasmid pJRMT7 digested with *HpaI*

Lane 4: plasmid pJRMT8 digested with *HpaI*

Lane 5: plasmid pJRMT9 digested with *BamHI/SacII*

Lane 6: plasmid pJRMT10 digested with *BamHI/SacII*

Lane M: 1 kb DNA marker

Fig 26:

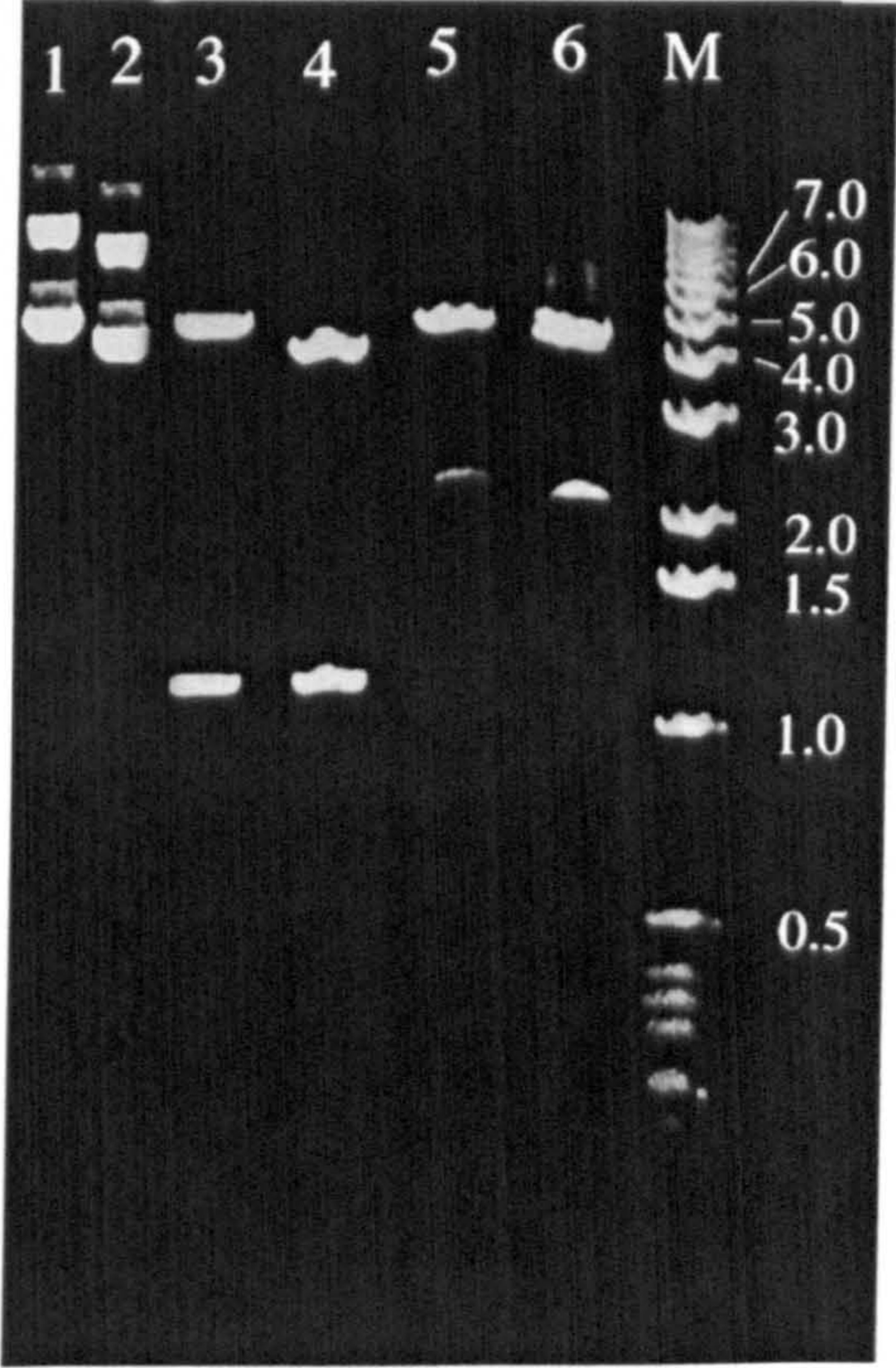
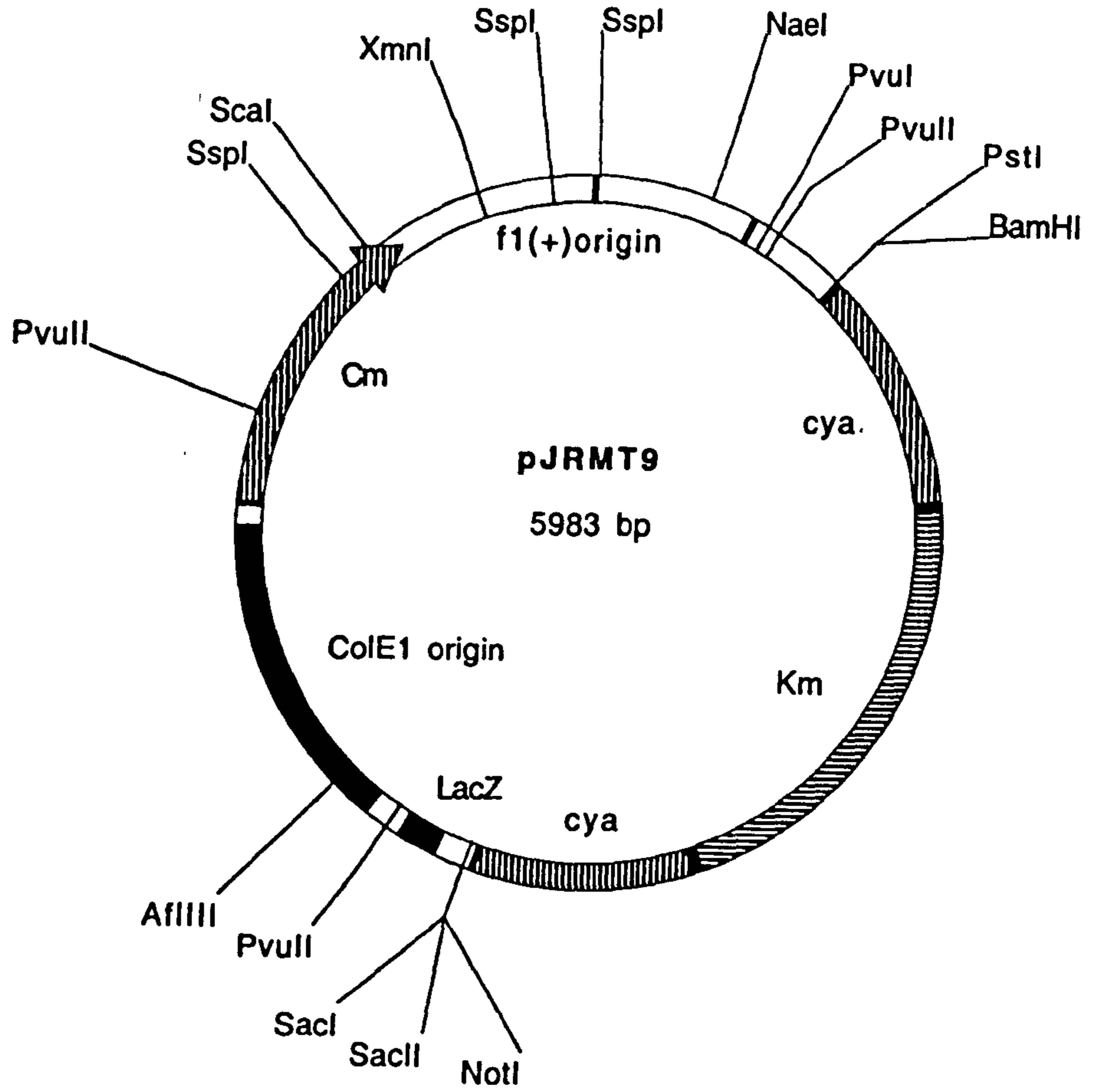


Fig 27: Map of plasmids pJRMT9 and pJRMT10

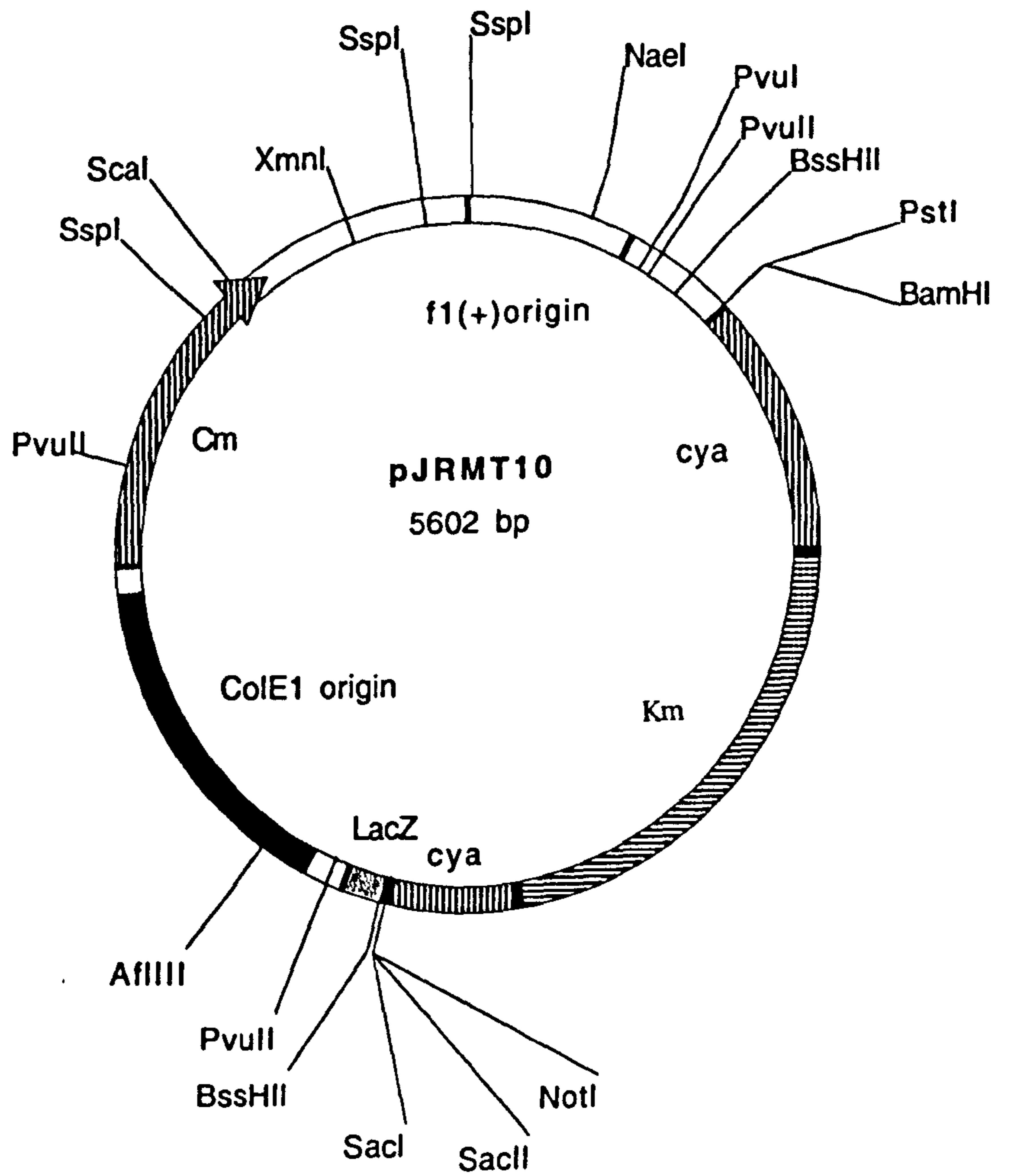
Plasmids pJRMT7 and pJRMT8 were digested with *HpaI*, to remove middle part of the *cya* gene which was replaced with the kanamycin resistance cassette (Km).

Fig 27:

A.



B.



plasmid pJRMT9 and pJRMT10 whereas it produced two fragments of ≈ 1.17 and ≈ 4.7 kb for plasmid pJRMT7 and two fragments of ≈ 1.17 and ≈ 4.4 kb for plasmid pJRMT8 (Fig. 26). Also digestion with *Bam*HI/*Sac*II produced two fragments of ≈ 3.4 and ≈ 2.55 kb for plasmid pJRMT9 and two fragments of ≈ 3.4 and ≈ 2.25 kb for plasmid pJRMT10 (Fig. 26). This was expected as these enzymes would remove the complete insert, leaving the plasmid vector (3.4 kb).

3. 1. 8. Allelic exchange

As the backbone of pCR-Script vector of plasmid pJRMT9 and pJRMT10 is a ColE1-based replication, it should not replicate in *Pasteurella* and so should act as a suicide vector. The plasmids pJRMT9 and pJRMT10 were introduced into *P. multocida* strains for allelic exchange by electroporation and bacteria spread onto BHI agar supplemented with 5% (v/v) sheep blood containing kanamycin. Some Km^R colonies were patched onto BHI agar containing kanamycin and then further subcultured onto BHI agar plates without kanamycin. After 10-100 subcultures on BHI agar without antibiotic, some clones from both 85020 and Quetta strains were inoculated into BHI broth and genomic DNA prepared. The genomic DNA was digested with *Acc*I restriction enzyme (the unique *Acc*I site at nt. 1459 in the *cya* gene sequence was removed by digestion with *Hpa*I). The digested materials were run in 0.6% (w/v) agarose gel and examined by Southern blot hybridisation. All samples when hybridised with *cya* probe (PCR amplimer from *P. multocida* strain 85020 using primers Cya4 and Cya5) showed four bands of ≈ 2.3 , ≈ 3.4 , ≈ 5.0 and ≈ 7.0 kb except the wild-type that just showed hybridisation to two bands of ≈ 2.3 and ≈ 5.0 kb (Fig. 28A). No hybridisation was detected with DNA from parent strains with Km^R probe (Fig. 28B), but the other samples showed two bands of ≈ 3.4 and ≈ 7.0 kb (Fig. 28B). Also as controls the plasmids pJRMT9 and pJRMT10 were digested with *Acc*I and run in an

Fig 28: Southern blot hybridisation of *P. multocida cya::Km^R* clones digested with *AccI* (size as kb)

A: Hybridisation with *cya* probe

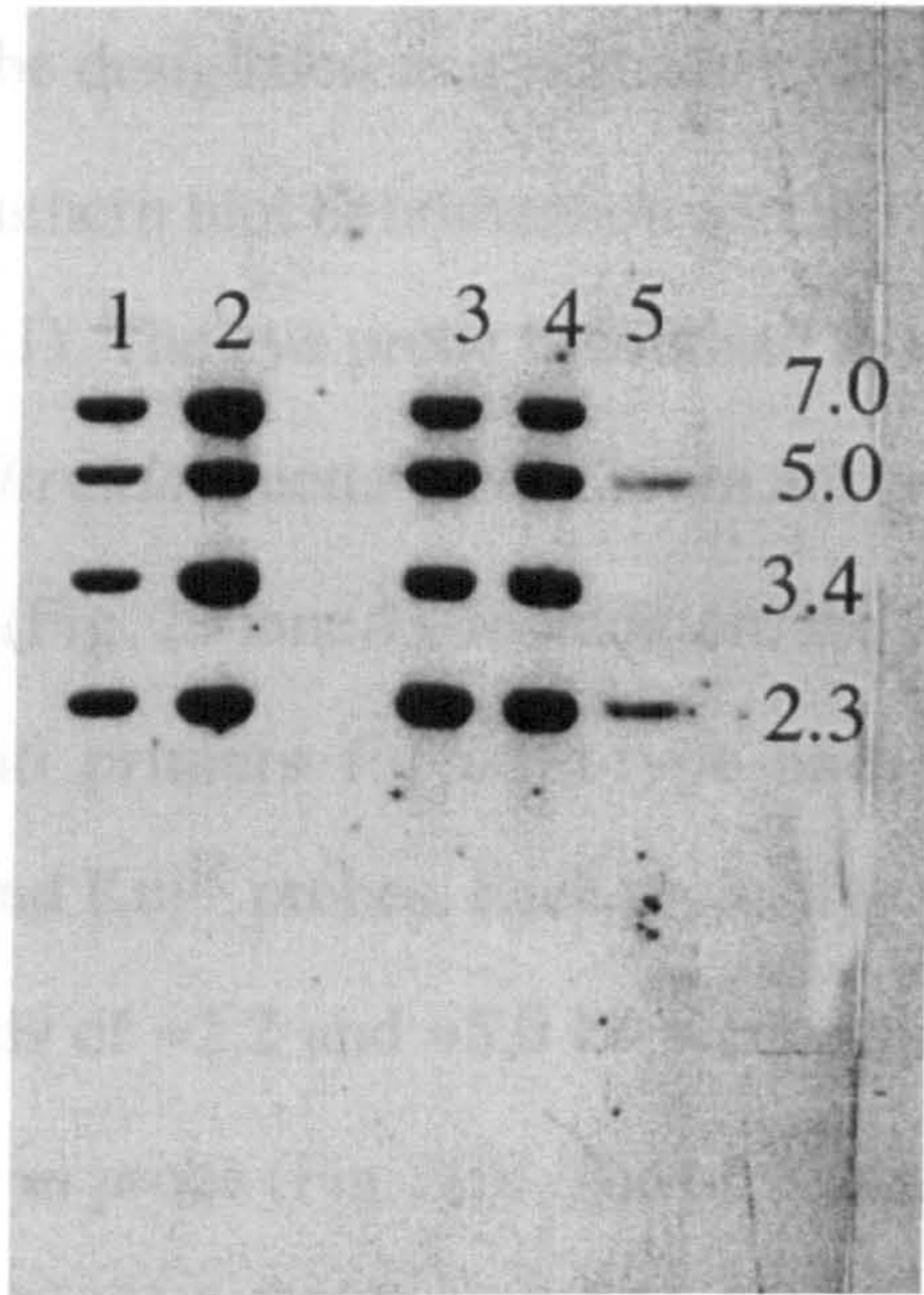
B: Hybridisation with *Km^R* probe

Lanes 1-4: *P. multocida* Quetta *cya:: Km^R* clones

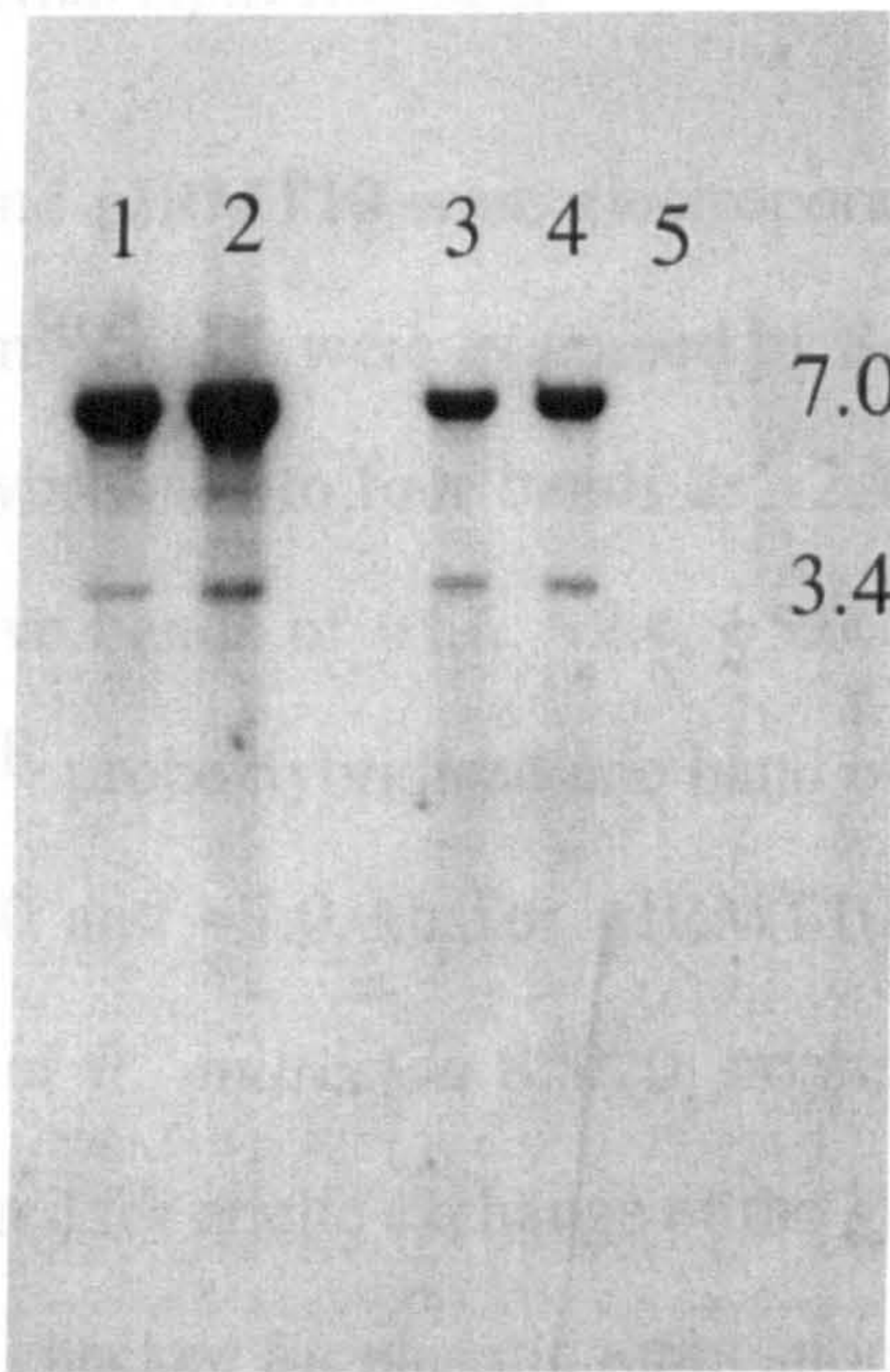
Lane 5: *P. multocida* Quetta wild type strain

Fig 28:

A.



B.



agarose gel for Southern blotting. When hybridised with the *cya* or Km^R probes, they showed two bands of ≈ 3.4 and ≈ 7.0 kb.

From more than 3000 single Km^R clones obtained in repeat experiments, about 300 were checked by Southern blot hybridisation and just one of them, derived from the *P. multocida* strain Quetta, showed the predicted size band (≈ 7.0 kb) that could be designated as a mutated *cya* gene. The mutant strain was confirmed by Southern blot hybridization and designated as *P. multocida* JRMT3 (Fig. 29 lane 1). The *cya* probe hybridised to two bands of ≈ 2.2 and ≈ 5.0 kb for the *P. multocida* Quetta parent strain and one band of ≈ 7.0 kb for *P. multocida* JRMT3 (Fig. 29 lane 1). In addition, the PCR products obtained with Cya5 and Cya6 primers for wild-type and JRMT3 strains were hybridised with *cya* and Km^R probes. Each probe hybridised to a ≈ 7.0 kb for JRMT3 and two bands of ≈ 2.2 and ≈ 5.0 kb were obtained with the Quetta parent strain and the *cya* probe (Fig. 30). The predicted hybridisation pattern was also obtained for genomic DNA; a ≈ 2.2 kb fragment for the Quetta parent strain and a similar sized fragment from JRMT3 as the ≈ 1.25 kb Km^R cassette replaced a ≈ 1.17 kb internal *Hpa*I fragment.

Plasmids pJRMT9 and pJRMT10 were electroporated into *P. multocida* strain 85020 and 60 Km^R clones were examined by Southern blotting. In all cases, the *cya* probe hybridised to four bands as ≈ 2.4 , ≈ 3.6 , ≈ 5.0 and ≈ 7.0 kb for pJRMT9 and five bands of ≈ 1.6 , ≈ 2.4 , ≈ 5.0 , ≈ 6.0 and ≈ 7.0 kb for pJRMT10, and the Km^R probe hybridised one band of ≈ 6.0 kb for pJRMT9 and two bands as ≈ 6.0 and ≈ 7.0 kb for pJRMT10 plasmids (Fig. 31). Therefore, in the case of *P. multocida* 85020, no strain demonstrated the correct gene type expected for allelic exchange of the *cya* allele. Also some of these Km^R clones were checked for plasmid replication with the Miniprep kit (QIAGEN) but they did not show any specific plasmid band.

Fig 29: Southern blot hybridisation of *P. multocida* Quetta *cya::Km^R* clones digested with *AccI* (size as kb)

A: Hybridisation with *cya* probe

B: Hybridisation with *Km^R* probe

Lanes 1-5: *P. multocida* Quetta *cya::Km^R* clones after transformation with plasmid pJRMT9

Lane 6: *P. multocida* Quetta wild-type strain

Only the digest in lane 1 shows the expected pattern. This strain was designated as *P. multocida* JRMT3.

Fig 29:

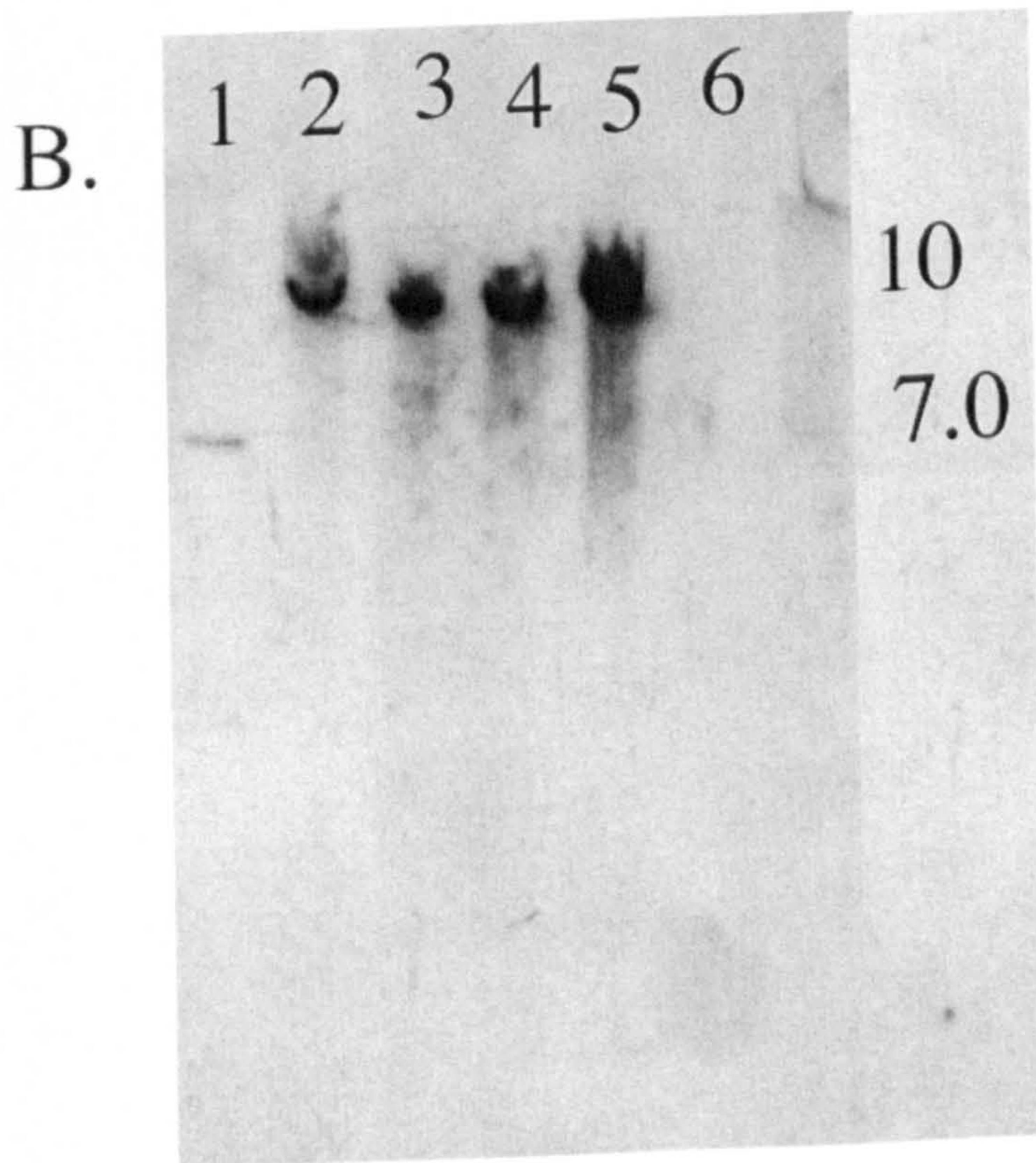
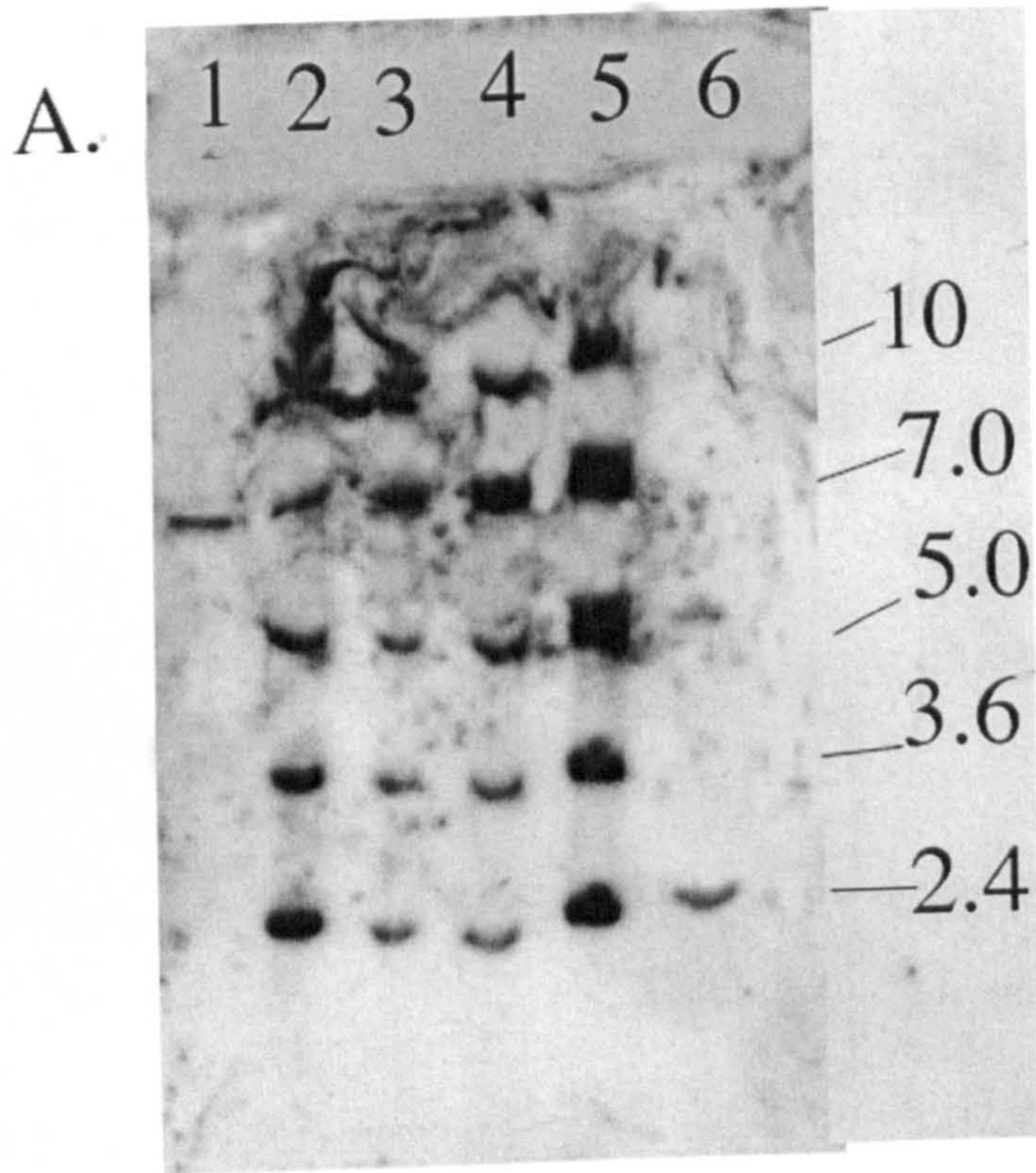


Fig 30: Southern blot hybridisation of *cya* mutated *P. multocida* strain

JRMT3 (size as kb)

A: Hybridisation with *cya* probe

B: Hybridisation with Km^R probe

**Lane 1: PCR product with *Cya* primers (*Cya5* and *Cya6*) from *P. multocida*
Quetta strain**

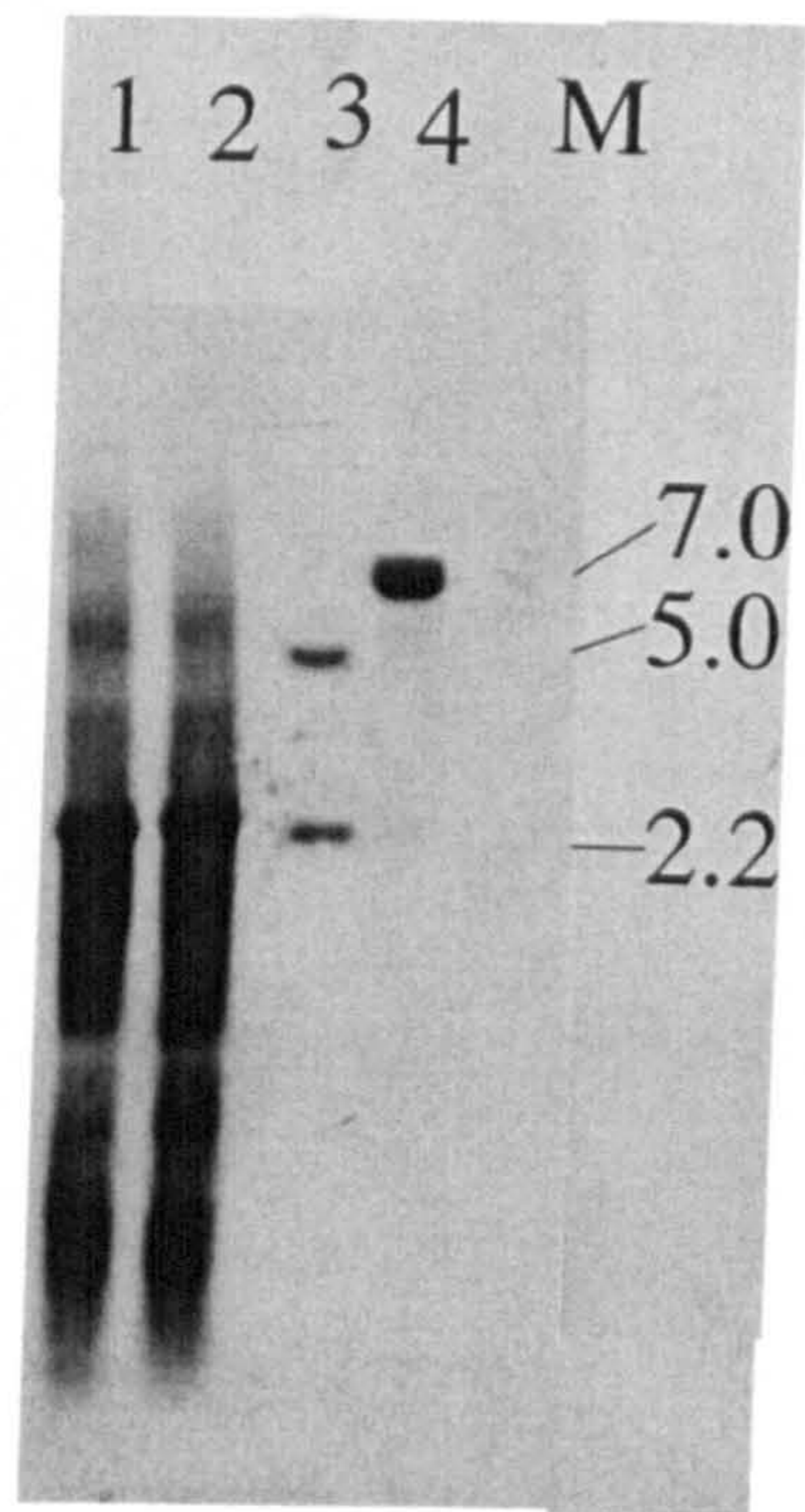
Lane 2: PCR product with *Cya5* and *Cya6* from *P. multocida* JRMT3

Lane 3: *P. multocida* strain Quetta genomic DNA digested with *AccI*

Lane 4: *P. multocida* strain JRMT3 genomic DNA digested with *AccI*

Fig 30:

A.



B.

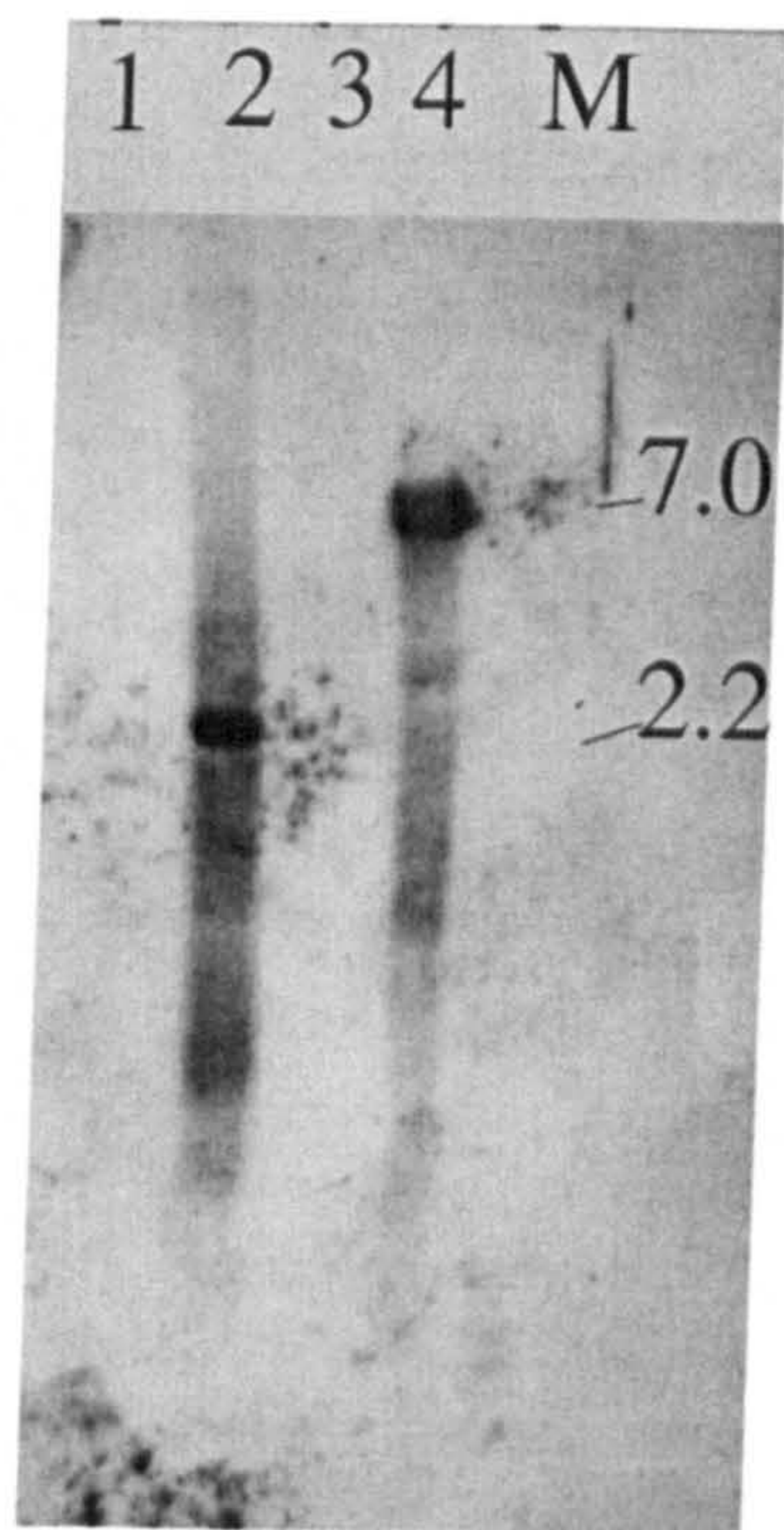


Fig 31: Southern blot hybridisation of *P. multocida* strain 85020 transformed with plasmids pJRMT9 and pJRMT10 and DNA digested with *AccI* (size as kb)

A: Hybridisation with *cya* probe

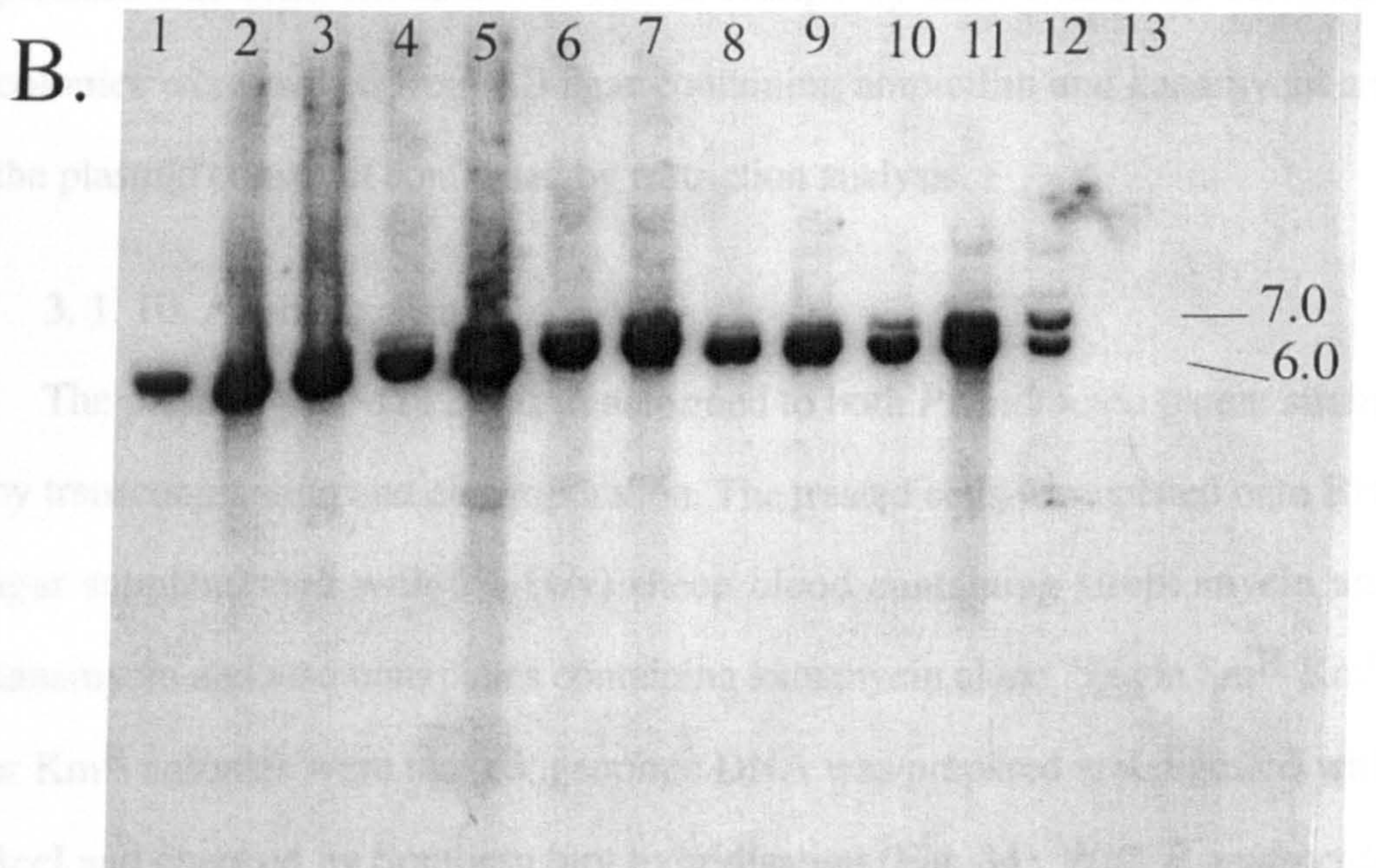
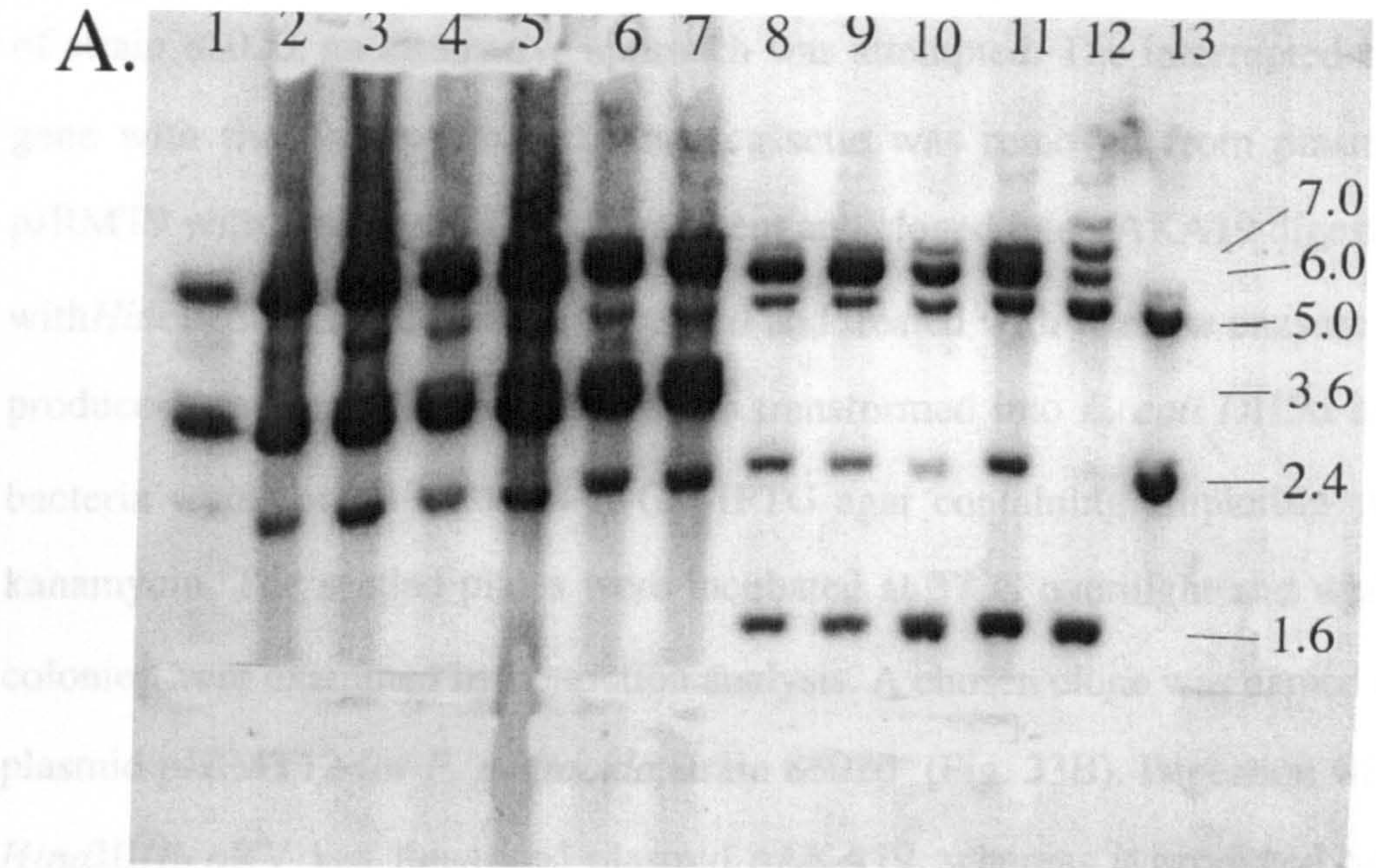
B: Hybridisation with Km^{R} probe

Lanes 1-7: *P. multocida* 85020 *cya::Km^R* clones transformed with plasmid pJRMT9

Lanes 8-12: *P. multocida* 85020 *cya::Km^R* clones transformed with plasmid pJRMT10

Lane 13: *P. multocida* strain 85020 wild-type

Fig 31:



3. 1. 9. Subcloning of interrupted *cya* gene into the vector pAKA19 for conjugative transfer to *P. multocida*

Success at creating a defined *P. multocida cya* mutant was apparently only achieved with the Quetta strain (JRMT3). In order to derive a similar derivative of strain 85020, an alternative approach was attempted. The interrupted *cya* gene with the kanamycin resistance cassette was removed from plasmid pJRMT9 with *PvuII* as a ≈ 3.0 kb fragment and cloned into pAKA19 digested with *HincII/EcoRI* and dephosphorylated and treated with Klenow enzyme to produce blunt ends. Plasmid DNA was transformed into *E. coli* DH5 α and bacteria were spread onto LB-X-Gal-IPTG agar containing ampicillin and kanamycin. The seeded plates were incubated at 37°C overnight and white colonies were examined by restriction analysis. A chosen clone was named as plasmid pJRMT12 for *P. multocida* strain 85020 (Fig. 33B). Digestion with *HindIII/EcoRV* just linearised plasmid pAKA19, whereas it produced two fragments of ≈ 3.0 and ≈ 5.0 kb for plasmid pJRMT12 (Fig. 32). Then this plasmid was transformed into *E. coli* SM10 λ pir by electroporation. Ten single colonies were picked from LB agar containing ampicillin and kanamycin and the plasmid construct confirmed by restriction analysis.

3. 1. 10. Allelic exchange

The plasmid pJRMT12 was transformed to both *P. multocida* parent strains by transconjugation and electroporation. The treated cells were plated onto BHI agar supplemented with 5% (v/v) sheep blood containing streptomycin and kanamycin and also onto plates containing kanamycin alone. Single Sm^R-Km^R or Km^R colonies were picked, genomic DNA was prepared and digested with *AccI* and checked by Southern blot hybridisation (Fig. 34). With *P. multocida* 85020 the *cya* probe hybridised to three bands of ≈ 1.7 , ≈ 2.3 and ≈ 4.0 kb, but for the Quetta strain it hybridised to three bands of ≈ 1.3 , ≈ 3.0 and ≈ 4.0 kb. The kanamycin resistance probe hybridised to two or three bands of ≈ 1.7 , ≈ 3.0 and ≈ 4.0 kb with *P. multocida* 85020 and to one band of ≈ 3.0 kb with the

Fig 32:

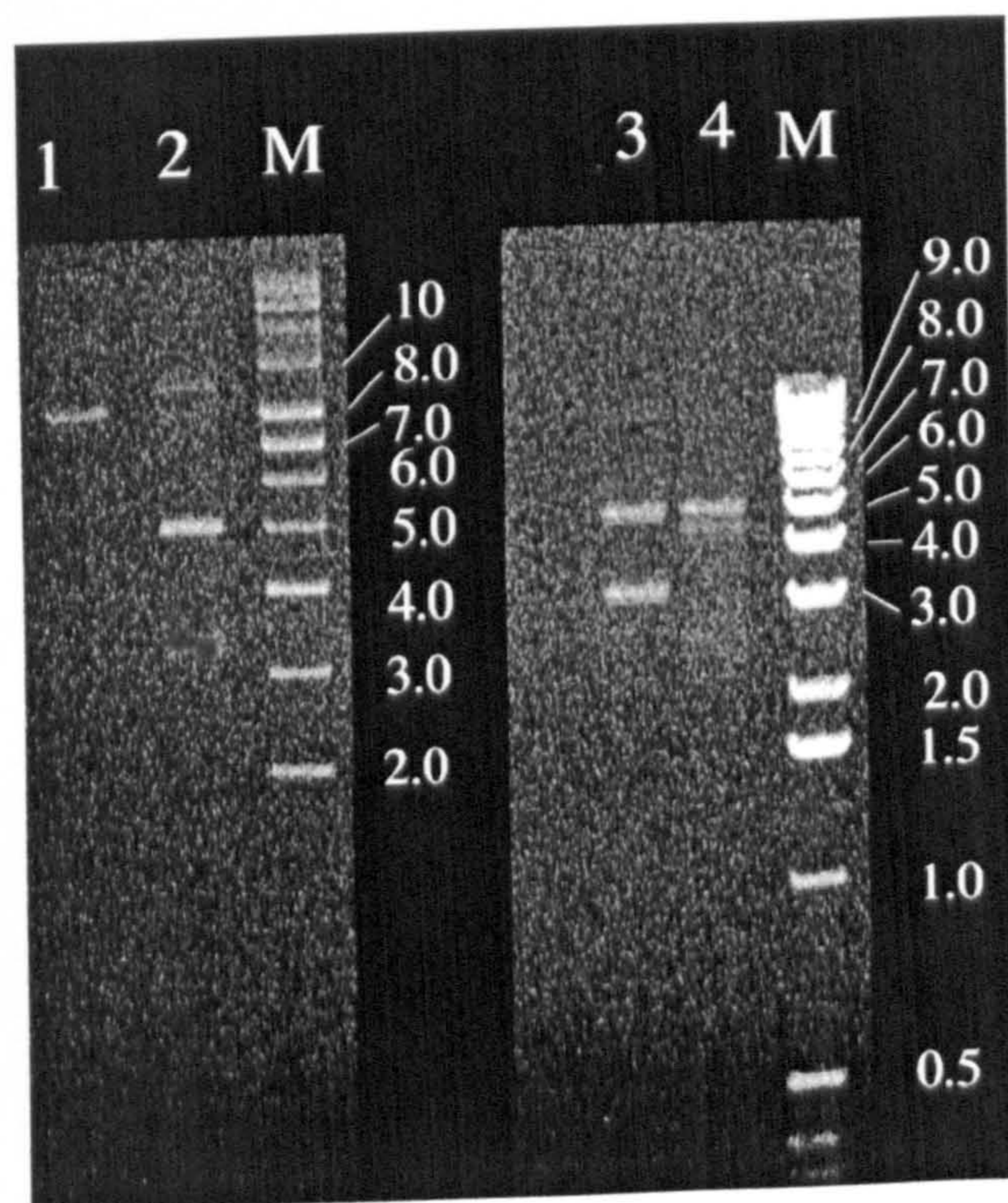


Fig 32: Restriction analysis of plasmid pJRMT12 (size as kb)

Lane 1: plasmid pJRMT12 undigested

Lane 2: plasmid pAKA19 undigested

Lane M: supercoiled DNA marker

Lane 3: plasmid pJRMT12 digested with *HindIII/EcoRV*

Lane 4: plasmid pAKA19 digested with *HindIII/EcoRV*

Lane M: 1 kb DNA marker

Fig 33: Map of plasmids pJRMT11 and pJRMT12

A: The ampicillin resistance cassette (Ap^R) removed from plasmid pAKA16 and cloned into plasmid pACYC184 to create plasmid pJRMT11.

B: The Km^R -interrupted *cya* gene was removed from plasmid pJRMT9 and cloned into plasmid pAKA19 to create plasmid pJRMT12.

Fig 33:

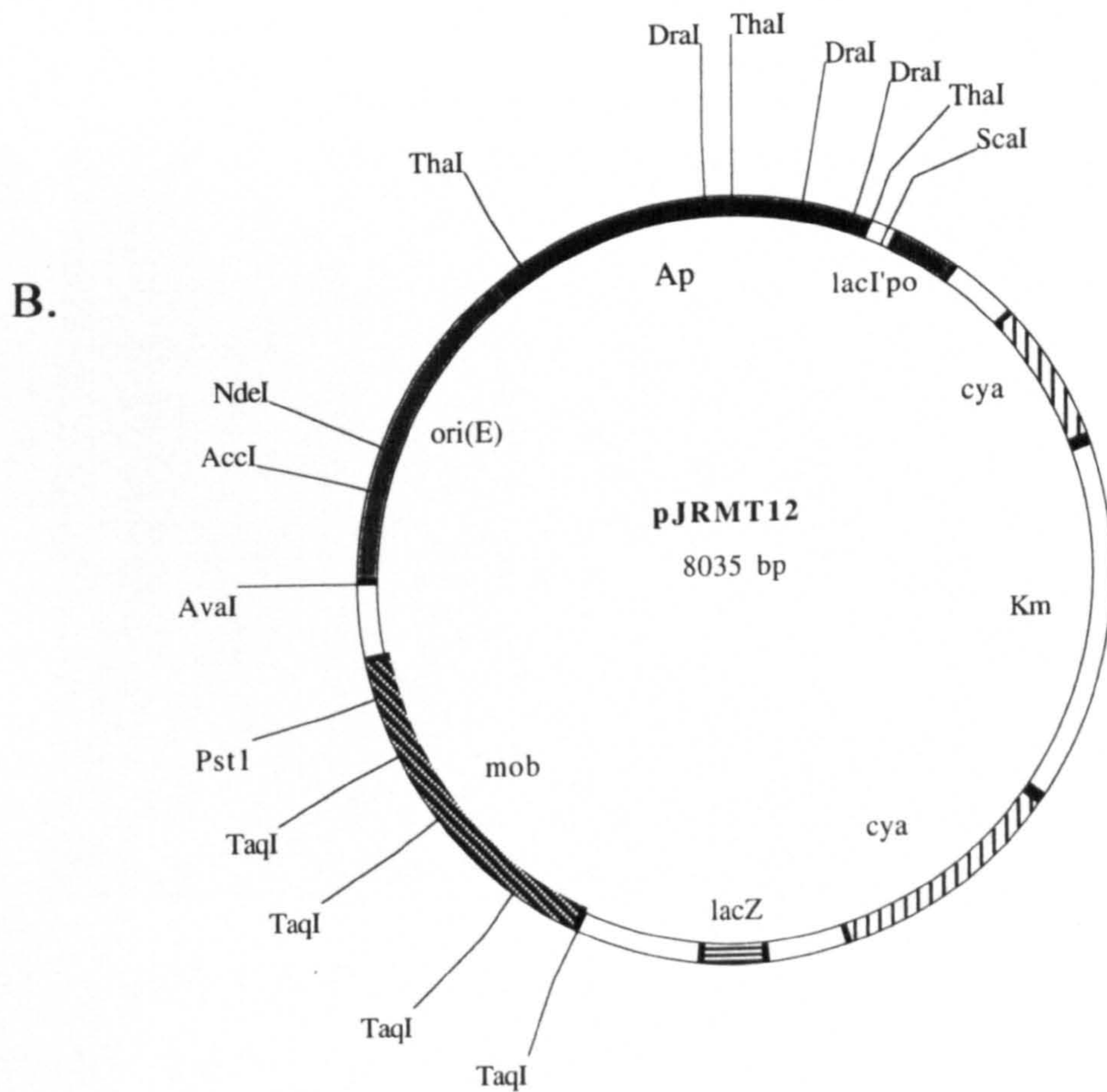
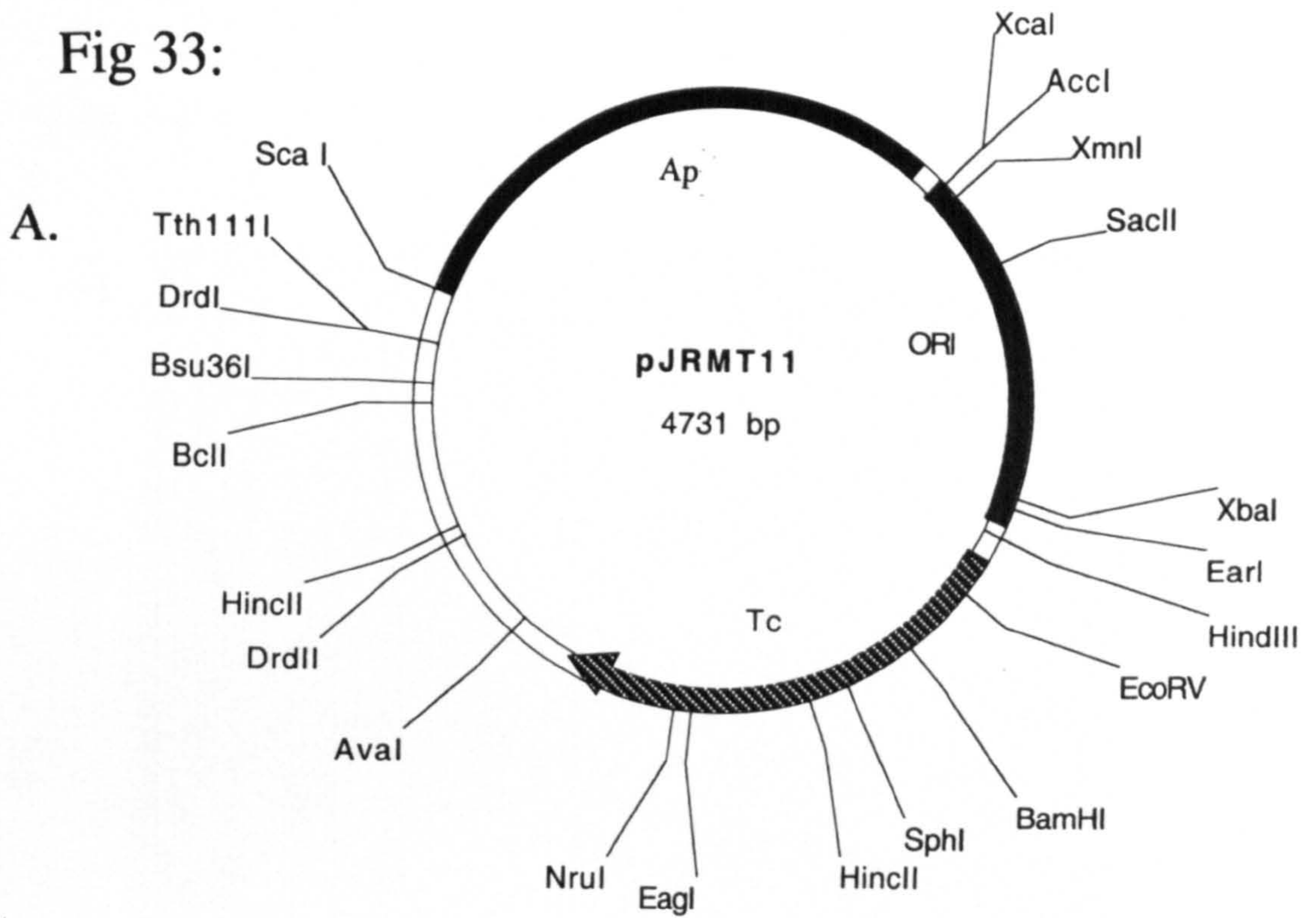


Fig 34: Southern blot hybridisation of *P. multocida* *cya*::Km^R clones transformed with plasmid pJRMT12 by transconjugation and genomic DNA digested with *AccI* (size as kb)

A: Hybridisation with *cya* probe

B: Hybridisation with Km^R probe

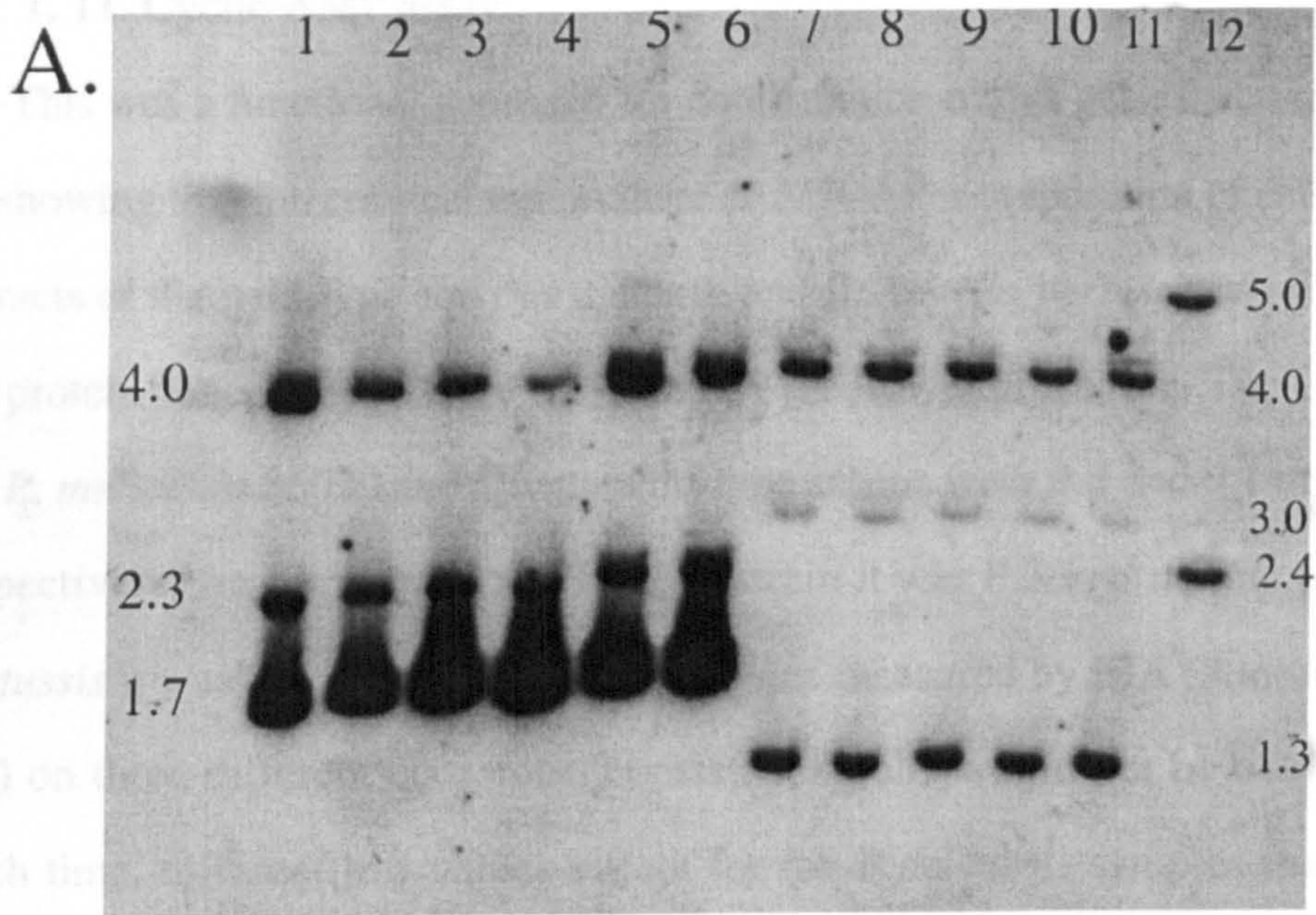
Lanes 1-6: *P. multocida* strain 85020 clones

Lanes 7-11: *P. multocida* strain Quetta clones

Lane 12: *P. multocida* strain 85020 wild-type

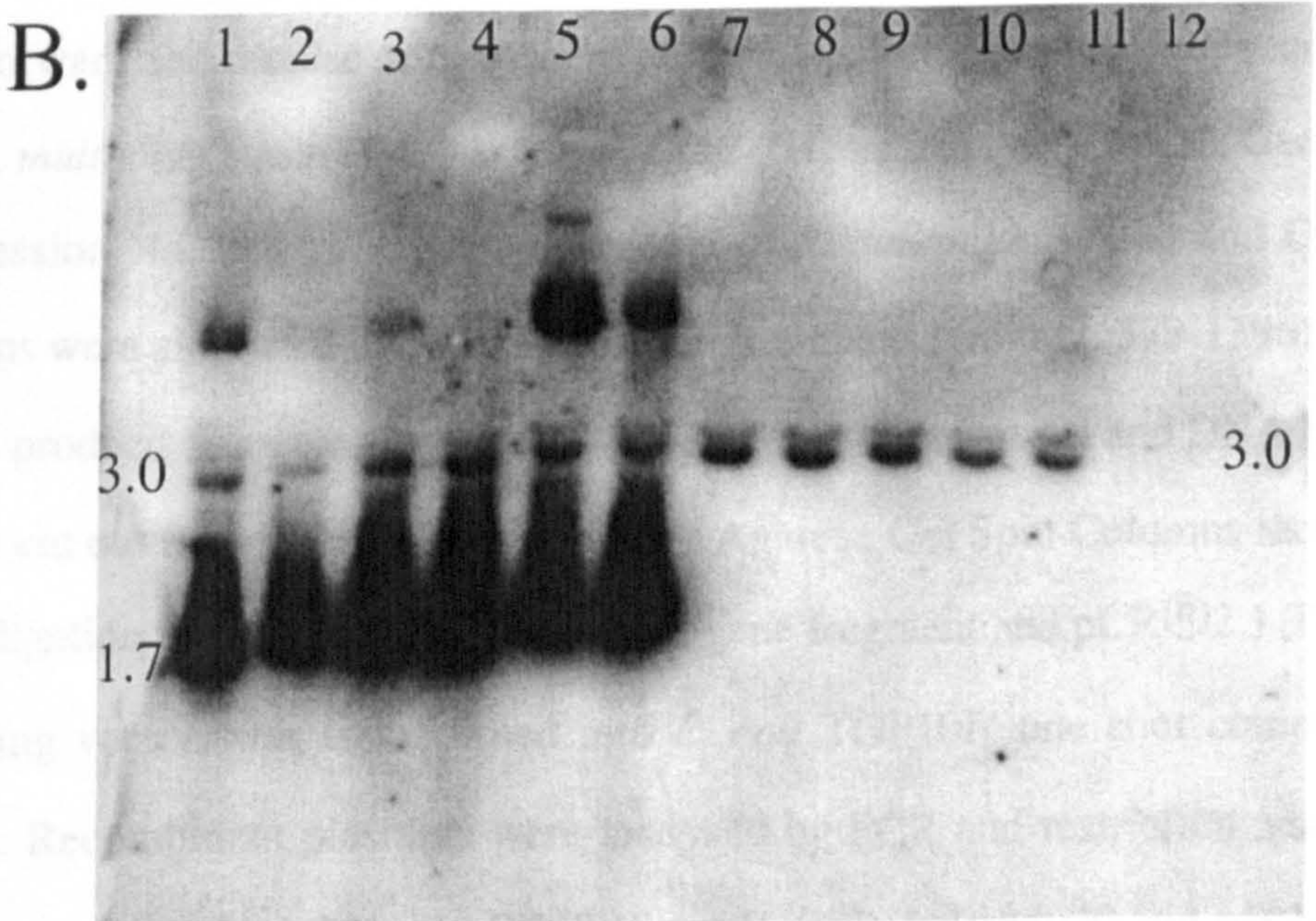
Quetta strain. Therefore, upon digestion with *Bst*I, when the DNA hybridization with the *pacA* and *pacB* probes gave the expected pattern for *P. maltorum*.

Fig 34:



show consistent evidence of identity (Table 4 and 10).

3.1.12 Cloning of *pacB* gene



for *P. maltorum* Quetta strain (GenBank accession number FJ411411). The PCR products were digested with *Bst*I and *Xba*I to create a 2.5 kb fragment on each side of the cloned *pacB* gene. The 10 kb fragment was inserted into the vector fragment of 8.5 kb as produced by *Bst*I and *Xba*I digestion of the

Quetta strain. Therefore, again, no colonies were found where DNA hybridisation with the *cya* and Km^R probes gave the expected pattern for allelic exchange at the *cya* locus.

3. 1. 11. Cyclic AMP assay

This was a functional approach for confirmation of *cya* gene inactivation, by showing that the cells cannot produce cAMP. After preparation of cell-free extracts of the wild-type and *cya* mutants, and *Bordetella pertussis* as control, the protein concentration were measured by the Lowry procedure. The values for *P. multocida* 85020 and Quetta wild-type strains were 9.8 and 9.1 mg/ ml respectively, for the *cya* mutant JRMT3 strain it was 8.3 mg/ ml and for *B. pertussis* it was 8.2 mg/ ml. When cAMP was measured by EIA (Amersham Kit) on three different occasions, consistent results would not be obtained. Each time, different low values except for the *B.pertussis* samples that did show consistent evidence of activity (Table 9 and 10).

3. 1. 12. Cloning of *galE* gene

Forward and reverse primers were designed according to the *galE* sequence of *P. multocida* strain PM25 serotype D:12 (Henestrosa *et al*, 1997; Genbank Accession No. U76617). The *galE* genes of *P. multocida* 85020 and Quetta strains were amplified by PCR as 1017 bp fragments (from nt. 379-1396). The PCR products were electrophoresed in 1.0% (w/v) agarose gel and DNA bands were cut out from gel and purified by the Agarose Gel Spin Columns method. The ligation mixture containing the *galE* gene fragment and pCR[®]2.1-TOPO cloning vector, was transformed into *E. coli* TOP10F' one shot competent cells. Recombinant plasmids were analysed by PCR and restriction analysis and were named as plasmid pJRMT13 for *P. multocida* 85020 and pJRMT14 for *P. multocida* Quetta strains (Fig. 36). Digestion with *EcoRI* which cuts on each side of the cloned insert produced a 1.0 kb fragment (*galE* gene) and a vector fragment of ≈2.5 kb as predicted (Fig. 35A). PCR analysis with *galE*

Table 9: Adenylate cyclase assay by enzyme immunoassay kit

Incubation time (min)	P. m. 85020 cell extract				<i>B. pertu ssis</i> cell extract			
	untreated		boiled*		untreated		boiled*	
	%B/B0	activity†	%B/B0	activity	%B/B0	activity	%B/B0	activity
0	88.4	40	90.0	33	75.8	180	85.0	60
30	63.3	65	89.1	38	58.2	500	83.2	65
60	62.5	180	87.9	45	45.6	750	80.4	80
120	58.9	480	87.0	47	37.1	1000	78.2	85

* Cell free extract were heated at 100°C in water bath for 10 min to destroy adenylate cyclase activity then used in cAMP assay reaction as controls

† The adenylate cyclase activity as fmol/well of samples can be read from graph (Fig.14).

Table 10: Adenylate cyclase assay of *P. multocida* strains 85020, Quetta, their *aroA* mutants and *cya* mutants by enzyme immunoassay kit

Strains	Experiment 1		Experiment 2		Experiment 3	
	%B/B0	Activity*	%B/B0	Activity	%B/B0	Activity
P. m. 85020 (W.T.)	68.9	280	66.1	380	58.3	550
" " <i>cya</i> ::Km ^R	79.5	90	68.1	320	64.2	400
" " <i>cya</i> ::Km ^R	72.1	210	74.3	180	70.3	280
" " <i>cya</i> ::Ap ^R	73.0	200	74.8	175	71.8	210
P. m. JRMT1 (<i>aroA</i> ⁻)	74.2	180	68.4	320	69.4	290
" " <i>cya</i> ::Ap ^R	76.1	160	71.5	210	78.0	105
" " <i>cya</i> ::Ap ^R	73.4	200	76.0	160	69.5	290
P. m. Quetta (W.T.)	61.4	470	68.2	320	67.1	350
" " <i>cya</i> ::Km ^R	72.1	210	69.8	300	74.5	175
" JRMT3 (<i>cya</i> ::Km ^R)	68.6	320	73.7	200	81.0	90
P. m. Quetta <i>cya</i> ::Ap ^R	71.6	210	71.4	210	76.4	160
P. m. JRMT1 (<i>aroA</i> ⁻)	82.1	70	69.4	290	67.8	340
" " <i>cya</i> ::Ap ^R	78.3	105	73.1	200	70.2	280
" " <i>cya</i> ::Ap ^R	74.8	175	74.3	175	72.5	200
ATP+ cell extract no incubation	88.1	43	80.4	90	82.3	70
ATP + Boiled extract	74.2	180	75.2	170	88.5	40

* The adenylate cyclase activity as fmol/well of samples can be read from graph (Fig.14).

Fig 35: Analysis of plasmids pJRMT13 and pJRMT14 (size as kb)

A: Restriction analysis:

Lane M: 1 kb DNA marker

Lane 1: plasmid pJRMT13 digested with *EcoRI*

Lane 2: plasmid pJRMT13 undigested

Lane 3: plasmid pJRMT14 digested with *EcoRI*

Lane 4: plasmid pJRMT14 undigested

B: PCR analysis with Gale primers (GaleE1 and GaleE2):

Lane 1: plasmid pJRMT13 PCR product

Lane 2: plasmid pJRMT14 PCR product

Lane 3: plasmid pJRMT15 PCR product

Lane 4: plasmid pJRMT16 PCR product

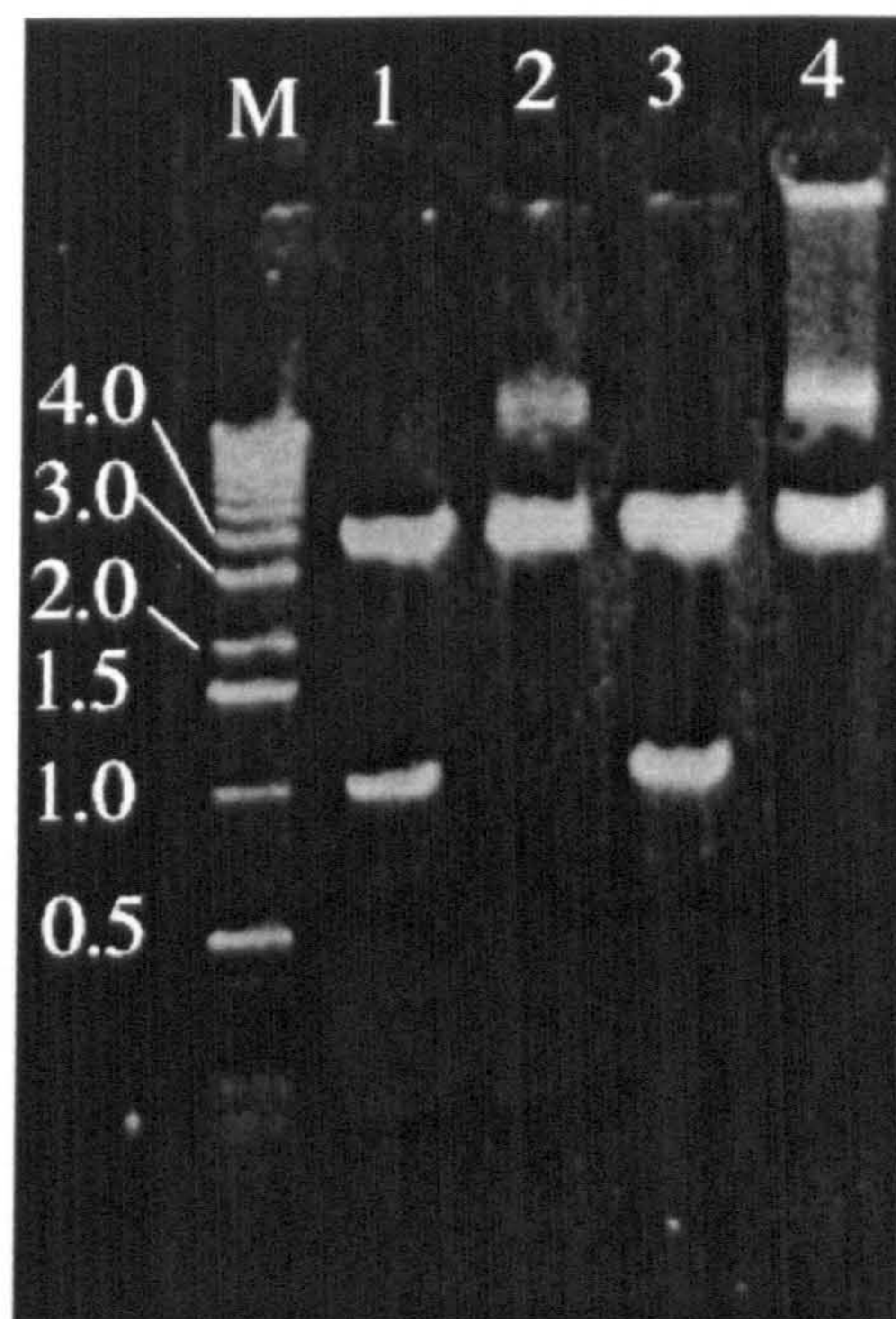
Lane 5: *P. multocida* 85020

Lane 6: *P. multocida* Quetta

Lane M: 1 kb DNA marker

Fig 35 :

A.



B.

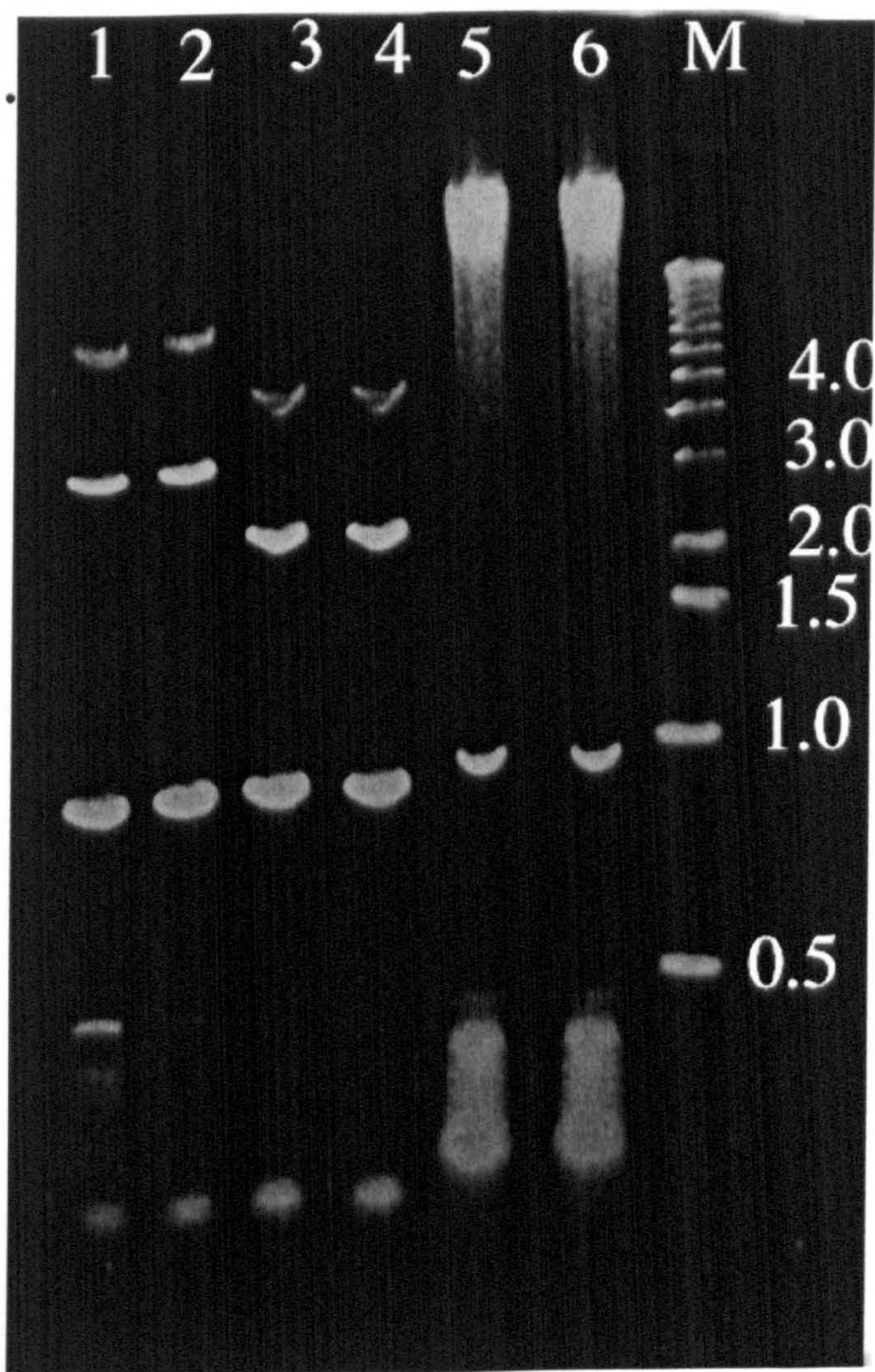
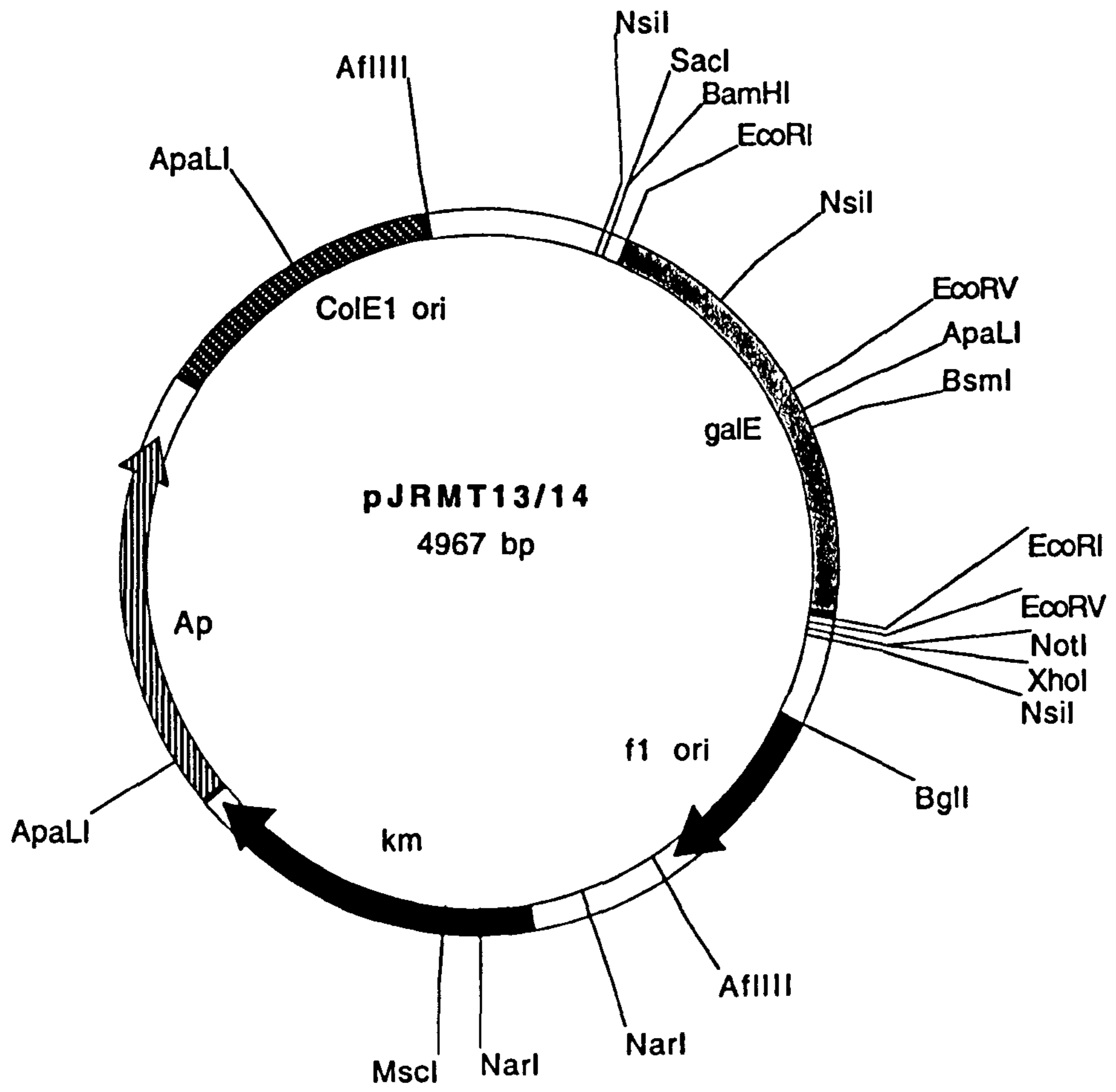


Fig 36: Map of plasmids pJRMT13 and pJRMT14

The amplified *galE* genes of the *P. multocida* strains were cloned into pCR®2.1-TOPO vector.

Fig 36:



primers produced the expected ≈ 1.0 kb amplicon (*galE* gene) (Fig. 35B, lane 1 and 2). Larger bands visible on the gel may represent plasmid template DNA.

3. 1. 13. Cloning of *galE* gene into plasmid pUC19

Plasmid pUC19 has a *ColE1* origin of replication and so should act as a suicide vector when transformed to *Pasteurella* strains. The *galE* genes were removed from plasmids pJRMT13 and pJRMT14 with *EcoRI* and plasmid pUC19 was digested with *EcoRI* and dephosphorylated. Gel-purified material was ligated and transformed into *E. coli* DH5 α competent cells by electroporation and the bacteria plated onto LB-X-Gal-IPTG agar containing ampicillin. After incubation for 24 hrs at 37°C, white colonies were picked and recombinant plasmids were confirmed by restriction analysis and were named as plasmids pJRMT15 and pJRMT16 for *P. multocida* 85020 and Quetta strains, respectively (Fig. 38A). *PvuII*-digestion of plasmid pUC19 produced two fragments of ≈ 0.32 and ≈ 2.37 kb, but for plasmids pJRMT15 and pJRMT16 two fragments of ≈ 1.34 and ≈ 2.37 kb were produced as predicted (Fig. 38A). Digestion with *EarI* produced three fragments of ≈ 0.39 , ≈ 0.49 and ≈ 1.8 kb for pJRMT15 and for pJRMT16 four fragments of ≈ 0.48 , ≈ 0.49 , ≈ 0.93 and ≈ 1.8 kb were produced (Fig. 37B, the two fragments of ≈ 0.48 and ≈ 0.49 overlapped). PCR analysis with *GalE* primers (Table 7) amplified the expected 1.0 kb band for plasmid pJRMT15 and pJRMT16 (Fig. 35B, lanes 3 and 4).

3. 1. 14. Insertion of the Km^R cassette into the *galE* gene

The plasmids pJRMT15 and pJRMT16 were digested with *NsiI* (at nt. 615 Fig. 38A) and dephosphorylated. The kanamycin resistance cassette was cut out from plasmid pUC4K with *PstI* (nt.417-1657). Digested material was run in a 0.8% (w/v) agarose gel and DNA bands purified, ligated and transformed into *E. coli* DH5 α competent cells by electroporation. Recombinant plasmids were confirmed by restriction analysis and designated as pJRMT17 and

Fig 37: Restriction analysis of plasmids pJRMT15 and pJRMT16 (size as kb)

A:

Lane M: supercoiled DNA marker

Lane 1: plasmid pUC19 undigested

Lane 2: plasmid pJRMT15 undigested

Lane 3: plasmid pJRMT16 undigested

Lane M: supercoiled DNA marker

B:

Lane M: 1 kb DNA marker

Lane 1: plasmid pUC19 digested with *PvuII* (≈ 0.32 and ≈ 2.37 kb fragments)

Lane 2: plasmid pUC19 digested with *EarI* (≈ 0.39 , ≈ 0.49 and ≈ 1.8 kb fragments)

Lane 3: plasmid pJRMT15 digested with *PvuII* (≈ 1.34 and ≈ 2.37 kb fragments)

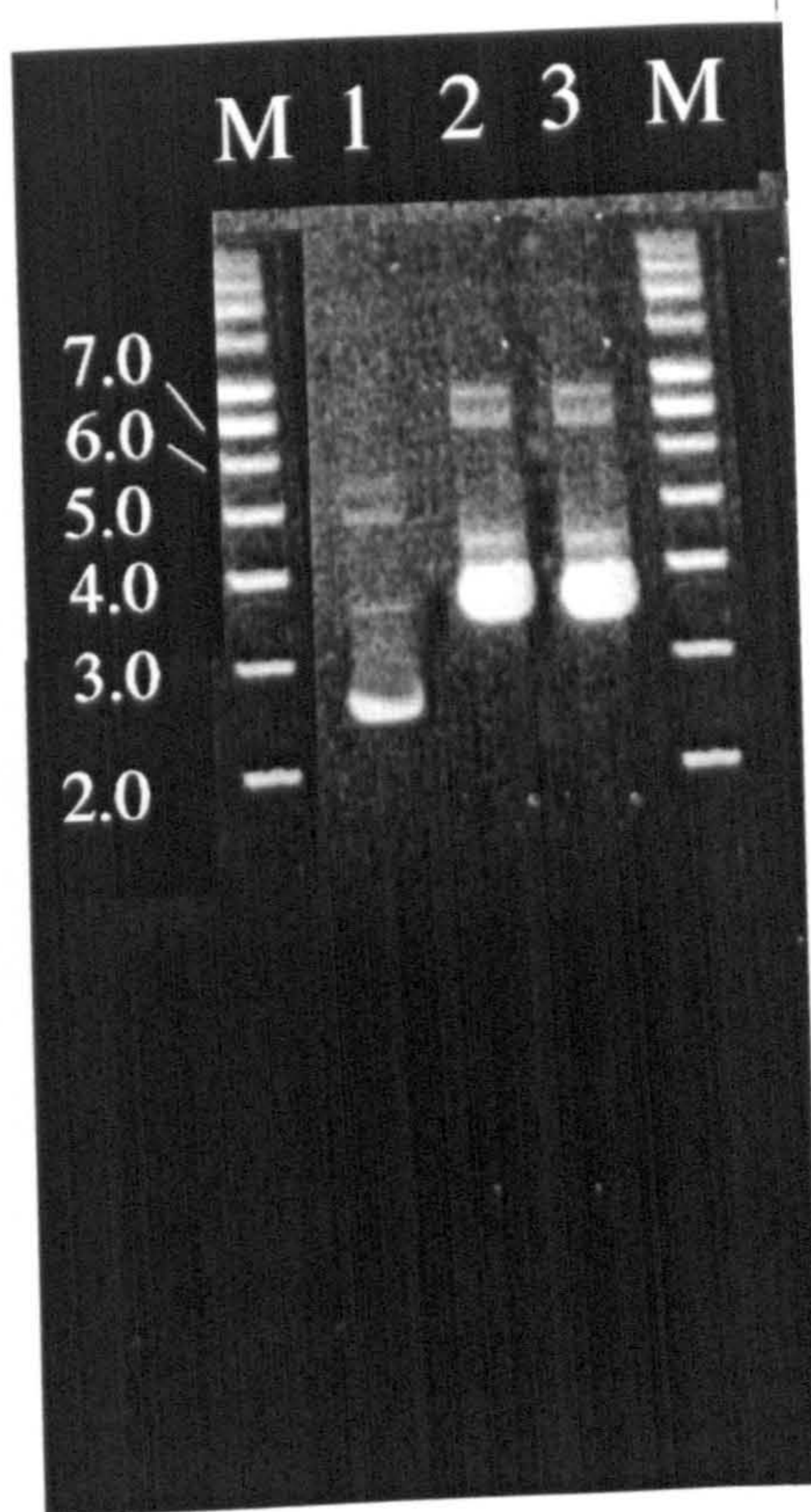
Lane 4: plasmid pJRMT15 digested with *EarI* (≈ 0.48 , ≈ 0.49 , ≈ 0.93 and ≈ 1.8 kb fragments) (two fragments of ≈ 0.48 and ≈ 0.49 overlapped)

Lane 5: plasmid pJRMT16 digested with *PvuII* (≈ 1.34 and ≈ 2.37 kb fragments)

Lane 6: plasmid pJRMT16 digested with *EarI* (≈ 0.48 , ≈ 0.49 , ≈ 0.93 and ≈ 1.8 kb fragments)

Fig 37:

A.



B.

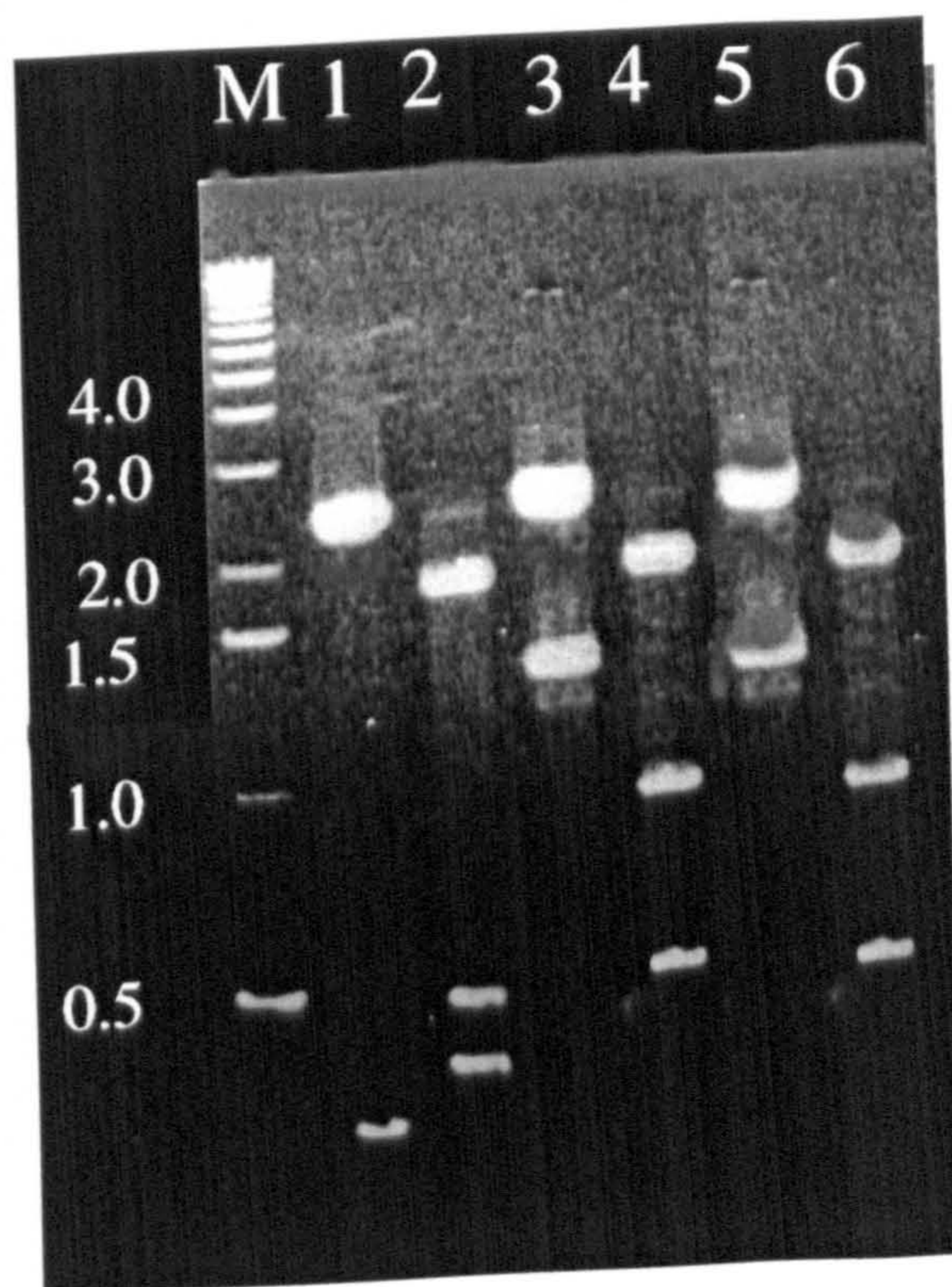


Fig 38:

A: Map of plasmids pJRMT15 and pJRMT16

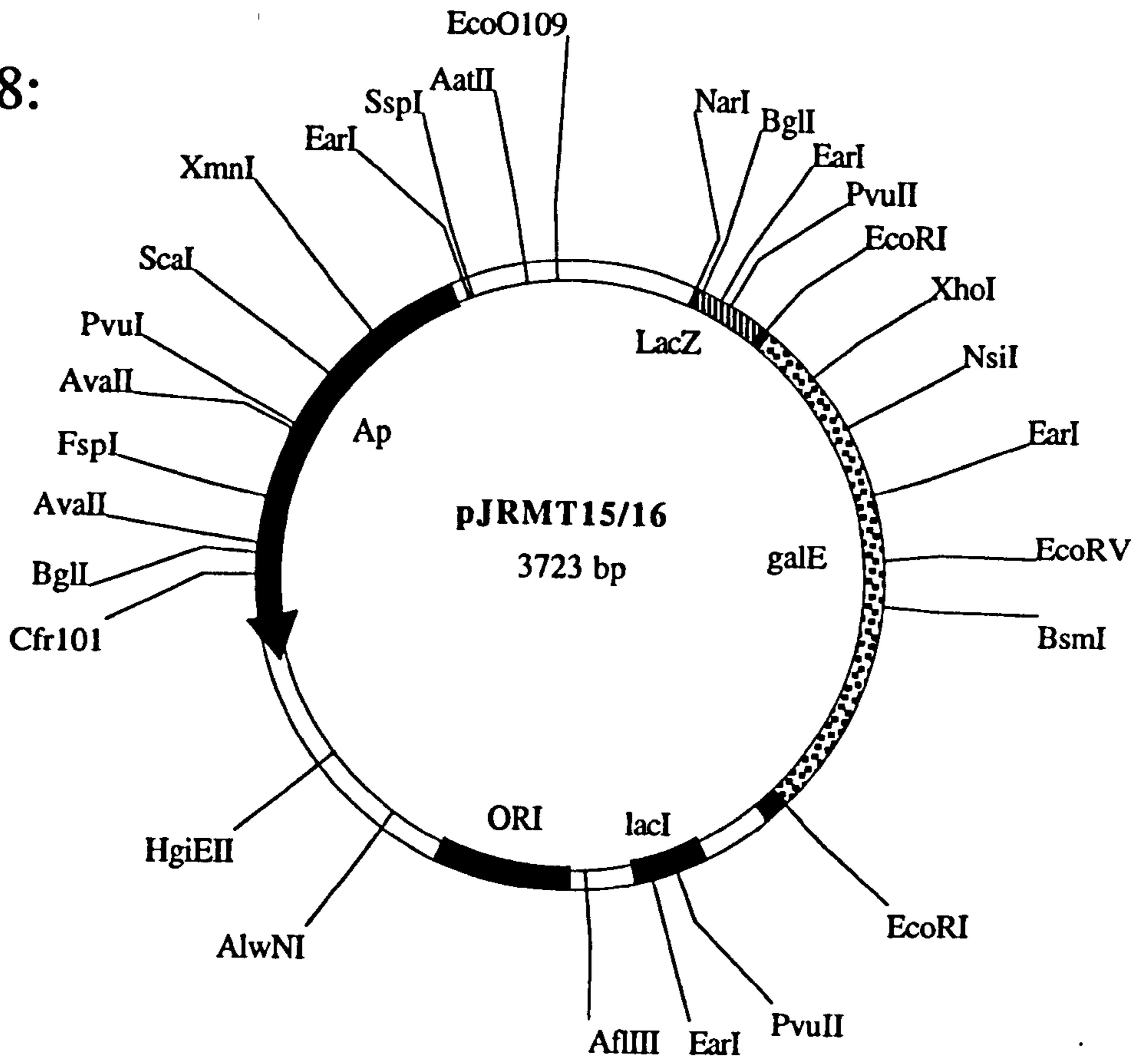
The *galE* gene was removed from plasmid pJRMT13 and pJRMT14 with *EcoRI* and cloned into pUC19.

B: Plasmid map of plasmids pJRMT17 and pJRMT18

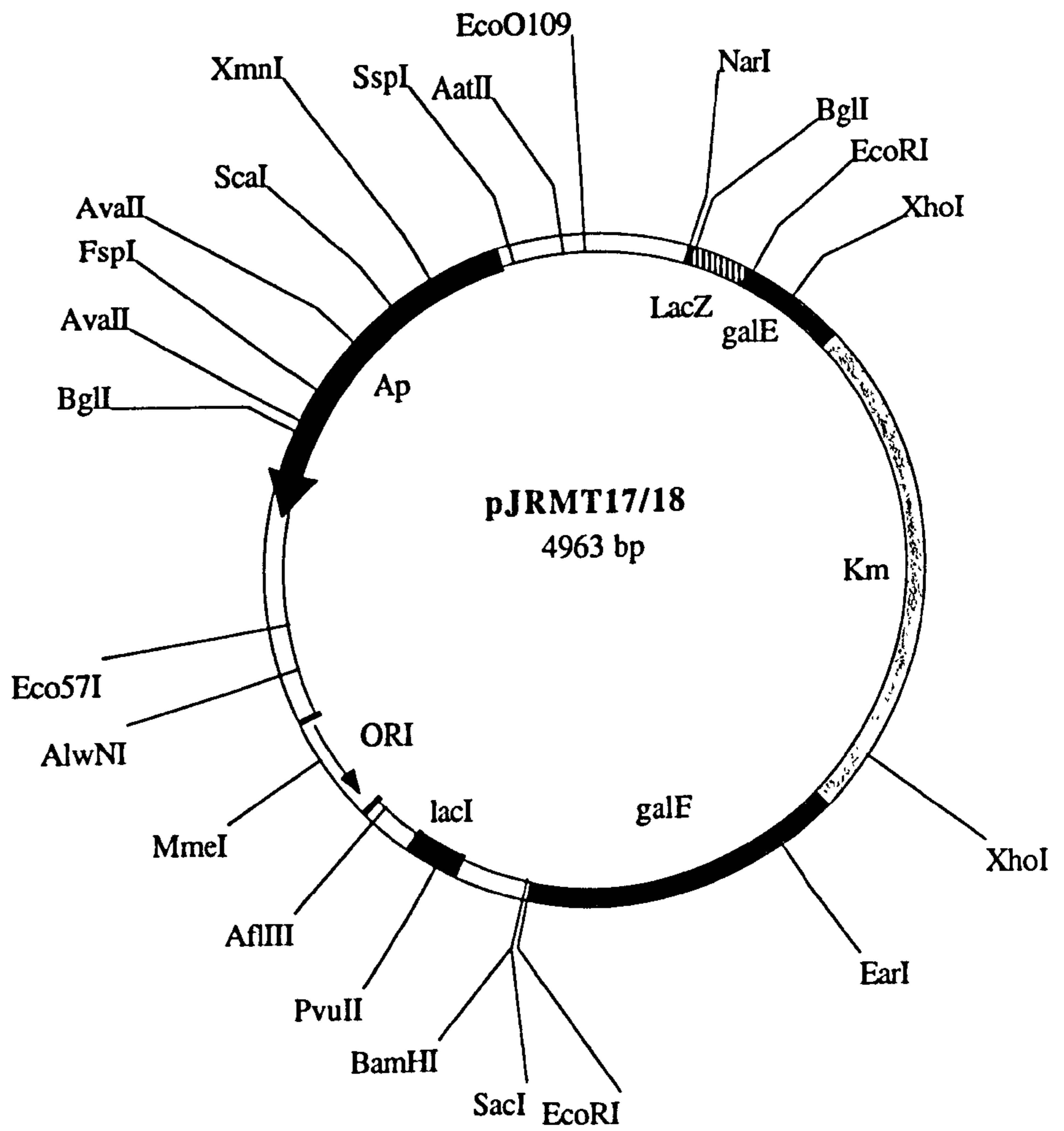
The plasmids pJRMT15 and pJRMT16 were digested with *NsiI* and ligated with the kanamycin resistance fragment (Km^R) from pUC4K.

Fig 38:

A.



B.



pJRMT18, for *P. multocida* 85020 and Quetta strains respectively (Fig. 38B). As expected *XhoI* digestion of plasmid pJRMT15 and pJRMT16 just linearised them (≈ 3.72 kb fragment) but for pJRMT17 and pJRMT18 two fragments of ≈ 1.3 and ≈ 3.66 kb were produced (Fig. 39B lanes 5 and 6). Digestion of plasmids pJRMT15 and pJRMT16 with *EarI* produced four fragments of ≈ 0.48 , ≈ 0.49 , ≈ 0.95 and ≈ 1.8 kb but for pJRMT17 and pJRMT18 five fragments of ≈ 0.48 , ≈ 0.49 , ≈ 0.95 , ≈ 1.2 and ≈ 1.8 kb were produced as predicted (Fig 39B; the two fragments of ≈ 0.48 and ≈ 0.49 overlapped).

3. 1. 15. Allelic exchange

The plasmids pJRMT17 and pJRMT18 were transformed to the *P. multocida* strains for allelic exchange by electroporation and were plated onto BHI agar supplemented with 5% (v/v) sheep blood containing kanamycin. After incubation for 48 hrs at 37°C, Km^R colonies were picked and patched onto BHI agar containing kanamycin. After ten subcultures, however, all the Km^R clones examined contained plasmid of the size of plasmid pJRMT17 (≈ 4.9 kb). PCR analysis of the Km^R clones with *galE* primers showed two or three bands as ≈ 1.0 kb (wild-type *galE* gene) and a second band as ≈ 2.2 kb (interrupted *galE* gene with Km^R cassette) (Fig. 40).

3. 1. 16. Cloning of interrupted *galE* gene into the vector pAKA19

Because of the apparent replication of plasmids pJRMT17 and pJRMT18 (*ColE1* origin) in *P. multocida* strains, it was decided to use pAKA19 as a suicide vector because although it also had a *ColE1*-based origin, it was used successfully before. The plasmids pJRMT17 and pJRMT18 were digested with *EcoRI* and the Km^R-interrupted *galE* gene ligated into pAKA19 which had been digested with *EcoRI* and dephosphorylated. The ligation mixture was transformed into *E. coli* DH5 α competent cells by electroporation and plated out onto LB-X-Gal-IPTG agar containing ampicillin and also LB agar containing ampicillin and kanamycin. White colonies from LB-X-Gal-IPTG

Fig 39: Analysis of plasmids pJRMT17 and pJRMT18 (size as kb)

A: Undigested plasmid DNA

Lane M: supercoiled DNA marker

Lane 1: plasmid pJRMT15

Lane 2: plasmid pJRMT17

Lane 3: plasmid pJRMT16

Lane 4: plasmid pJRMT18

Lane M: supercoiled DNA marker

B: Restriction analysis

LaneM: 1 kb DNA marker

Lane 1: plasmid pJRMT15 digested with *EarI* (4 fragments, 3 bands)

Lane 2: plasmid pJRMT15 digested with *XhoI* (linearised)

Lane 3: plasmid pJRMT15 digested with *BamHI/XhoI* (linearised)

Lane 4: plasmid pJRMT17 digested with *EarI* (5 fragments, 4 bands)

Lane 5: plasmid pJRMT17 digested with *XhoI* (2 fragments)

Lane 6: plasmid pJRMT17 digested with *BamHI/XhoI* (2 fragments)

Lane 7: plasmid pJRMT16 digested with *EarI* (4 fragments, 3 bands)

Lane 8: plasmid pJRMT16 digested with *XhoI* (linearised)

Lane 9: plasmid pJRMT16 digested with *BamHI/XhoI* (linearised)

Lane 10: plasmid pJRMT18 digested with *EarI* (5 fragments, 4 bands)

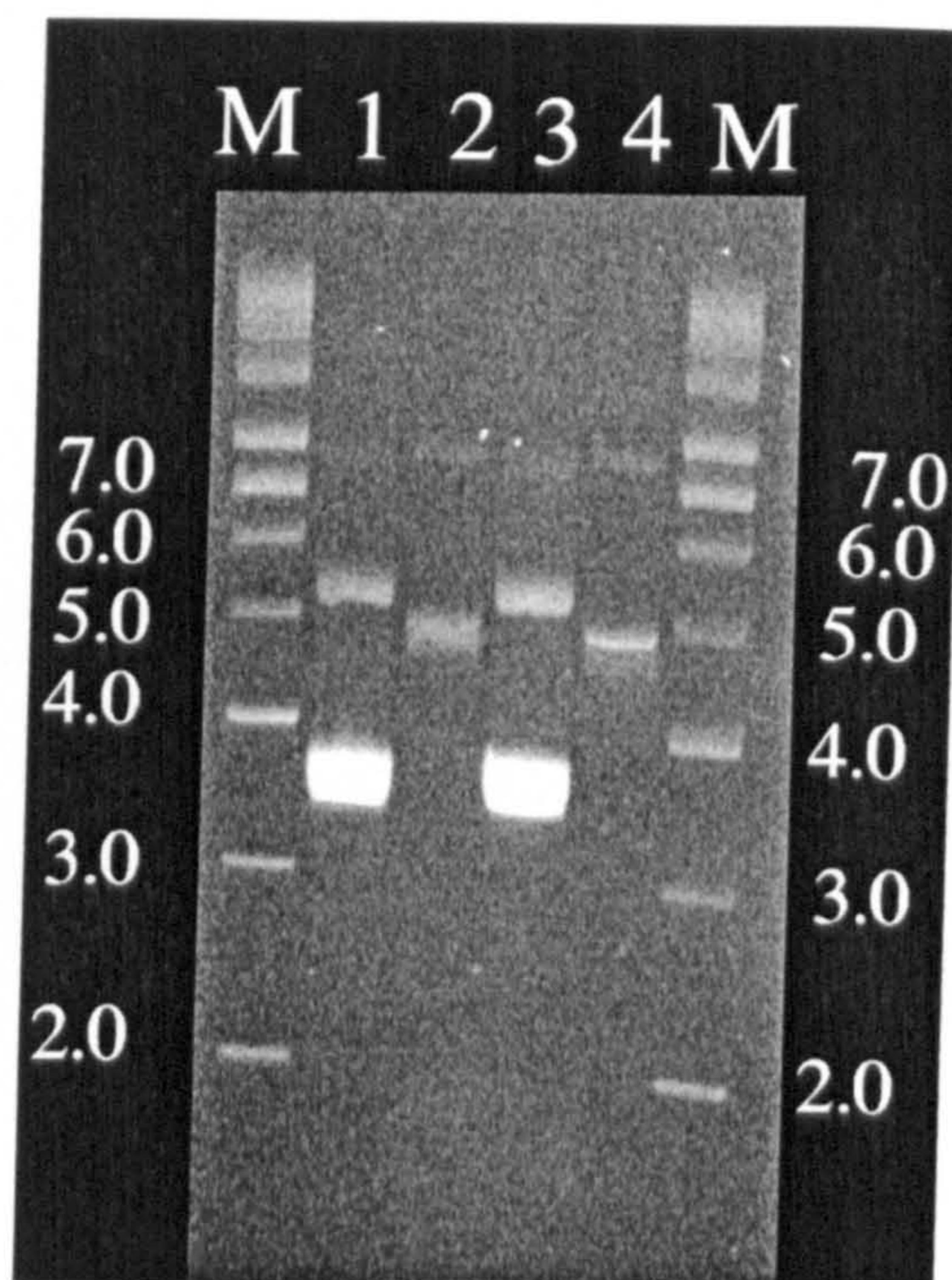
Lane 11: plasmid pJRMT18 digested with *XhoI* (2 fragments)

Lane 12: plasmid pJRMT18 digested with *BamHI/XhoI* (2 fragments)

LaneM: 1 kb DNA marker

Fig 39:

A.



B.



Fig 40: PCR analysis of *P. multocida galE::Km^R* clones transformed with plasmids pJRMT17 and pJRMT18 (size as kb)

A:

Lanes 1-7: transformed clones

Lane 8: *P. multocida* 85020

Lane M: 1 kb DNA marker

For all clones the GalE primers amplified two bands, of ≈ 1.0 kb representing the wild-type gene and ≈ 2.2 kb as the mutated gene.

B:

Lanes 1-11: transformed clones

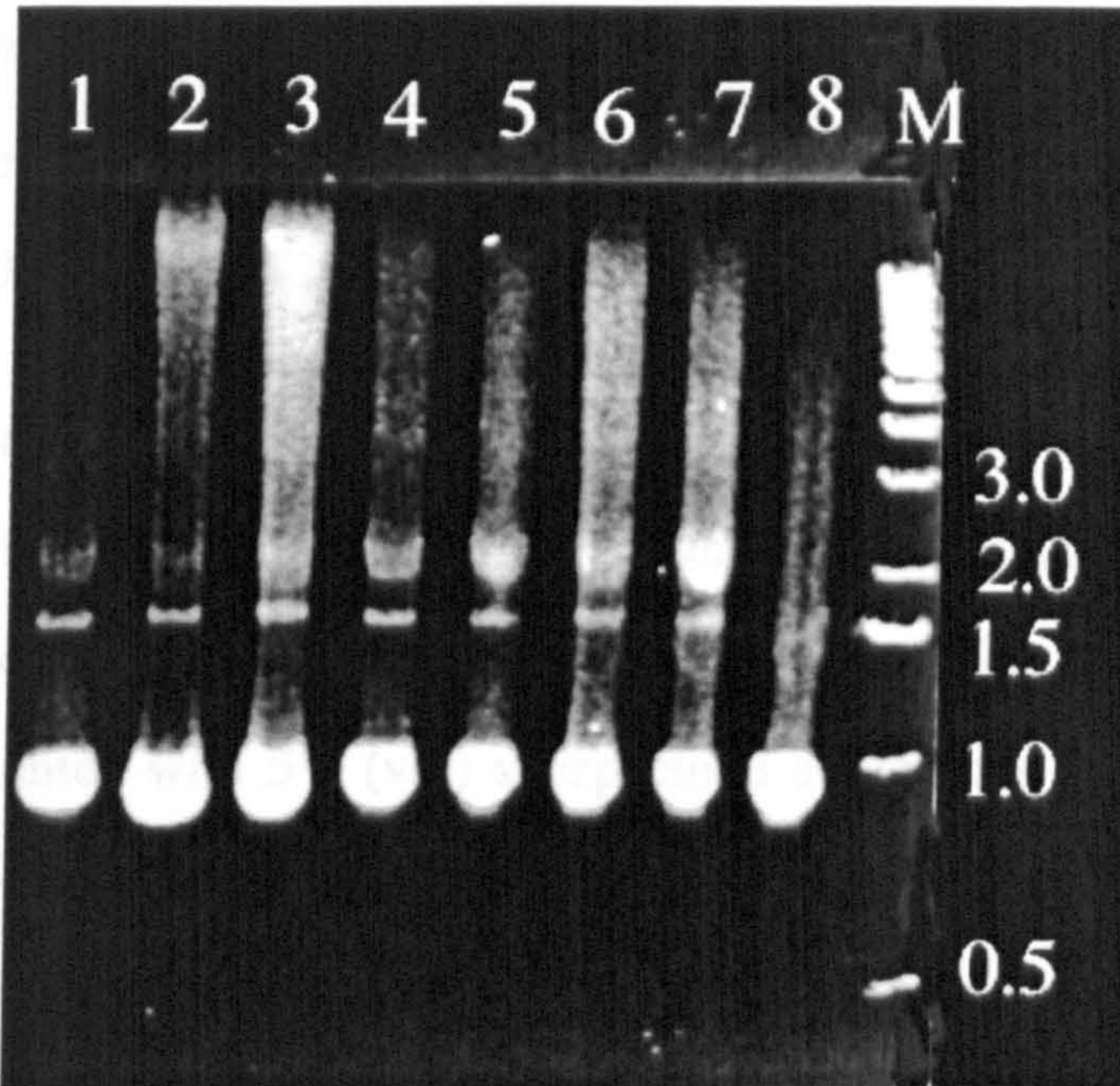
Lane 12: *P. multocida* Quetta

Lane M: 1 kb DNA marker

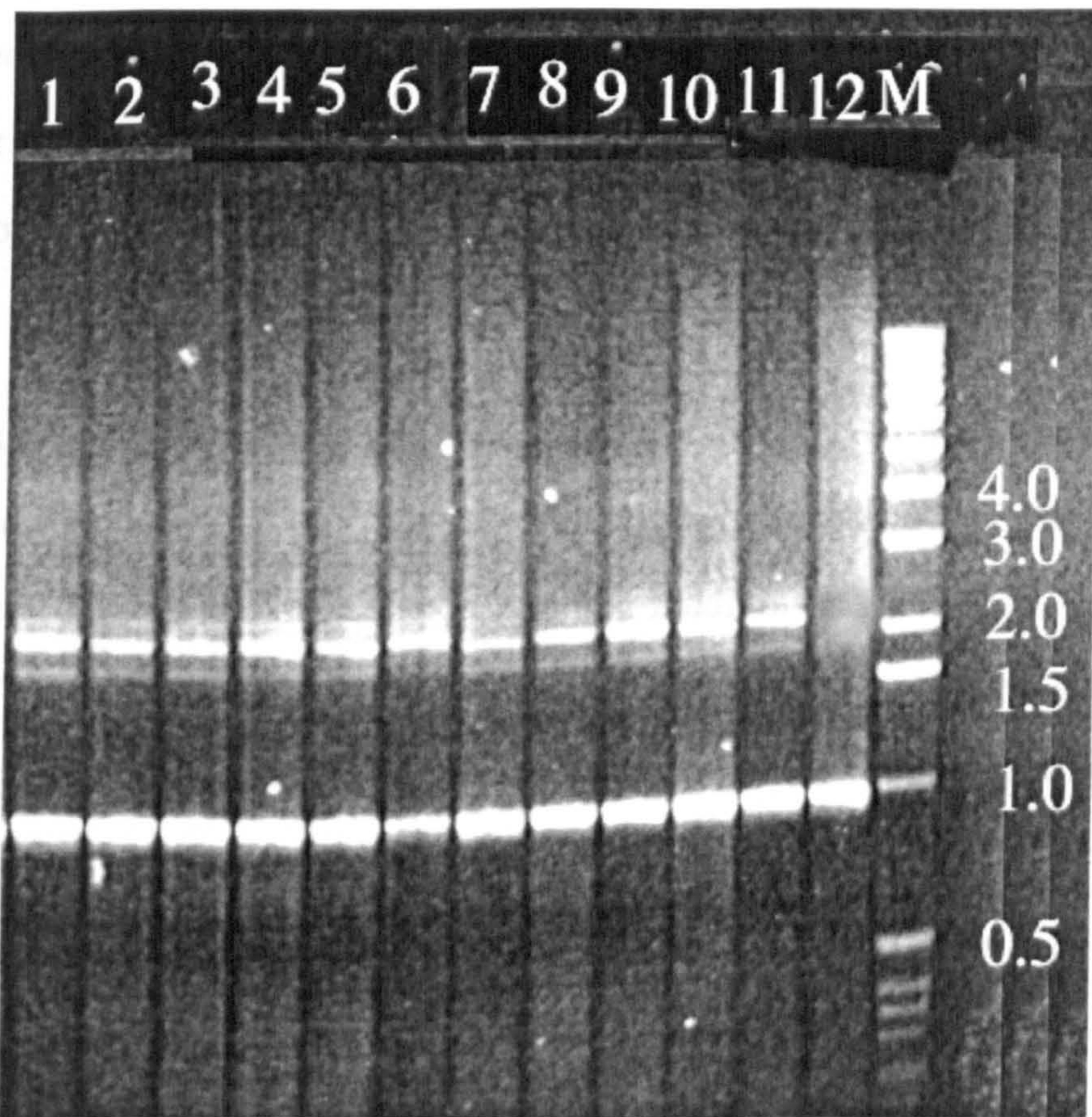
For all clones the GalE primers amplified two bands, of ≈ 1.0 kb representing the wild-type gene and ≈ 2.2 kb as the mutated gene.

Fig 40:

A.



B.



agar or single colonies from LB agar containing ampicillin and kanamycin were examined by restriction analysis and designated as pJRMT19 and pJRMT20 for *P. multocida* 85020 and Quetta strains respectively (Fig. 42A). Digestion with *EcoRI* just linearised plasmid pAKA19 whereas it produced two bands, of 2.2 kb (*galE* gene interrupted with Km^R cassette) and 5.0 kb for the vector for plasmids pJRMT19 and pJRMT20 (Fig. 41).

3. 1. 17. Allelic exchange

The plasmids pJRMT19 and pJRMT20 were transferred to the *P. multocida* strains for allelic exchange by electroporation and conjugation and plated onto BHI agar supplemented with 5% (v/v) sheep blood containing appropriate antibiotics. Single colonies were picked and patched onto kanamycin plates. After three subcultures in the presence of Km, the Km^R clones were checked by PCR but most of them showed two bands representing the wild-type *galE* gene and the mutated gene with Km^R cassette insertion. When some of them were checked for plasmid replication, as was found for the previous *ColE1*-based vector, the plasmids pJRMT19 and pJRMT20 did not appear to act as suicide vectors in *P. multocida* strains and were maintained in the presence of kanamycin.

The possibility was considered that galactose present in BHI agar supplemented with sheep blood could produce problems for growth of *galE* mutants (see section 1. 4. 6. 8). Thus a minimal medium was prepared, as Mohammad Tabatabaei (MT) minimal medium (Appendix 5. 15). This was a modification of the PMM medium (Appendix 5.10). After electroporation, 0.5 ml of MT minimal medium was added and the transformed cells incubated for recovery. After 4 hrs 100 µl aliquots were spread on MT minimal agar containing kanamycin and incubated at 37°C for 48 hrs. Some single colonies were grown, but when checked by PCR they showed the same pattern as above.

Fig 41: Restriction analysis of plasmids pJRMT19 and pJRMT20 (size as kb)

A:

Lanes 1-2: plasmid pJRMT19 digested with *EcoRI* (≈ 2.2 and ≈ 5.0 kb fragments)

Lanes 3-4: plasmid pJRMT20 digested with *EcoRI* (≈ 2.2 and ≈ 5.0 kb fragments)

Lane 5: plasmid pAKA19 digested with *EcoRI* (linearised plasmid DNA)

Lane M: 1 kb DNA marker

B: Undigested plasmid DNA

Lanes 1-2: plasmid pJRMT19

Lanes 3-4: plasmid pJRMT20

Lane 5: plasmid pPAKA19

Lane M: supercoiled DNA marker

Fig 41:

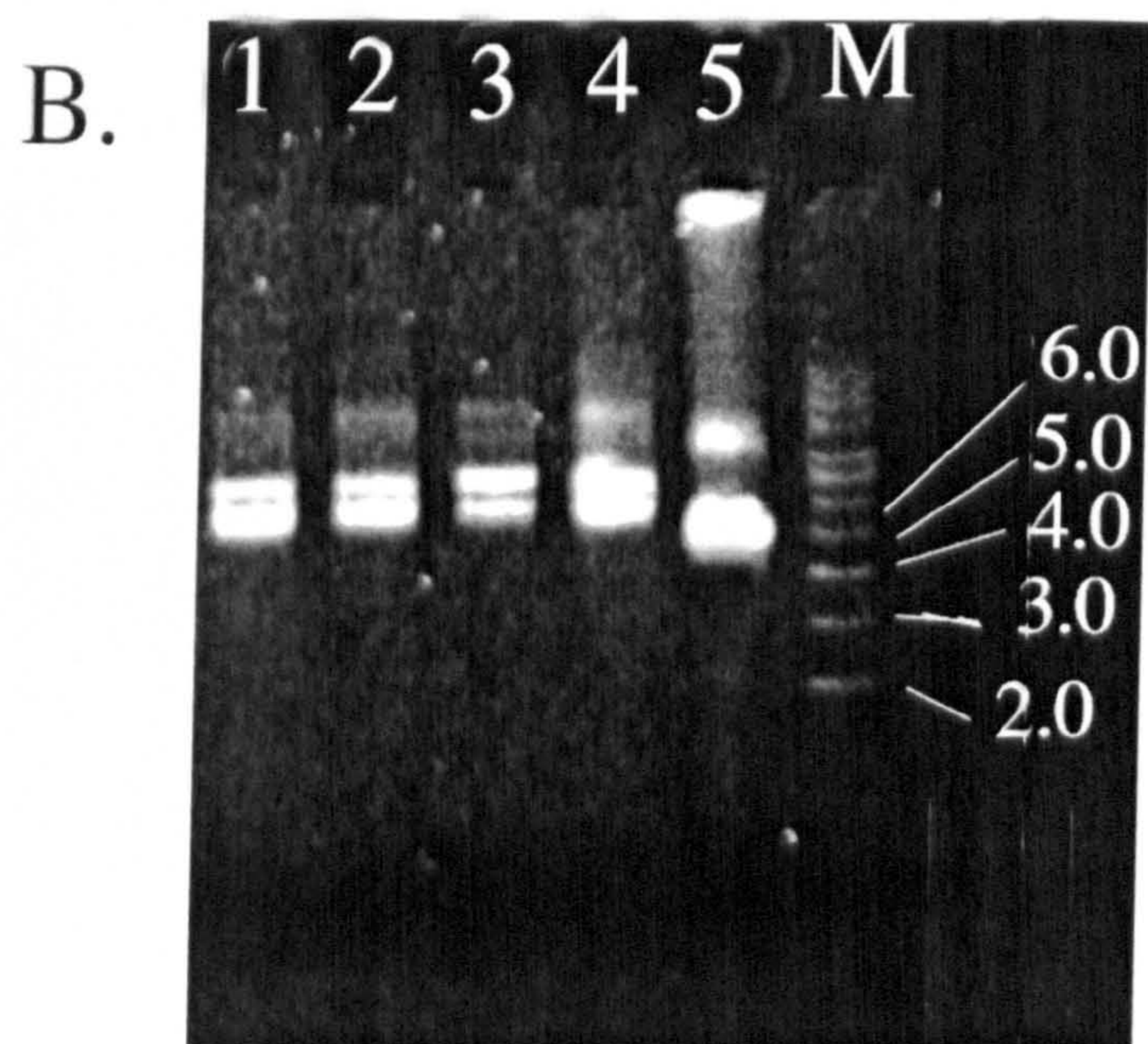
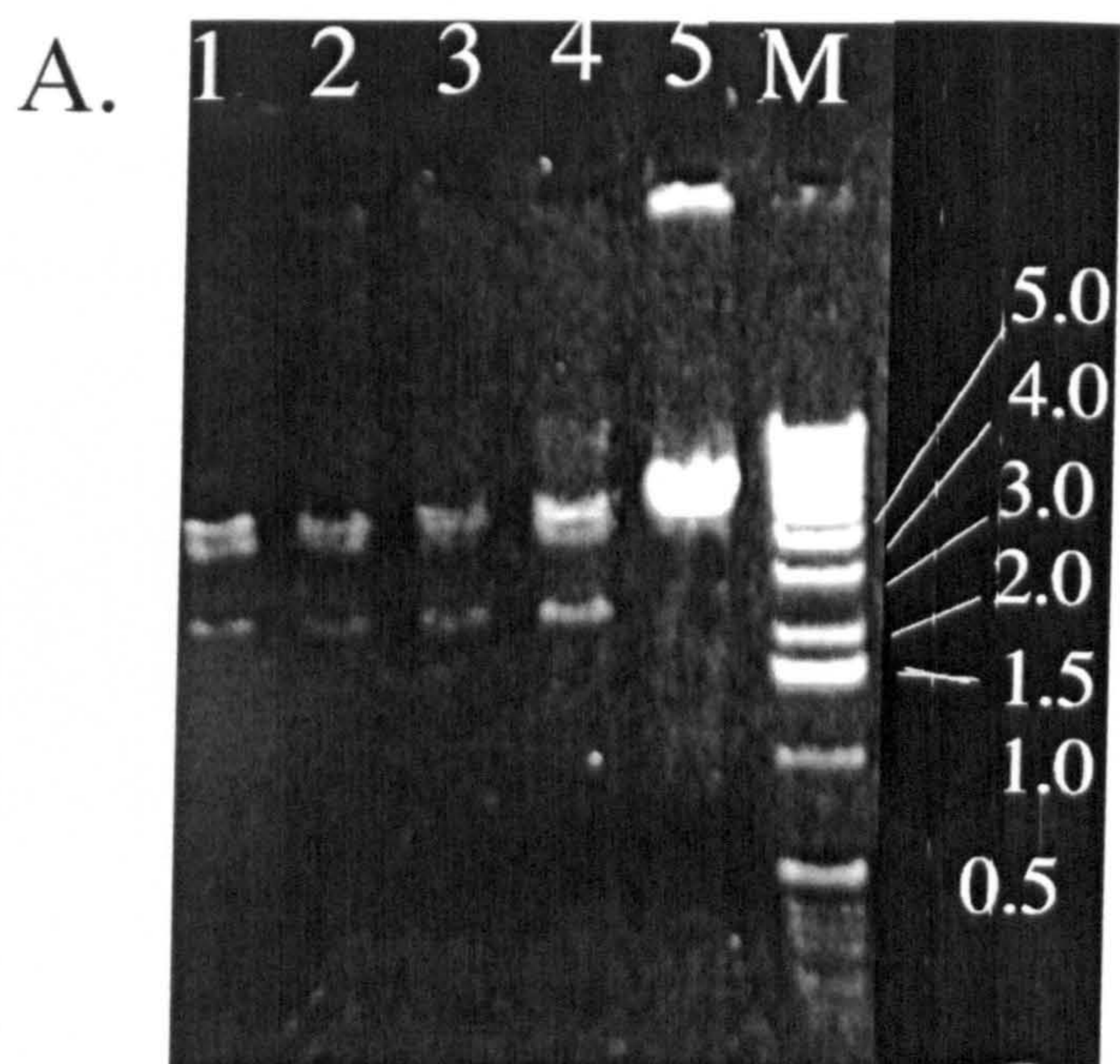


Fig 42:

A: Map of plasmids pJRMT19 and pJRMT20

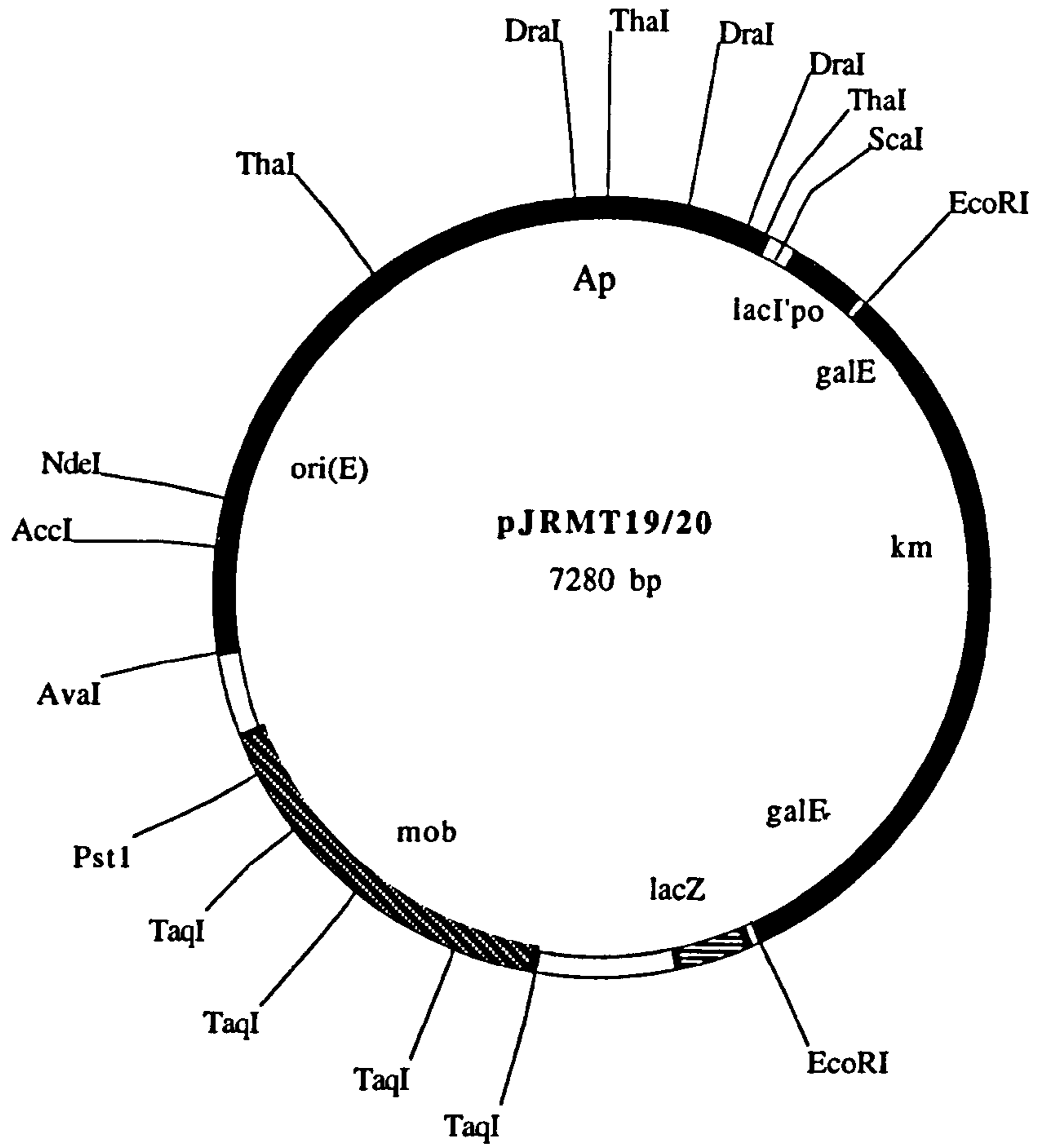
The Km^R-interrupted *galE* genes were cut out from plasmid pJRMT17 and pJRMT18 with *EcoRI* and cloned into pAKA19 which had been digested with *EcoRI* and dephosphorylated .

B: Map of plasmids pJRMT21 and pJRMT22

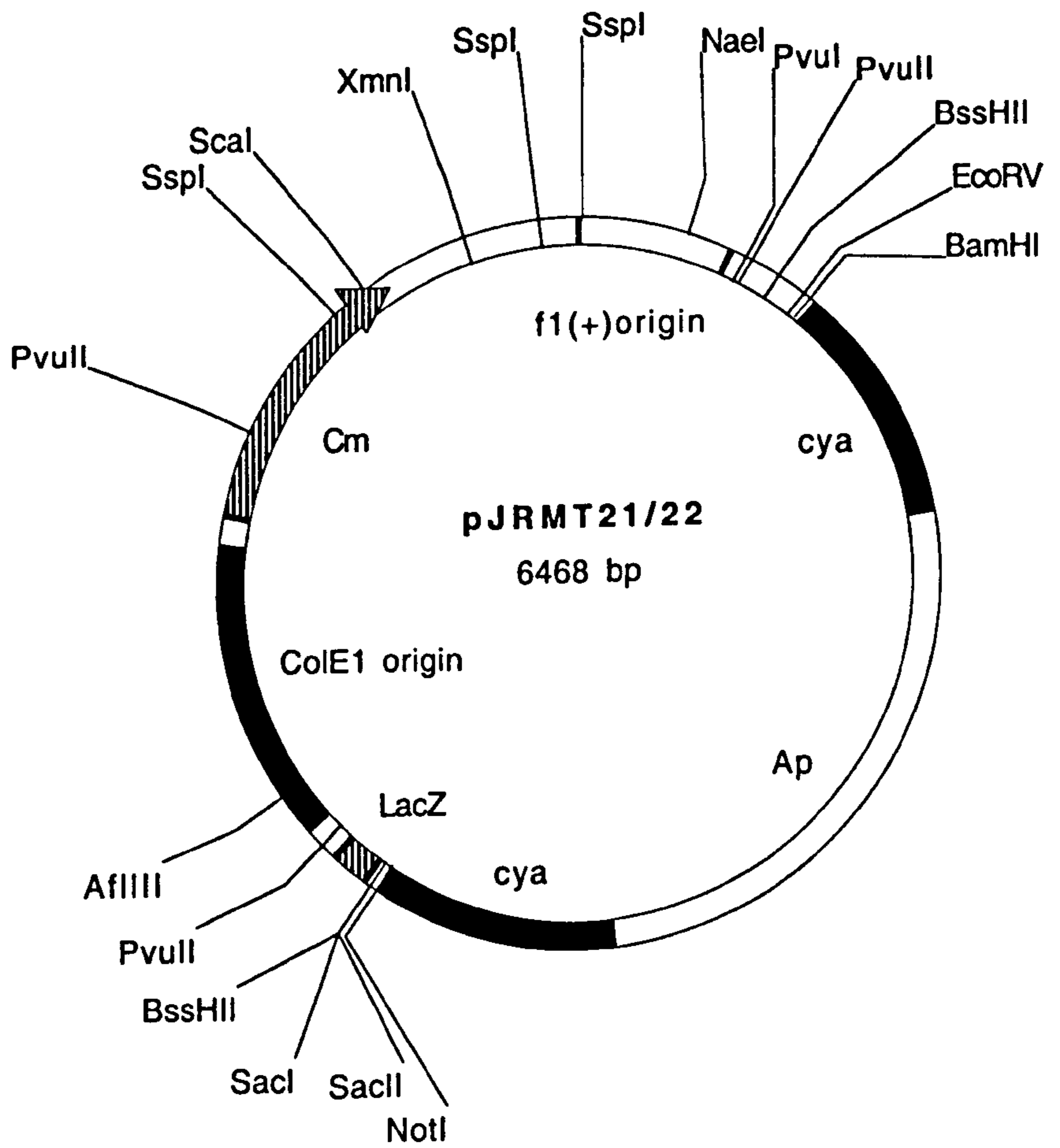
The ampicillin resistance cassette was cut out with *ScaI/SacII* from plasmid pJRMT11 and cloned into pJRMT7 and pJRMT8 which had been digested with *HpaI* and dephosphorylated .

Fig 42:

A.



B.



3. 2. Construction of double mutants (*aroA::km^R* and *cya::Ap^R*)

The data of Homchampa *et al.* (1992) indicated that strains with a single attenuating mutation may be too virulent at high doses to confer protection in a single dose, but they suggested that introduction of a second attenuating mutation may overcome this problem. This assumption was examined in mouse experiments reported later (Section 3. 5).

Double mutants were constructed by using the *aroA::Km^R* (or *aroA* deletion) (Section 3. 3. 1) strain as recipient and plasmid containing *cya::Ap^R* (or *cya* deletion) (Section 3. 3. 3). Selection of inheritance of *Ap^R* by the recipient will indicate exchange of the *cya::Ap^R* sequence with the *cya* sequence in chromosome. For the deletion derivatives, initial selection used the resistance marker encoded by the plasmid.

3. 2. 1. Mutation of *cya* gene by insertion of an *Ap^R* cassette

For construction of a double mutant derived from *aroA::Km^R* strain, it was necessary to use the *cya* gene interrupted by a sequence encoding resistance to an antibiotic other than kanamycin.

3. 2. 1. 1. Cloning of ampicillin resistance cassette

The ampicillin resistance cassette was cut out with *ApalI/ScaI* from plasmid pAKA16 as a 1416 bp (from nt. 1865-3330) fragment, blunted by Klenow enzyme and cloned into *PvuII/ScaI* digested and dephosphorylated pACYC184 (919 bp removed from nt. 3850-515) (Table 4). This recombinant plasmid was confirmed by restriction analysis and named as plasmid pJRMT11 (Fig. 33A). Digestion of plasmid pACYC184 with *ScaI/SacII* produced two fragments of ≈ 0.9 and ≈ 3.0 kb as predicted whereas plasmid pJRMT11 produced two fragments of ≈ 1.7 and ≈ 3.0 kb (Fig. 43).

Fig 43: Restriction analysis of plasmid pJRMT11 (size as kb)

Lane M: 1kb DNA marker

Lane 1: plasmid pJRMT11 digested with *ScaI/SacII*

Lane 2: plasmid pACYC184 digested with *ScaI/SacII*

Lane 3: plasmid pJRMT11 undigested

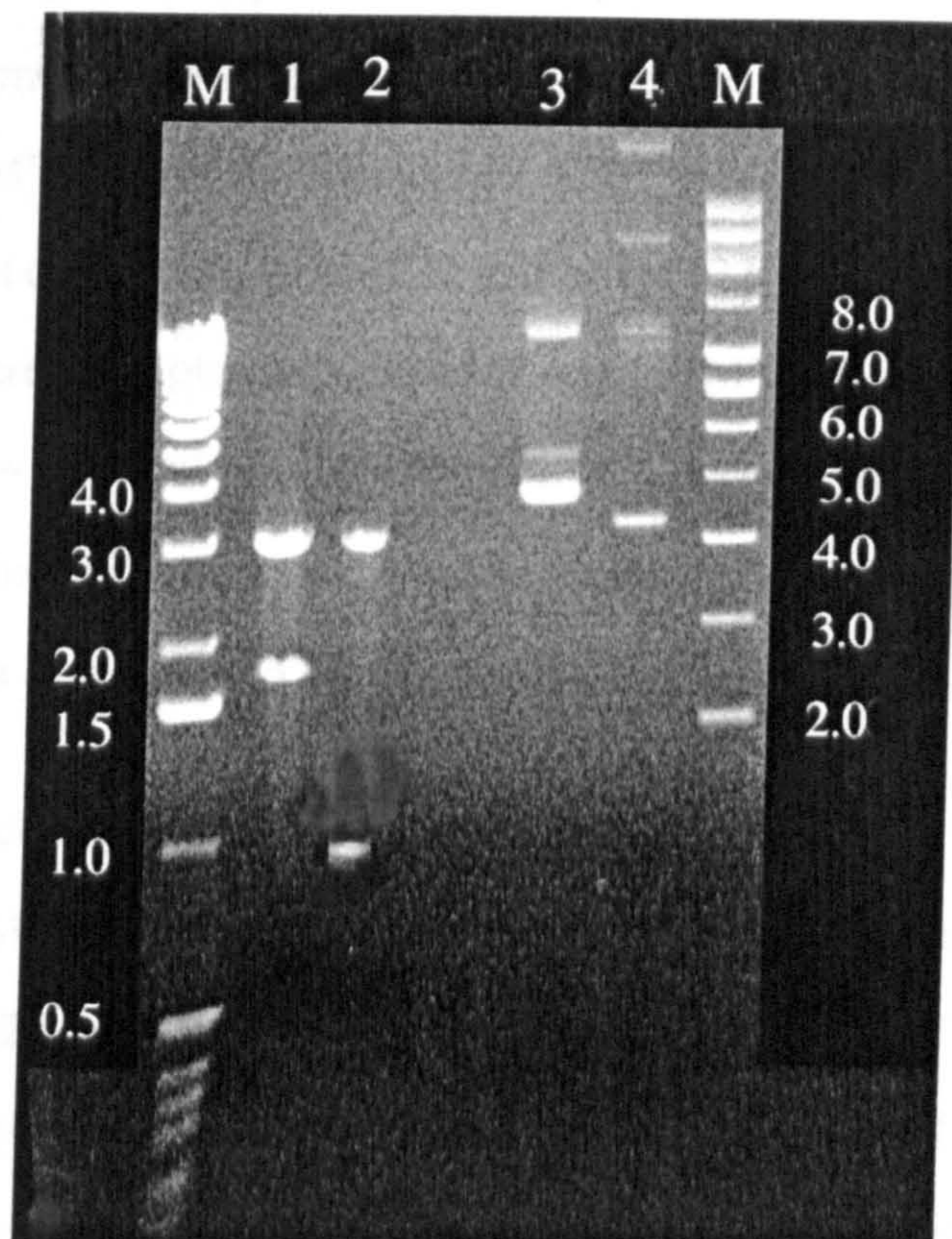
Lane 4: plasmid pACYC184 undigested

Lane M: supercoiled DNA marker

3.2.1.2. Identification of *P. mallosus* strains

The presence of *pIRMT2* was confirmed by PCR amplification of the *pIRMT2* gene from genomic DNA of multiple fragments from *P. mallosus* strains 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, 633, 634, 635, 636, 637, 638, 639, 640, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851, 852, 853, 854, 855, 856, 857, 858, 859, 860, 861, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 873, 874, 875, 876, 877, 878, 879, 880, 881, 882, 883, 884, 885, 886, 887, 888, 889, 890, 891, 892, 893, 894, 895, 896, 897, 898, 899, 900, 901, 902, 903, 904, 905, 906, 907, 908, 909, 910, 911, 912, 913, 914, 915, 916, 917, 918, 919, 920, 921, 922, 923, 924, 925, 926, 927, 928, 929, 930, 931, 932, 933, 934, 935, 936, 937, 938, 939, 940, 941, 942, 943, 944, 945, 946, 947, 948, 949, 950, 951, 952, 953, 954, 955, 956, 957, 958, 959, 960, 961, 962, 963, 964, 965, 966, 967, 968, 969, 970, 971, 972, 973, 974, 975, 976, 977, 978, 979, 980, 981, 982, 983, 984, 985, 986, 987, 988, 989, 990, 991, 992, 993, 994, 995, 996, 997, 998, 999, 1000.

Fig 43:



3. 2. 1. 2. Insertion of Ap^R cassette into the *cya* gene

The plasmids pJRMT7 and pJRMT8 were digested with *Hpa*I and the 1168 bp middle fragment (from nt. 690-1857) of the *cya* gene removed, run in a 0.7% (w/v) agarose gel and the larger fragments purified as vector. The ampicillin resistance cassette was removed as a 1734 bp fragment with *Sca*I/*Sac*II from plasmid pJRMT11, treated with Klenow enzyme and ligated with *Hpa*I vector fragments of pJRMT7 and pJRMT8. The *E. coli* XL1 Blue competent cells were transformed by electroporation and the bacteria spread onto LB agar containing chloramphenicol and ampicillin. The recombinant plasmids were confirmed by restriction analysis and were named plasmid pJRMT21 and pJRMT22 for *P. multocida* 85020 and Quetta strains respectively. Treatment of plasmids pJRMT21 and pJRMT22 with *Hpa*I had no effect (not shown) whereas for plasmids pJRMT7 and pJRMT8, two fragments of ≈ 1.17 and ≈ 4.7 or ≈ 1.17 and ≈ 4.4 kb respectively, were produced (Fig. 44). The sequence of the Ap^R cassette was unknown, thus plasmids pJRMT21 and pJRMT22 were not digested with *Bam*HI/*Sac*II.

3. 2. 1. 3. Allelic exchange

The plasmids pJRMT21 and pJRMT22 were transformed to parent *P. multocida* strains 85020 and Quetta and *aroA* mutant strains JRMT1 and JRMT2 for allelic exchange by electroporation and plated onto BHI agar supplemented with 5% (v/v) sheep blood containing appropriate antibiotics. Selection was made for Ap^R for *P. multocida* 85020 and Quetta strains and Ap^R-Km^R for *P. multocida* JRMT1 and JRMT2 strains. Potential mutants were checked by Southern blot hybridisation and designated as *P. multocida* JRMT4 and JRMT5 from *P. multocida* strains 85020 and Quetta, respectively, and *P. multocida* JRMT6 and JRMT7 from *P. multocida* JRMT1 and JRMT2 strains, respectively. The genomic DNA of *P. multocida* double mutants and single Ap^R mutants were digested with *Acc*I and hybridised with *cya* and

Fig 44: Restriction analysis of plasmids pJRMT21 and pJRMT22 (size as kb)

Lane 1: plasmid pJRMT9 digested with *HpaI*

Lane 2: plasmid pJRMT10 digested with *HpaI*

Lane 3: plasmid pJRMT7 digested with *HpaI*

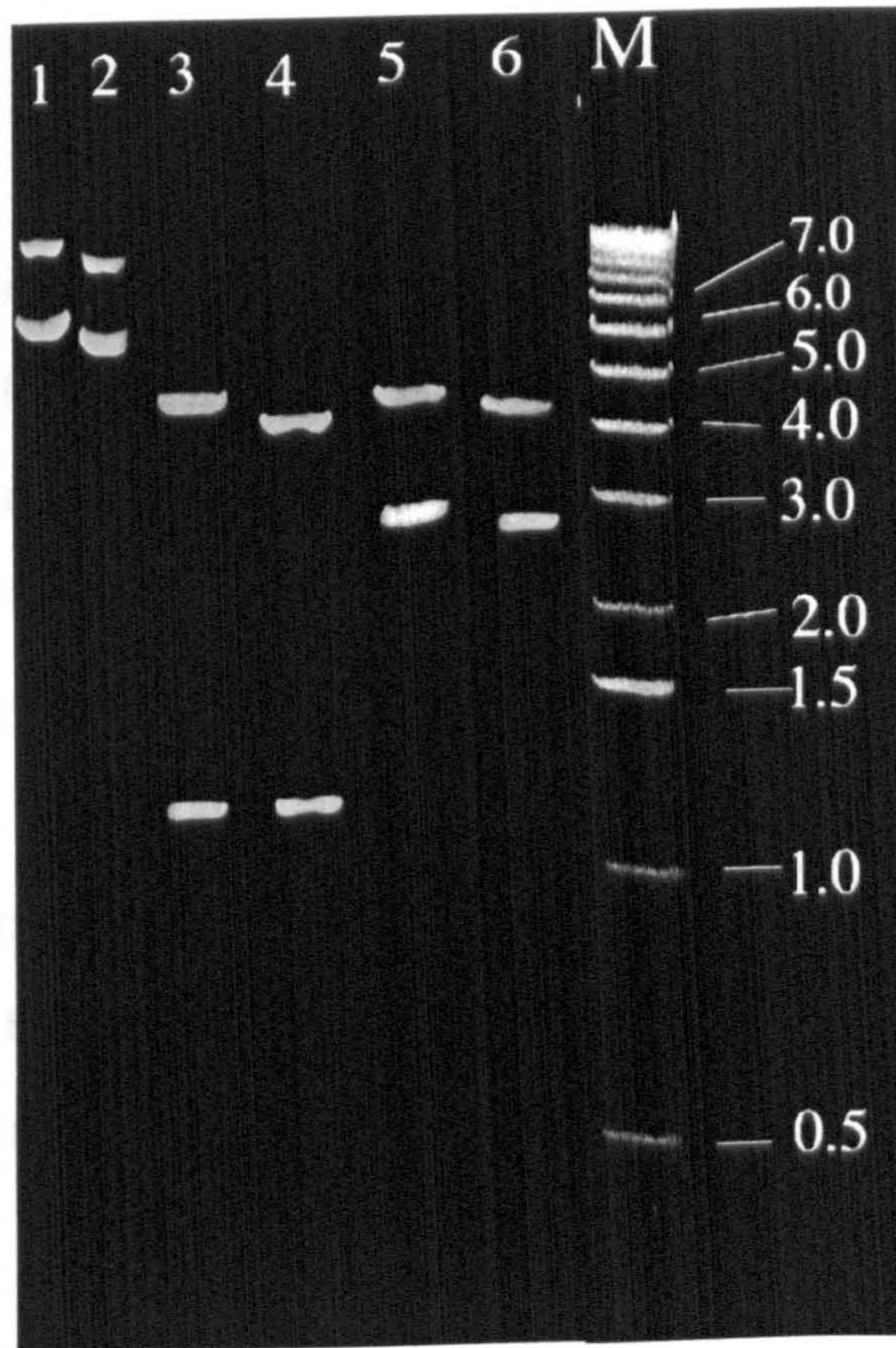
Lane 4: plasmid pJRMT8 digested with *HpaI*

Lane 5: plasmid pJRMT9 digested with *BamHI/SacII*

Lane 6: plasmid pJRMT10 digested with *BamHI/SacII*

Lane M: 1 kb DNA marker

Fig 44:



3. 3. Construction of markerless mutants

For use as vaccine strains, *S. Typhimurium* mutants should be devoid of any antibiotic resistance markers. To select such mutants the first step was the construction of a markerless selection for the *uidA* gene. This was achieved by the insertion of a *uidA* gene into the *uidA* locus of the *uidA* gene. In the second step, after several attempts, a markerless selection vector (second cloning event) was used to replace the *uidA* gene with a markerless selection.

ampicillin resistance (Ap^R) probes. The *cya* probe hybridised to two bands of ≈ 2.6 and ≈ 6.0 for the parent strains JRMT1 and JRMT2 and most of the putative double mutants (e.g. JRMT6 and JRMT7), except for one clone which showed hybridisation to three bands of ≈ 2.6 , ≈ 6.0 and ≈ 8.0 kb (Fig. 45). The Ap^R probe did not hybridise to any band for the parent JRMT1 and JRMT2 strains, but for the double mutants of JRMT1 (e.g. JRMT6) it hybridised to two bands as with the *cya* probe. Two bands were also obtained for the double mutants of JRMT2 (e.g. JRMT7) at ≈ 6.5 and ≈ 8.0 or ≈ 2.6 kb (Fig. 45). The *cya* probe with Quetta Ap^R (JRMT5 strains) hybridised to 3-4 bands of ≈ 2.6 , ≈ 3.0 , ≈ 6.0 and ≈ 7.0 kb (Fig. 46, lanes 1-5) and two bands of ≈ 2.6 and ≈ 6.0 kb for the Quetta parent strain (Fig. 46, lane 6). It hybridised to three bands for strain 85020 Ap^R (JRMT4) (Fig. 46, lane 7). The Ap^R probe did not hybridise to any bands for the parent strains and hybridised to two or three bands of ≈ 2.6 , ≈ 5.5 and ≈ 7.0 kb for the Ap^R mutants (e.g. JRMT4 and JRMT5). When plasmids pJRMT21 and pJRMT22 were digested with *AccI*, they hybridised to two fragments (data not shown), and this indicated that at least one *AccI* site is present in the Ap^R cassette. Thus the Ap^R probe for transformed cells should have hybridised to two band, but at different sizes to those in the wild-type. However, in these cells they hybridised to more than two bands with Ap^R or *cya* probes.

3. 3. Construction of marker-free mutants

For use as vaccine strains, attenuated mutants should not carry antibiotic resistance markers. To select such mutants, the first step (first crossing-over) involved selection for the antibiotic resistance marker that was carried by the vector and then, in the second step, either selection can be made against the vector (second crossing-over) or loss of the vector may be anticipated on repeated subculture.

Fig 45: Southern blot hybridisation of *P. multocida* double mutant strains

(size as kb)

A: Hybridisation with *cya* probe

B: Hybridisation with Ap^R probe

Lanes 1-7: *P. multocida* JRMT6 *aroA*::Km^R, *cya*::Ap^R

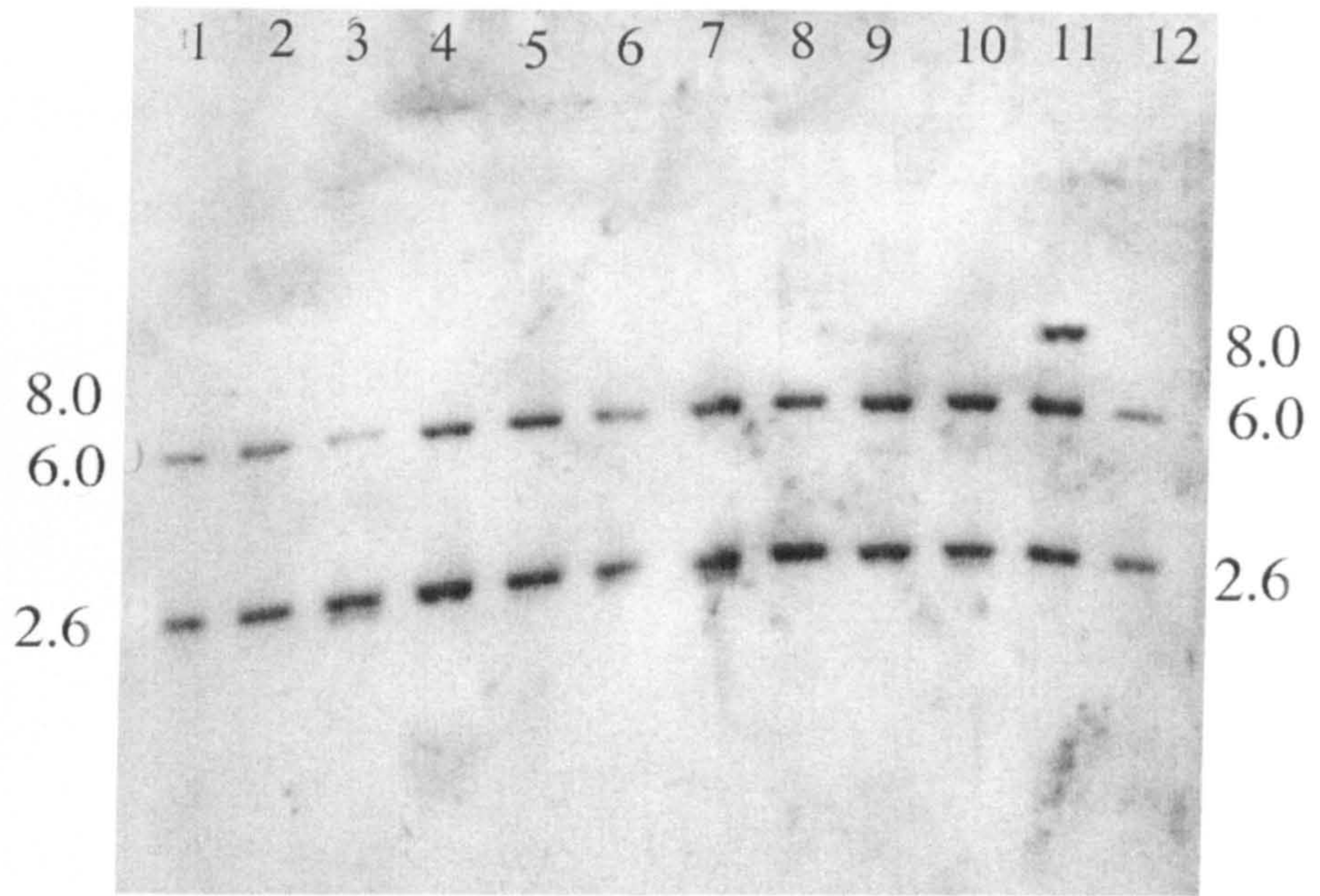
Lane 8: *P. multocida* JRMT1 *aroA*::Km^R

Lanes 9-11: *P. multocida* JRMT7 *aroA*::Km^R, *cya*::Ap^R

Lane 12: *P. multocida* JRMT2 *aroA*::Km^R

Fig 45:

A.



B.

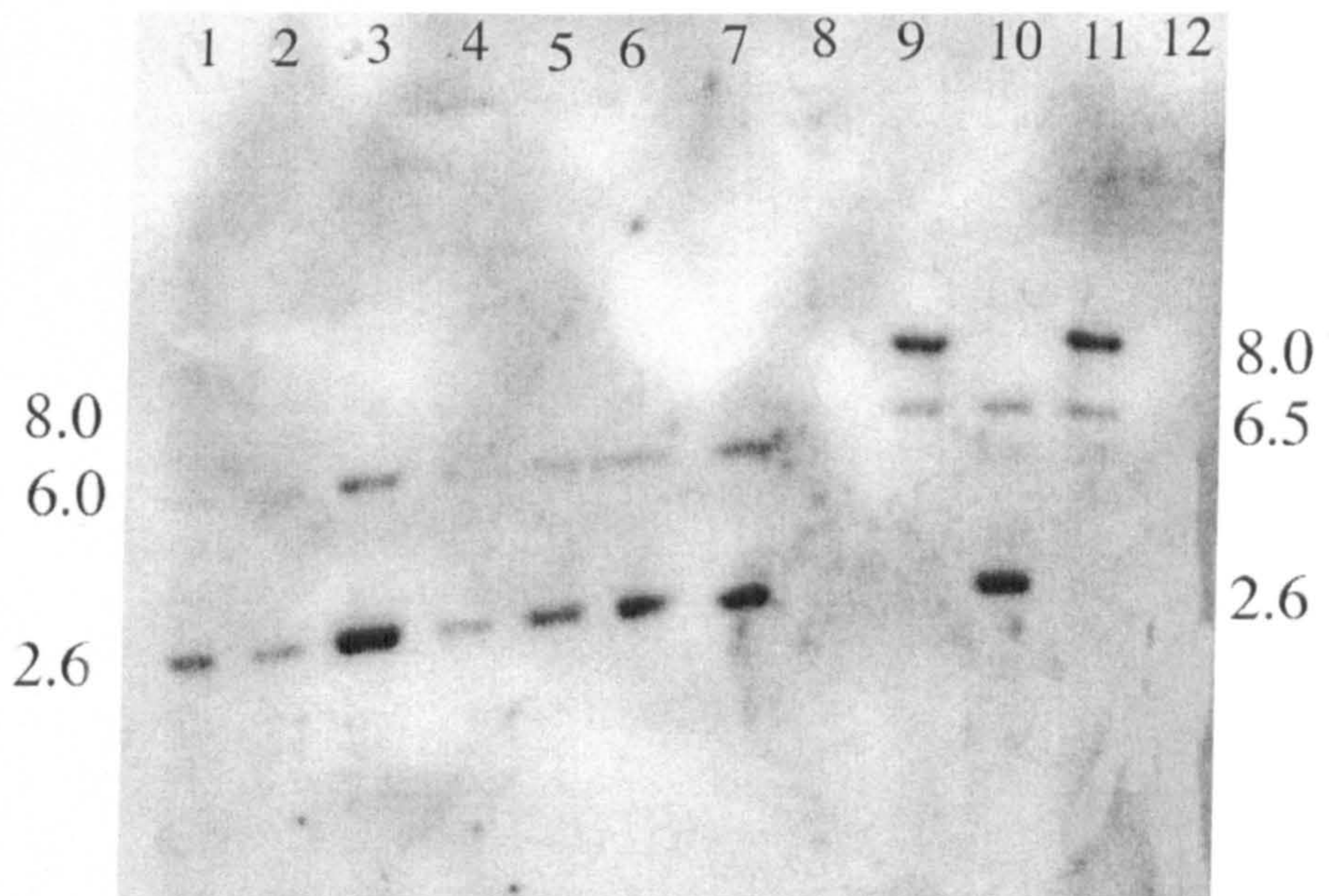


Fig 46: Southern blot hybridisation of *P. multocida* *cya::Ap^R* mutant strains transformed with plasmids pJRMT21 and pJRMT22 (size as kb)

A: Hybridisation with *cya* probe

B: Hybridisation with ampicillin probe

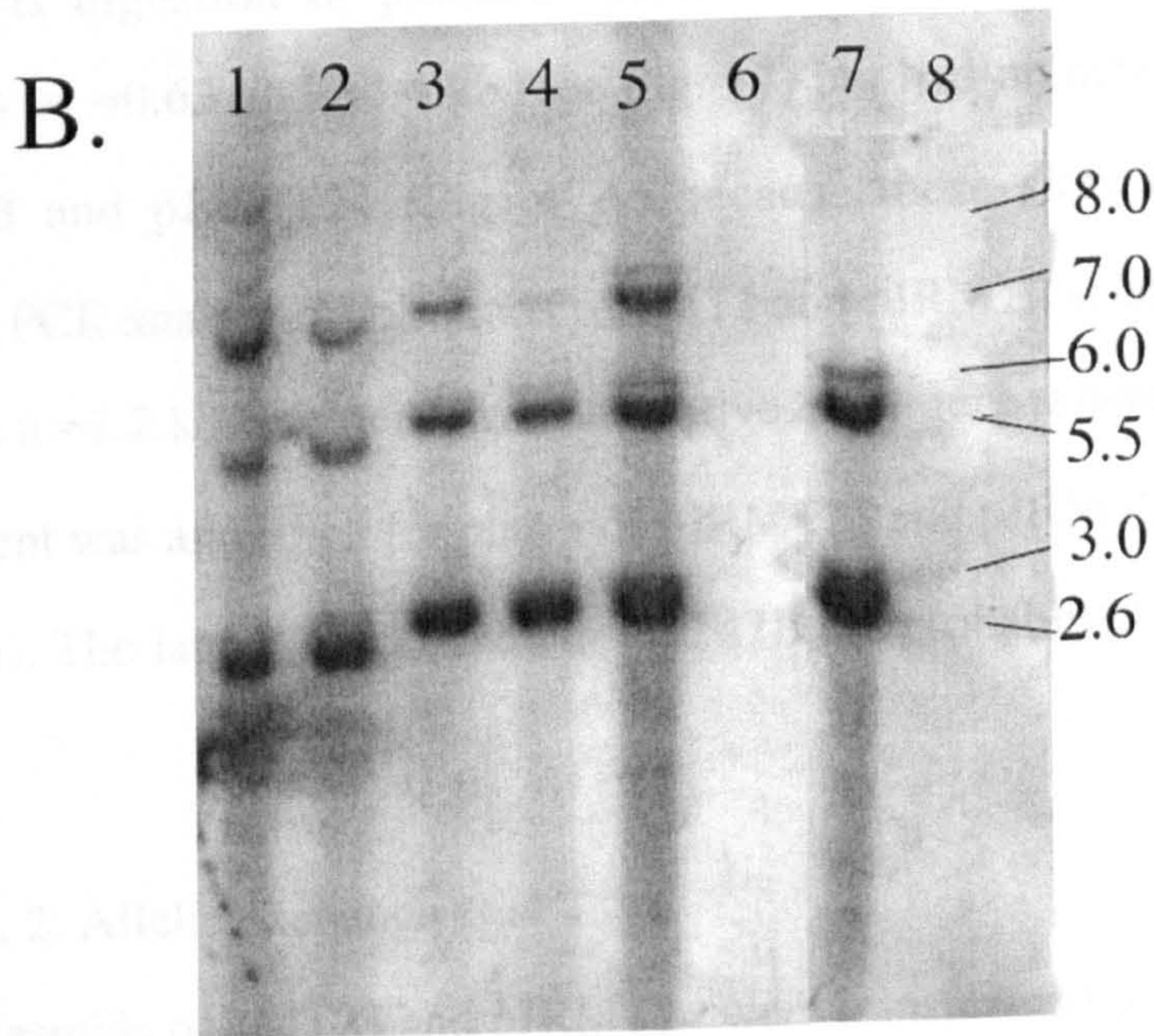
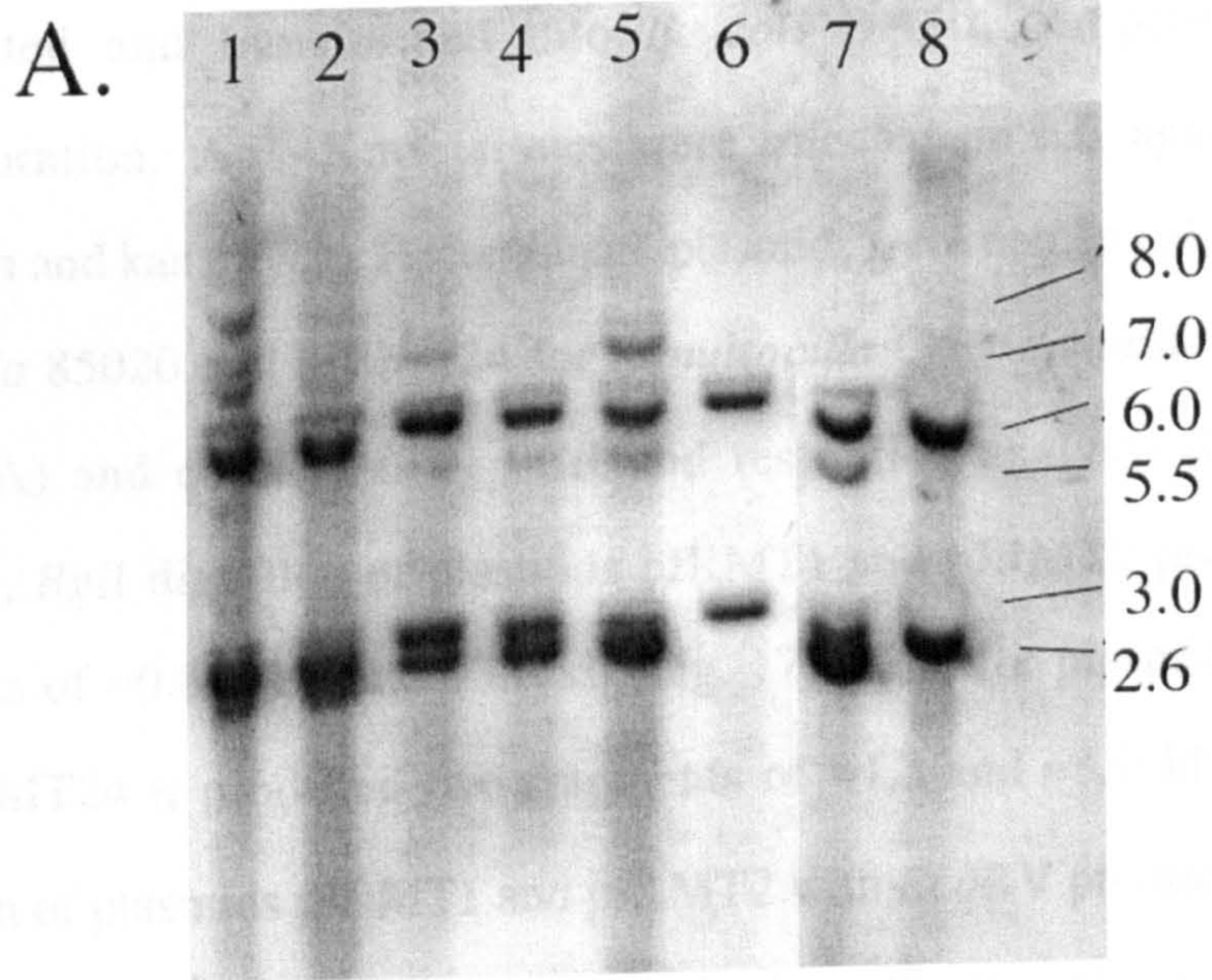
Lanes 1-5: *P. multocida* JRMT5 (Quetta) *cya::Ap^R*

Lane 6: *P. multocida* Quetta

Lane 7: *P. multocida* JRMT4 (85020) *cya::Ap^R*

Lane 8: *P. multocida* 85020

Fig 46:



3. 3. 1. Construction of marker-free *aroA* mutants

3. 3. 1. 1. Construction of deleted *aroA* gene (621 bp deletion)

The plasmids pJRMT1 and pJRMT2 were digested with *NdeI/SacII* (See Fig. 17) and a 621 bp middle section of the *aroA* gene (from nt. 459-1080) removed. The remaining portion was "polished" by T4 DNA polymerase, run in a 0.7% (w/v) agarose gel and the fragments (≈ 4.4 kb) purified from the gel, self ligated and transformed into *E. coli* DH5 α competent cells by electroporation. Ap^R-Km^R clones were selected on LB agar containing ampicillin and kanamycin. Recombinant plasmids were named pJRMT23 for *P. multocida* 85020 and pJRMT24 for *P. multocida* Quetta strains respectively (Fig. 48A) and confirmed by PCR and restriction analysis (Fig. 47). As expected, *BglI* digestion of plasmids pJRMT1 and pJRMT2 produced three fragments of ≈ 0.8 , ≈ 1.3 and ≈ 3.0 kb (Fig. 17) while for plasmids pJRMT23 and pJRMT24 it produced two fragments of ≈ 1.3 and ≈ 3.0 kb (Fig. 47A). Digestion of plasmids pJRMT1 and pJRMT2 with *EcoRV* produced linearised DNA of ≈ 5.1 kb (see Fig. 17) whereas it had no effect on plasmids pJRMT23 and pJRMT24 (Fig. 47A), because the *EcoRV* site had been removed. Also, *NdeI/SacII* digestion of plasmids pJRMT1 and pJRMT2 produced two fragments of ≈ 0.63 and ≈ 4.4 kb (see Fig. 17) but had no effect on plasmids pJRMT23 and pJRMT24 (Fig. 47A) because these sites had also been removed. PCR analysis of plasmids pJRMT1 and pJRMT2 with AroA primers amplified a ≈ 1.2 kb fragment from the native *aroA* gene whereas only a ≈ 0.6 kb fragment was amplified for plasmids pJRMT23 and pJRMT24, as predicted (Fig. 47B). The larger sized bands in Fig. 47B are probably plasmid template DNA.

3. 3. 1. 2. Allelic exchange

The plasmids pJRMT23 and pJRMT24 were transformed into *P. multocida* strains by electroporation and plated out onto BHI agar supplemented with 5%

Fig 47: Analysis of plasmids pJRMT23 and pJRMT24 (size as kb)

A: Restriction analysis:

Lane 1: 1 kb DNA marker

Lane 2: plasmid pJRMT23 digested with *Bgl*II (1.3 and 3.0 kb)

Lane 3: plasmid pJRMT23 digested with *Eco*RV (non-affected)

Lane 4: plasmid pJRMT23 digested with *Nde*I/*Sac*II (non-affected)

Lane 5: plasmid pJRMT23 undigested

Lane 6: plasmid pJRMT24 digested with *Bgl*II (1.3 and 3.0 kb)

Lane 7: plasmid pJRMT24 digested with *Eco*RV (non-affected)

Lane 8: plasmid pJRMT24 digested with *Nde*I/*Sac*II (non-affected)

Lane 9: plasmid pJRMT24 undigested

Lane 10: 1 kb DNA marker

Lane 11: plasmid pJRMT1 digested with *Bgl*II (0.8,1.3 and 3.0 kb)

Lane 12: plasmid pJRMT1 digested with *Eco*RV (linearised)

Lane 13: plasmid pJRMT1 digested with *Nde*I/*Sac*II (0.6 and 4.3 kb)

Lane 14: plasmid pJRMT1 undigested

Lane 15: supercoiled DNA marker

B: PCR analysis with AroA primers (AroA1 and AroA2)

Lane M: 1 kb DNA marker

Lane 1: plasmid pJRMT1

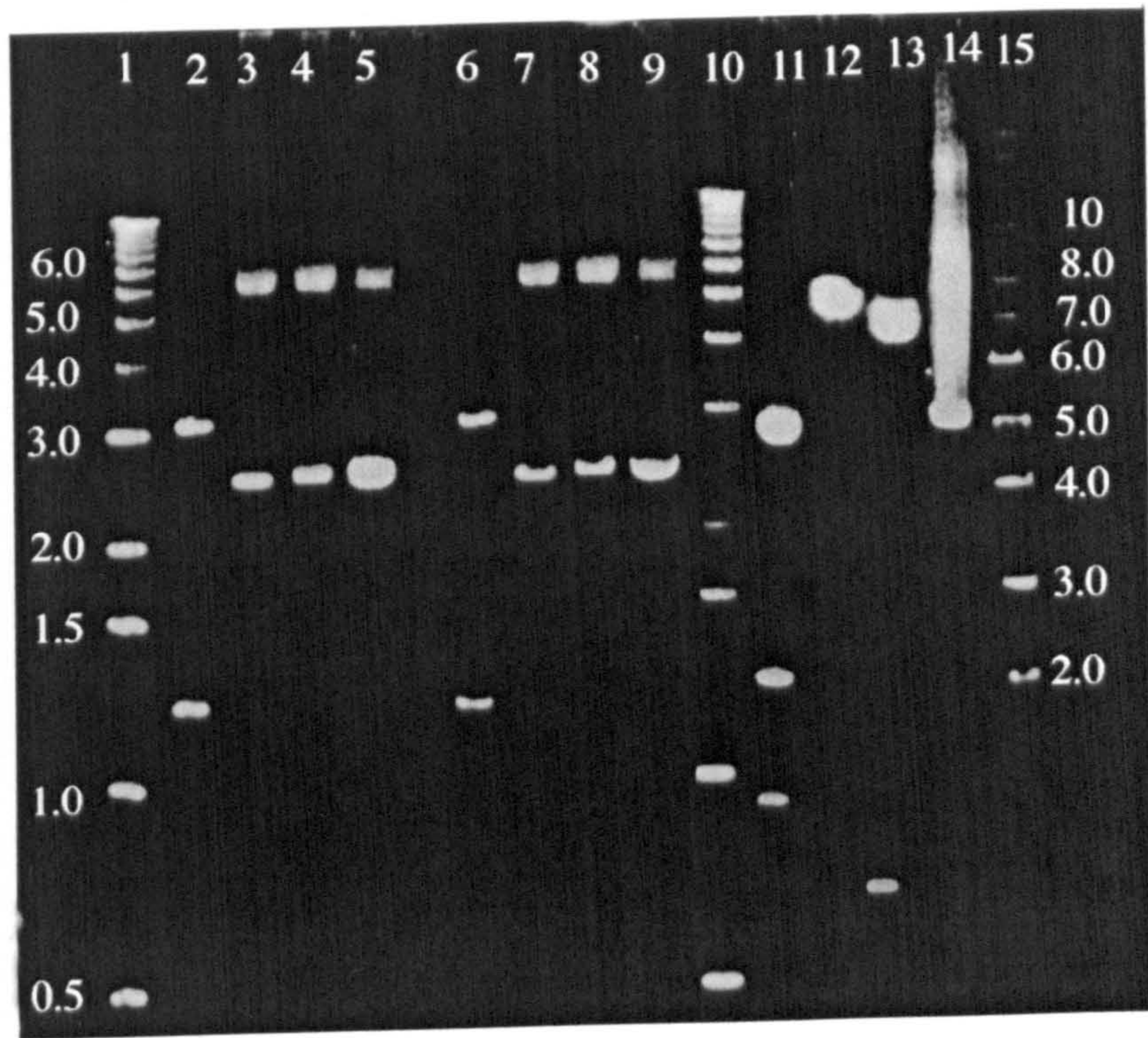
Lanes 2-8: plasmid pJRMT23 (different clones)

Lane 9: plasmid pJRMT2

Lanes 10-12: plasmid pJRMT24 (different clones)

Fig 47:

A.



B.

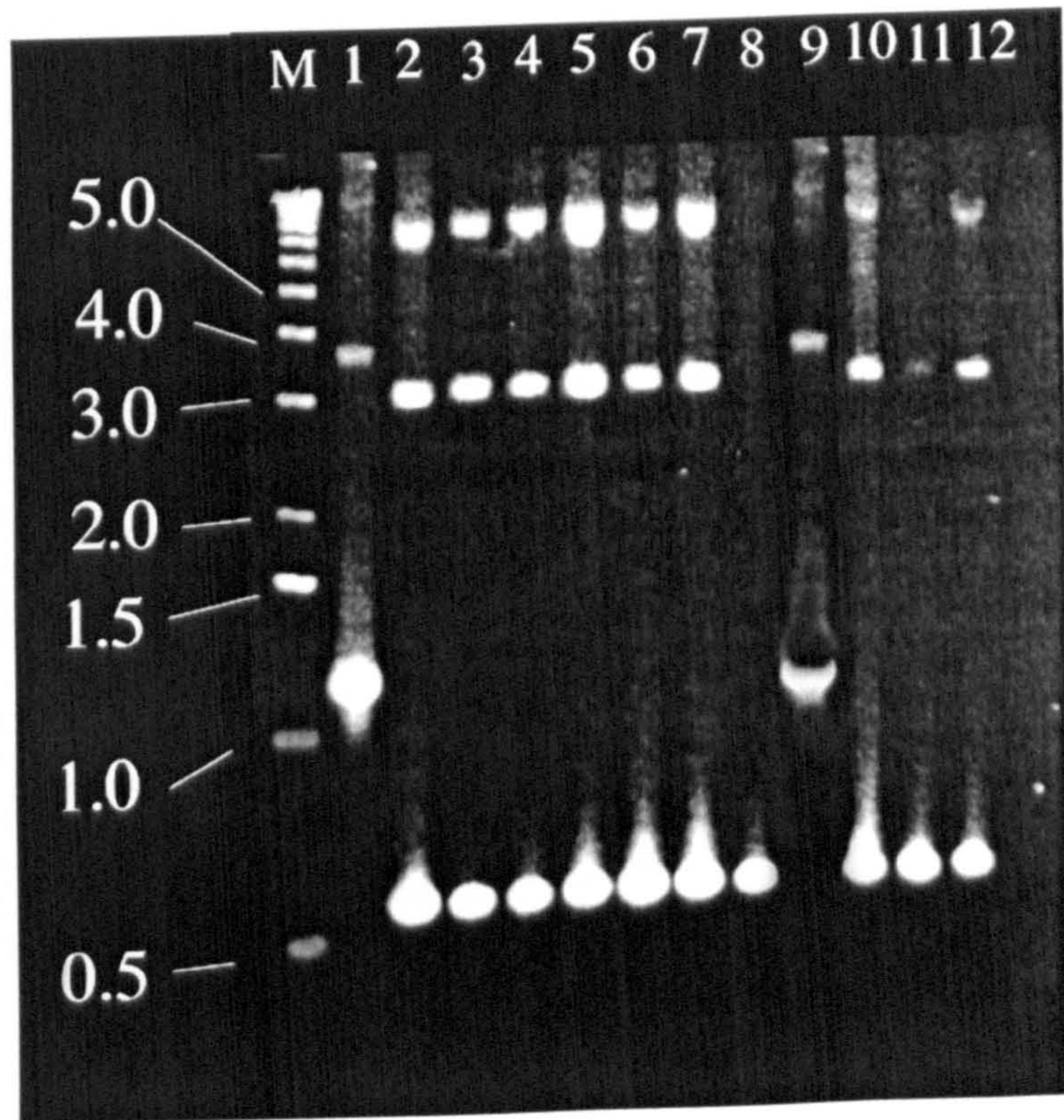


Fig 48:

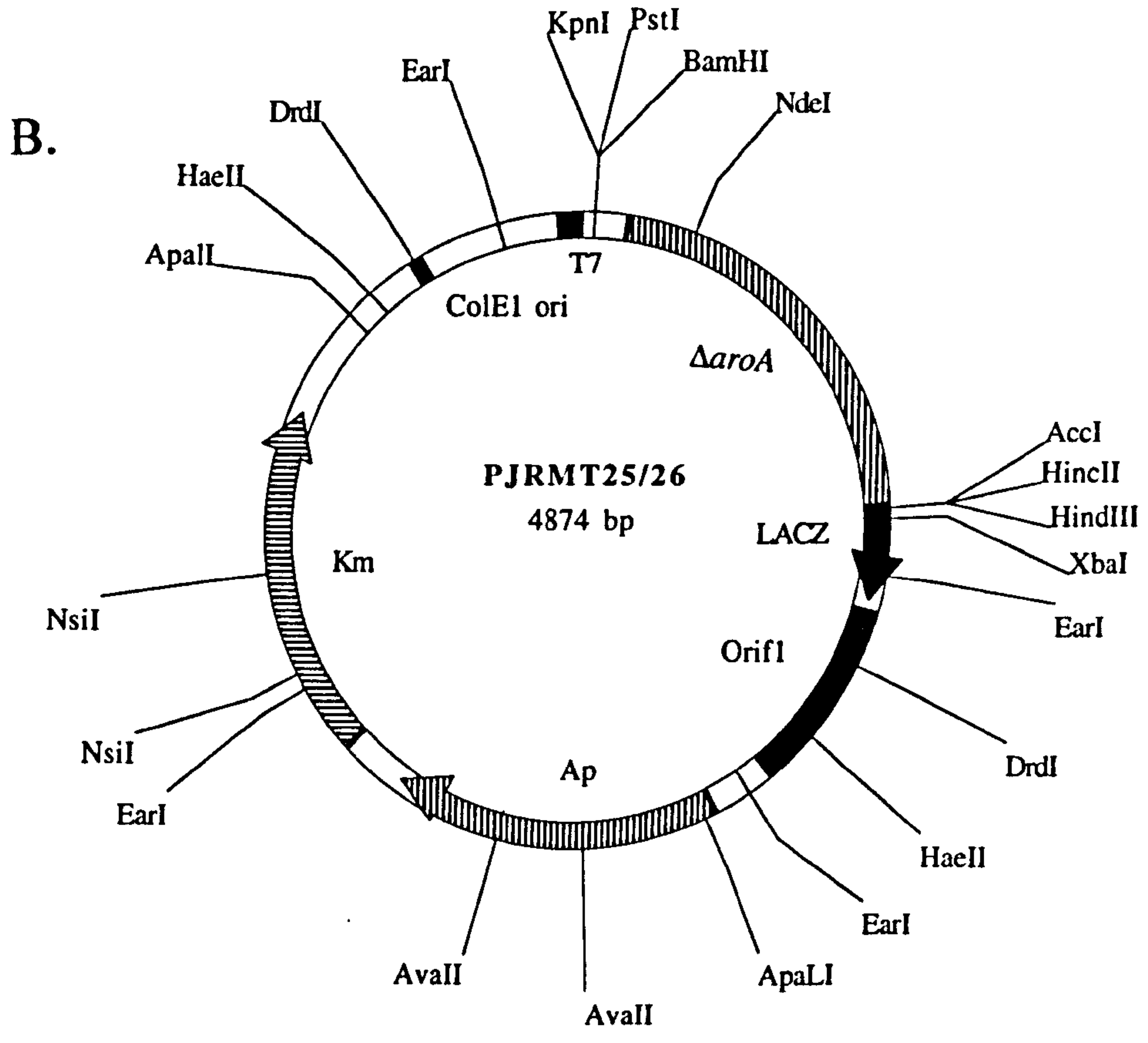
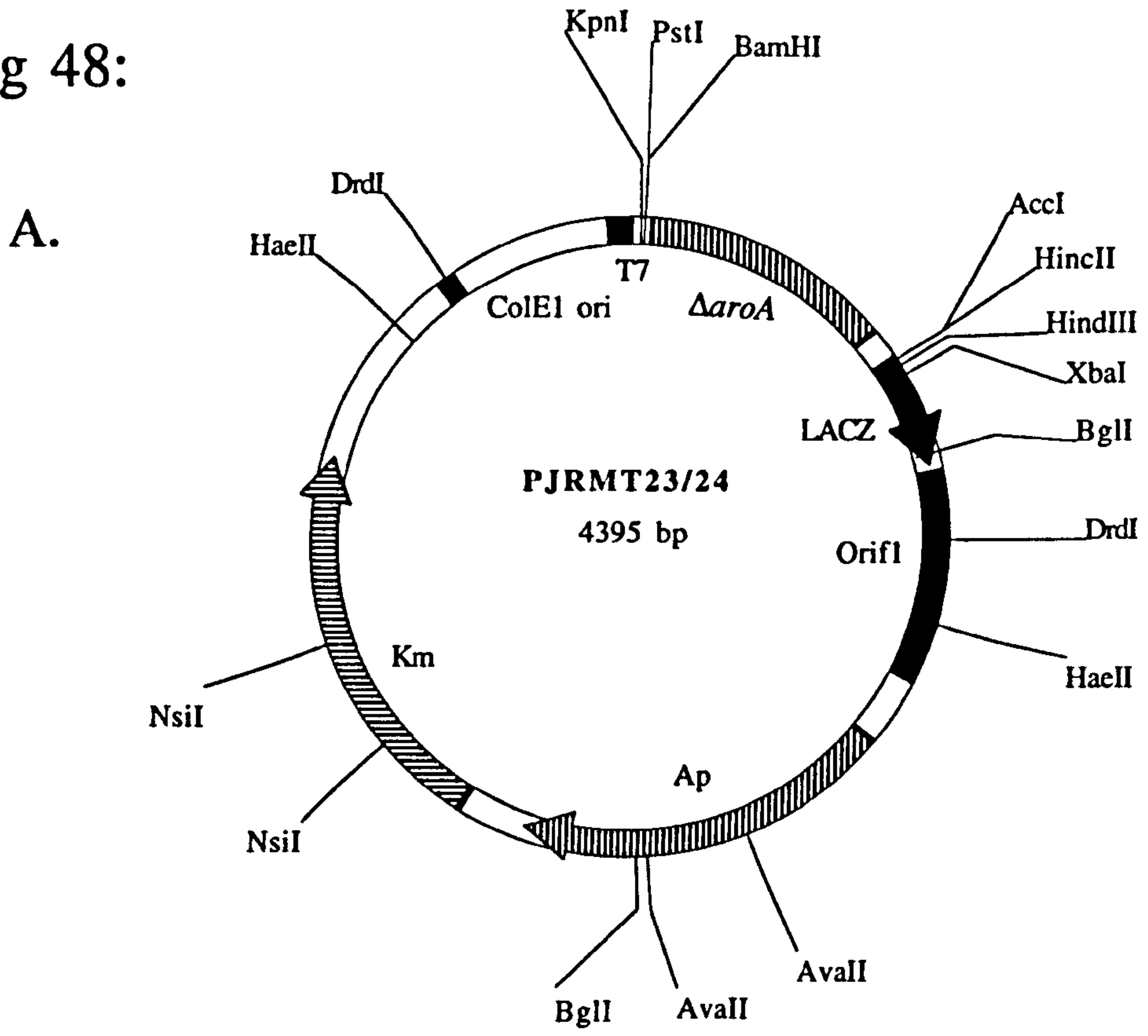
A: Maps of plasmids pJRMT23 and pJRMT24

The plasmids pJRMT1 and pJRMT2 were digested with *NdeI/SacII* to remove a 621 bp section of the *aroA* gene and the plasmid DNA self-ligated as plasmids pJRMT23 and pJRMT24.

B: Maps of plasmids pJRMT25 and pJRMT26

The plasmid pJRMT1 and pJRMT2 were digested with *AflIII/NsiI* to remove a 142 bp section of the *aroA* gene and the plasmids DNA self ligated.

Fig 48:



(v/v) sheep blood containing kanamycin. After incubation for 48 hrs at 37 °C, single Km^R colonies were patched onto BHI agar supplemented with 5% (v/v) sheep blood without any antibiotic. After incubation, this procedure was repeated until some Km^S clones were found. Some Km^S clones were inoculated into BHI broth and genomic DNA prepared for PCR with *aroA* primers. Some clones showed two bands, one of wild-type gene size and the other the size of the mutated gene (Fig. 49 & 50).

In another experiment, the procedure outlined by Homchampa *et al.* (1997) consisting of a combination of repeated subculturing and auxotrophic enrichment was evaluated. However, after passage in this way, when selected clones were checked by PCR, all of them showed the wild-type pattern.

3. 3. 1. 3. Construction of an alternative deleted *aroA* gene (142 bp deletion)

It was not clear why the Km^S clones above showed both native and deleted *aroA* amplicons. For this reason a smaller deletion was engineered which would leave longer flanking regions of the *aroA* gene for recombination with the native gene. For this purpose, plasmids pJRMT1 and pJRMT2 were digested with *Afl*III (at nt. 938) and *Sac*II (at nt. 1080) and a 142 bp middle fragment of the *aroA* gene removed (Fig. 17). The digested material was made blunt ended, religated and transformed into *E. coli* DH5 α . Recombinant plasmids were analysed by PCR and restriction analysis and they were designated pJRMT25 for *P. multocida* 85020 and pJRMT26 for *P. multocida* Quetta strains respectively (Fig. 48B). As expected *E*arI digestion of plasmid pJRMT1 and pJRMT2 produced five fragments of \approx 0.63, \approx 0.64, \approx 1.07, \approx 1.26 and \approx 1.42 whereas four fragments of \approx 0.64, \approx 1.26, \approx 1.42 and \approx 1.55 kb were produced for plasmids pJRMT25 and pJRMT26 because one *E*arI site had been removed from middle part of *aroA* gene. Digestion with *A*paLI produced two fragments of \approx 2.1 and \approx 2.9 kb for plasmids pJRMT1 and pJRMT2 whereas for plasmids pJRMT25 and pJRMT26, it produced two

Fig 49: PCR analysis with AroA primers of *P. multocida* 85020 clones transformed with plasmid pJRMT23 (size as kb)

A:

Lanes 1-7: transformed clones

Lane 8: *P. multocida* 85020

Lane 9: negative control

Lane M: 1 kb DNA marker

B:

Transformed clones after 10 subcultures:

Left:

Lanes 1-3: transformed clones

Lane 4: *P. multocida* 85020

Lane 5: negative control

Lane M: 1 kb DNA marker

Right:

Lane M: 1 kb DNA marker

Lanes 1-2: transformed clones

Lane 3: *P. multocida* 85020

Lane 4: negative control

Fig 49:

A.



B.

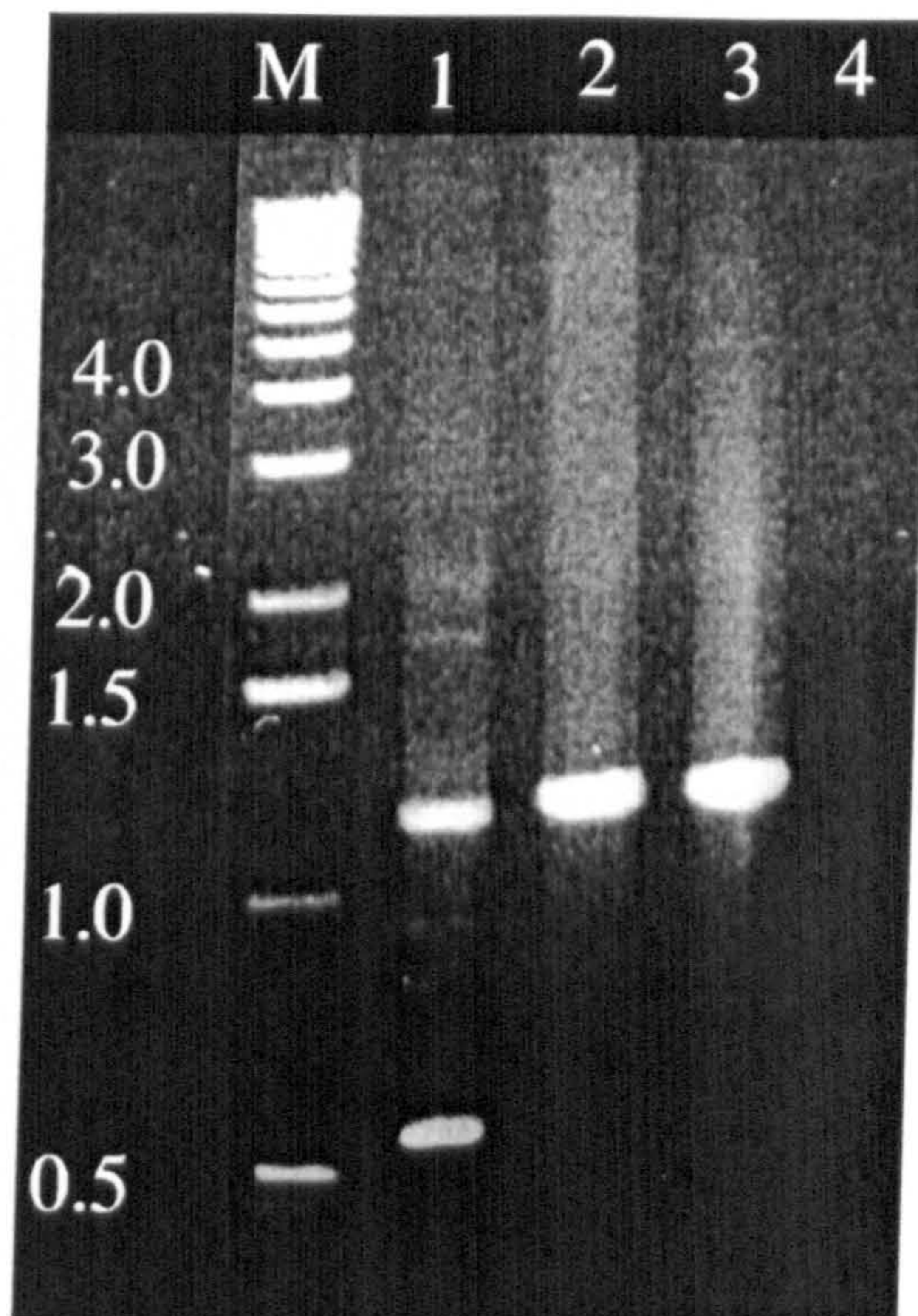
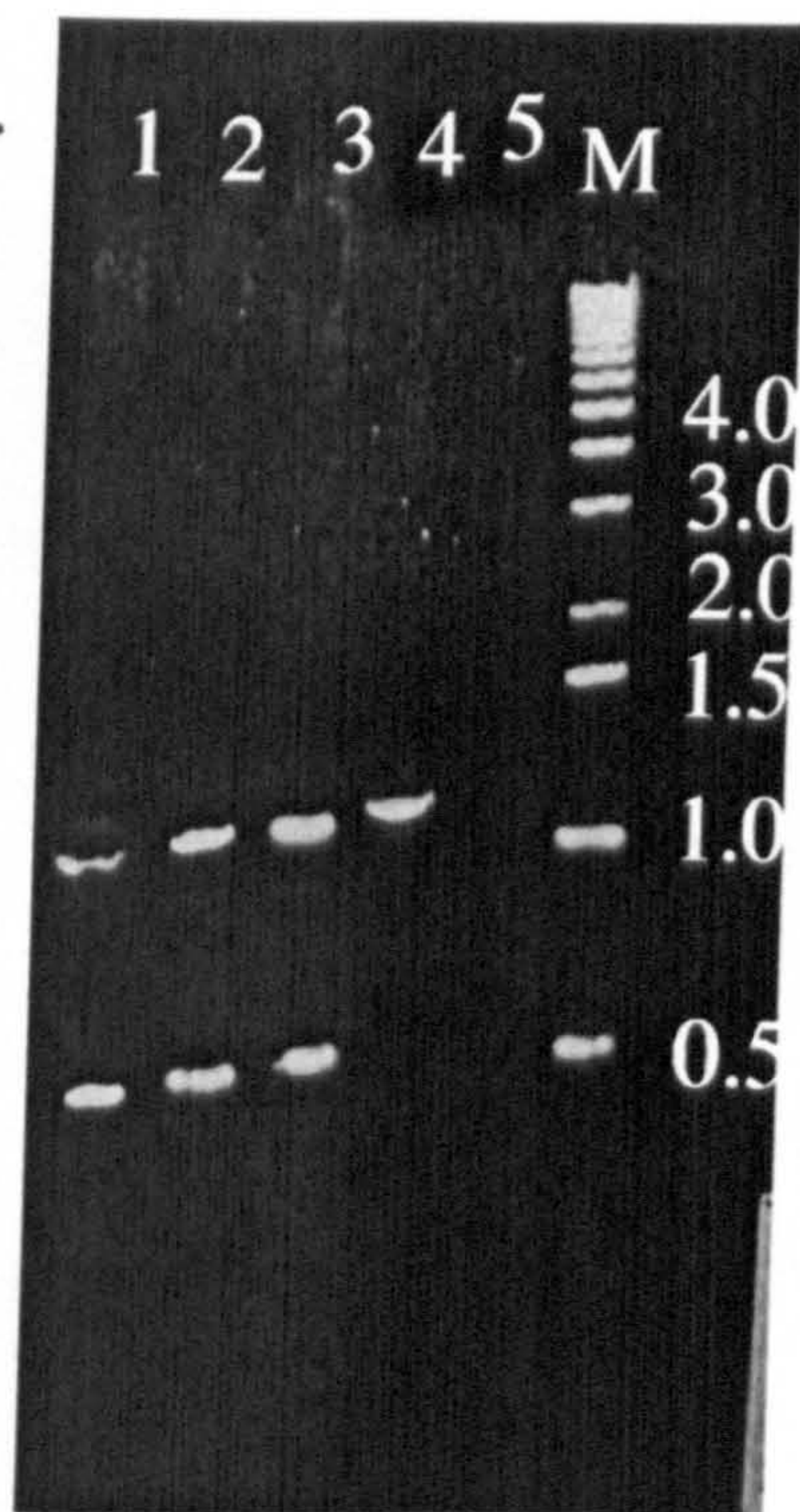


Fig 50: PCR analysis with AroA primers of *P. multocida* Quetta clones transformed with plasmid pJRMT24 (size as kb)

A:

Lanes 1-7: transformed clones

Lane 8: *P. multocida* Quetta (wild-type)

Lane 9: negative control

Lane M: 1 kb DNA marker

B: Transformed clones after 10 subcultures

Lanes 1-3: transformed clones

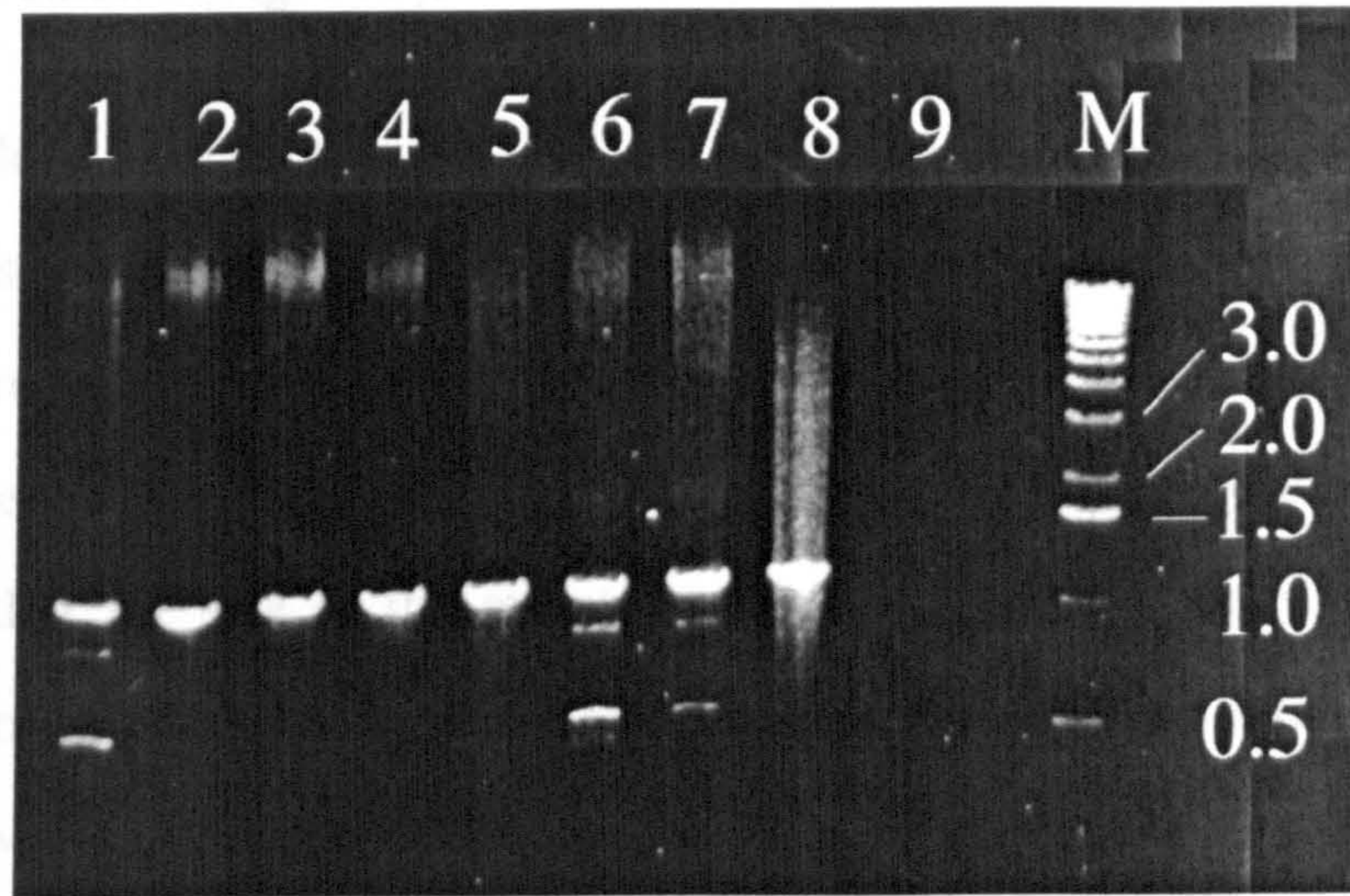
Lane 4: *P. multocida* Quetta (wild-type)

Lane 5: negative control

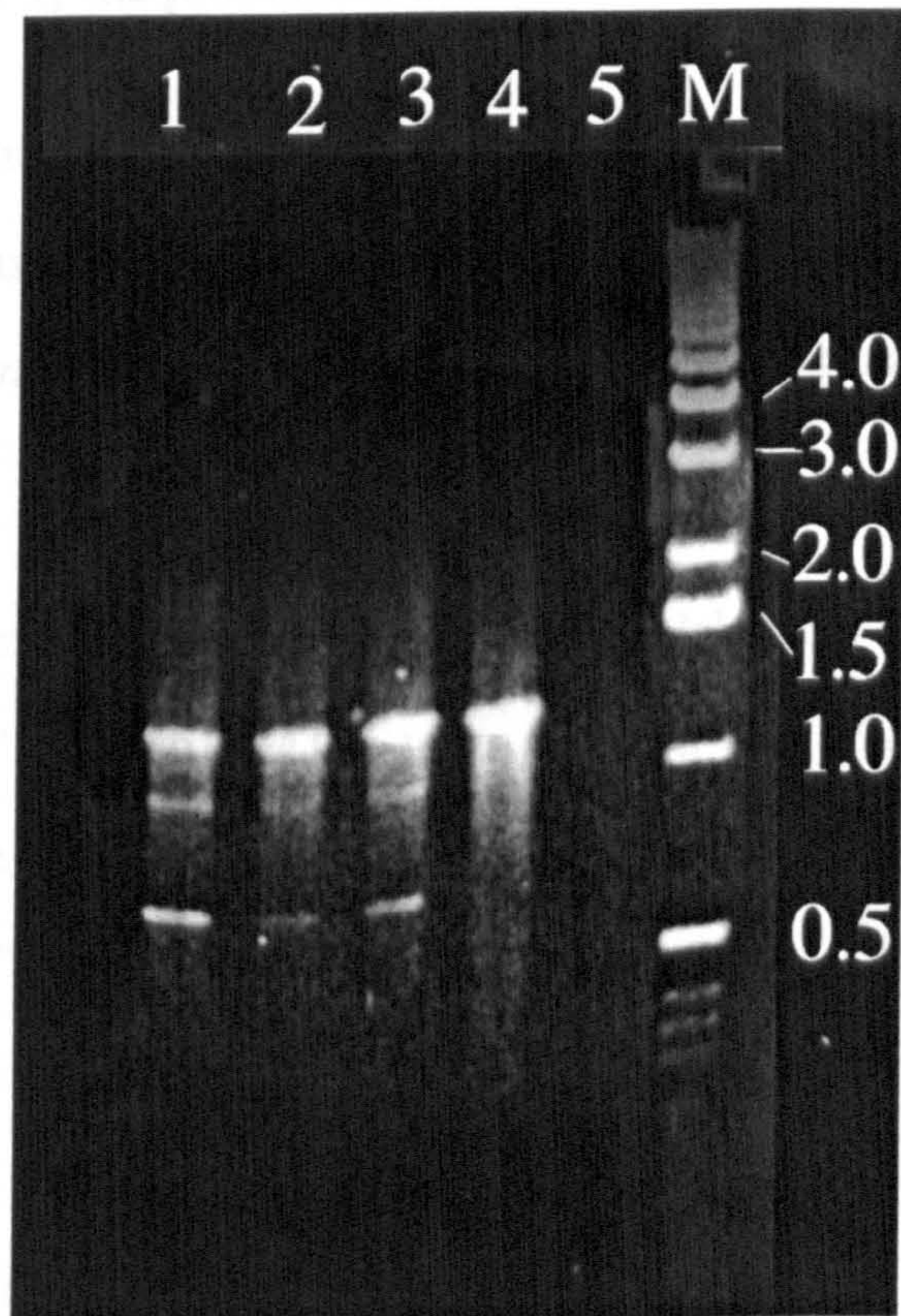
Lane M: 1 kb DNA marker

Fig 50:

A.



B.



fragments of ≈ 2.1 and ≈ 2.75 kb (Fig. 51A). PCR analysis of plasmid DNA with *aroA* primers amplified bands of 1.2 kb for plasmids pJRMT1 and pJRMT2 whereas bands of 1.05 kb were amplified for plasmids pJRMT25 and pJRMT26, as predicted (Fig. 51B).

3. 3. 1. 4. Allelic exchange

The plasmids pJRMT25 and pJRMT26 were transformed into *P. multocida* strains by electroporation and Km^R-Ap^R clones selected. After PCR analysis with *aroA* primers, a few clones showed two bands, one of the wild-type gene size and the other the size of the mutated gene (Fig. 52) and these were subcultured into BHI broth without any antibiotic for induction of a second crossing-over event. After ten subcultures some clones were checked by PCR, but all of them showed the wild-type gene pattern.

The last two experiments for construction of marker-free *aroA* mutants were found to be unsuccessful by the procedures used.

3. 3. 2. Construction of marker-free *galE* mutants

In further experiments to construct marker-free mutants for use in double mutation constructs, it was decided to construct deleted *galE* mutants without any antibiotic marker.

3. 3. 2. 1. Construction of deleted *galE* gene (250 bp deletion)

The plasmids pJRMT15 and pJRMT16 were digested with *NsiI/EcoRV* to remove a 250 bp middle part of the *galE* gene (from nt. 615-865; Fig. 38A), and self ligated as pJRMT41 and pJRMT42 for *P. multocida* 85020 and Quetta *galE* gene respectively (Fig. 54). The recombinants were confirmed with restriction analysis and PCR. PCR analysis with *GalE* primers of plasmids pJRMT15 and pJRMT16 amplified a ≈ 1.0 kb fragment whereas a ≈ 0.75 kb fragment was amplified for plasmids pJRMT41 and pJRMT42 (Fig. 53B left). When digested with *EcoRI*, that cuts at both ends of the *galE* gene inserted into

Fig 51: Analysis of plasmids pJRMT25 and pJRMT26 (size as kb)

A: Restriction analysis

Lanes 1-2: plasmid pJRMT25 digested with *EcoRI*

Lane 3: plasmid pJRMT1 digested with *EcoRI*

Lanes 4-5: plasmid pJRMT26 digested with *EcoRI*

Lane 6: plasmid pJRMT2 digested with *EcoRI*

Lane M: 1 kb DNA marker

Lanes 7-8: plasmid pJRMT25 digested with *ApaI*

Lane 9: plasmid pJRMT1 digested with *ApaI*

Lanes 10-11: plasmid pJRMT25 digested with *ApaI*

Lane 12: plasmid pJRMT2 digested with *ApaI*

Lane M: 1 kb DNA marker

B: PCR analysis with AroA primers

Lane 1: plasmid pJRMT1

Lane 2: plasmid pJRMT25

Lane 3: plasmid pJRMT2

Lane 4: plasmid pJRMT26

Lane M: 1 kb DNA marker

Fig 51:

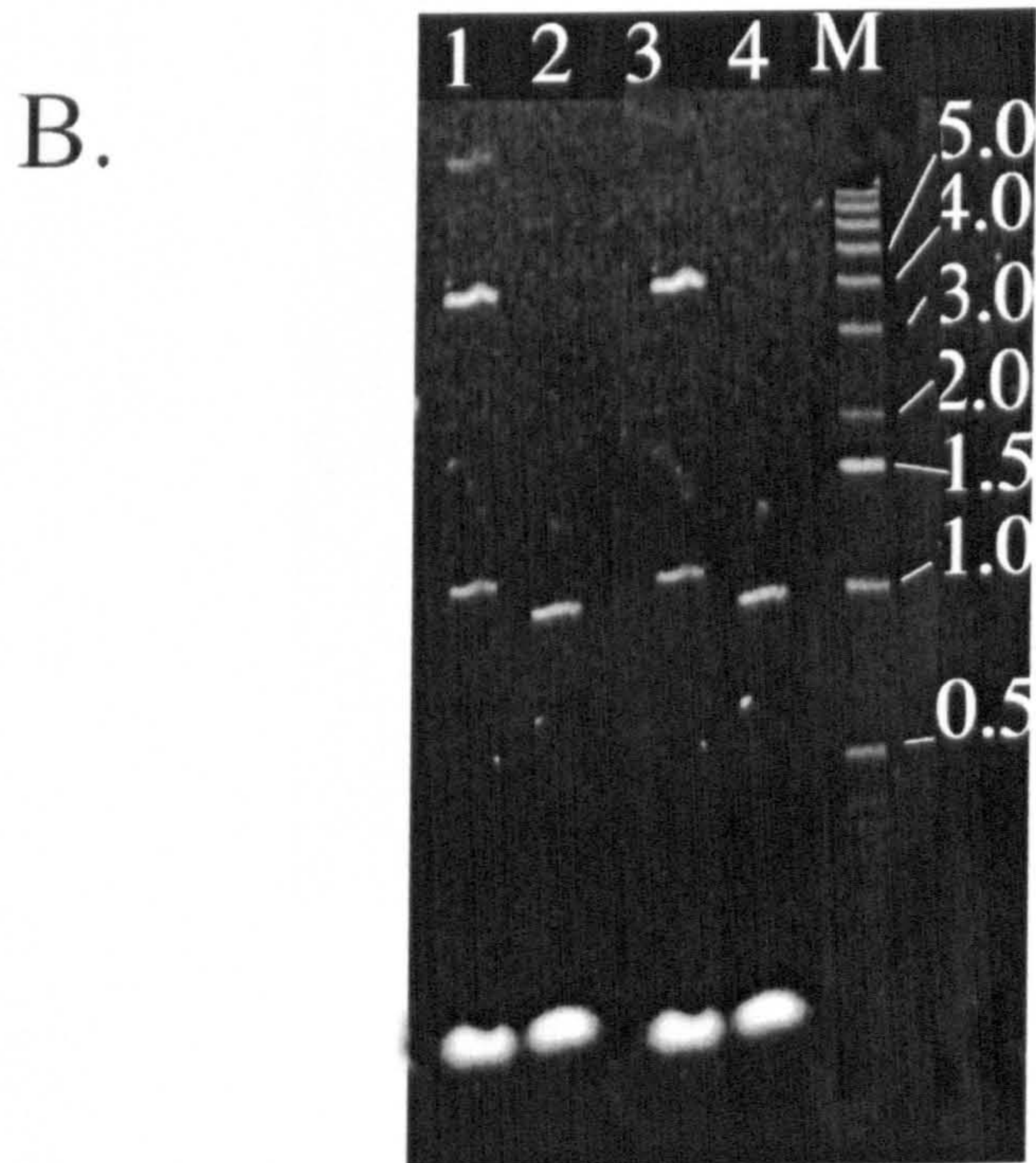
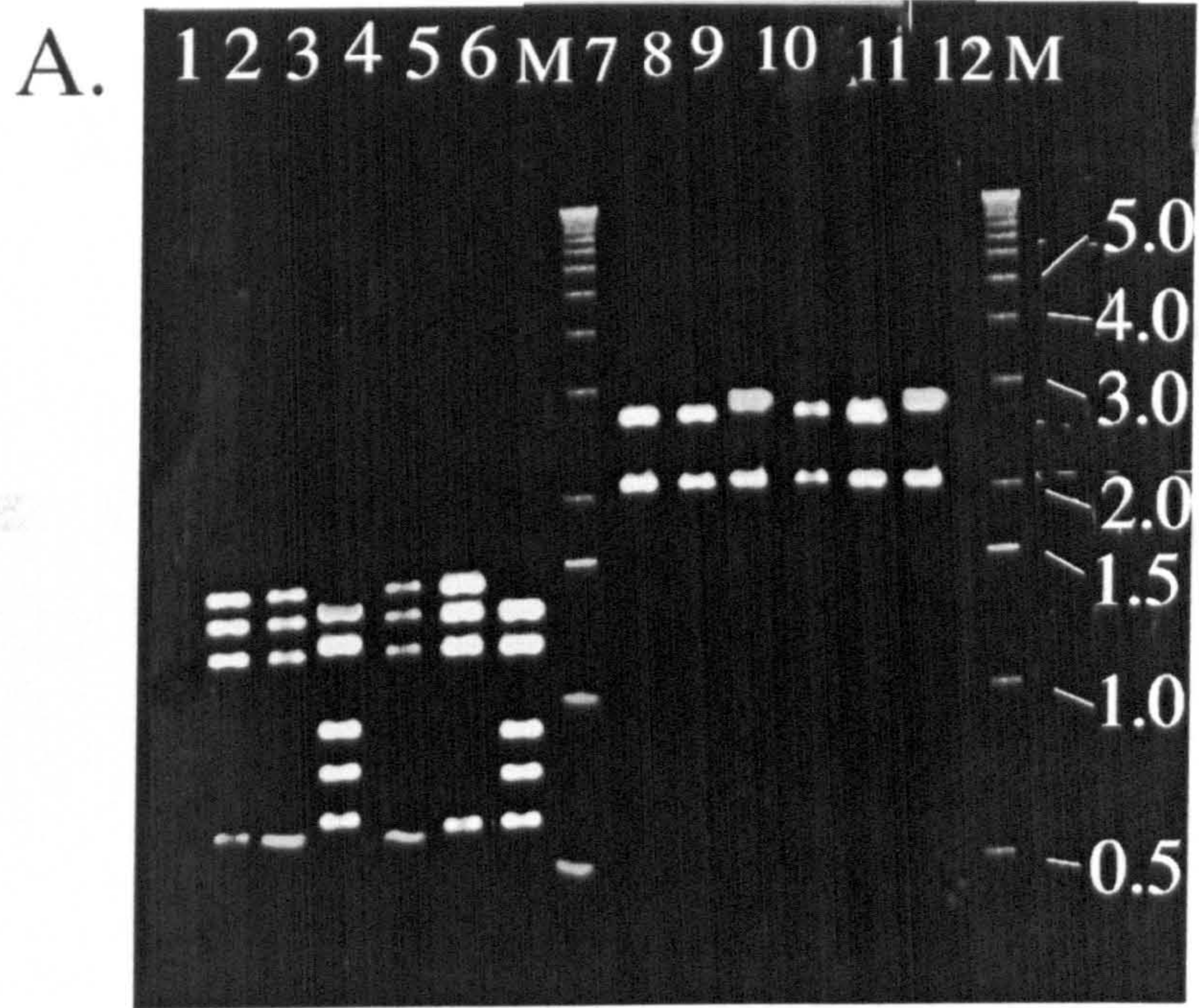


Fig 52: PCR analysis with *aroA* primers of *P. multocida* 85020 clones transformed with plasmid pJRMT25 (size as kb)

A:

Lanes 1-10: transformed clones

Lane 11: *P. multocida* 85020 (wild-type)

Lane 12: negative control

Lane M: 1 kb DNA marker

B:

Lanes 1-8: transformed clones

Lane 9: *P. multocida* 85020 (wild-type)

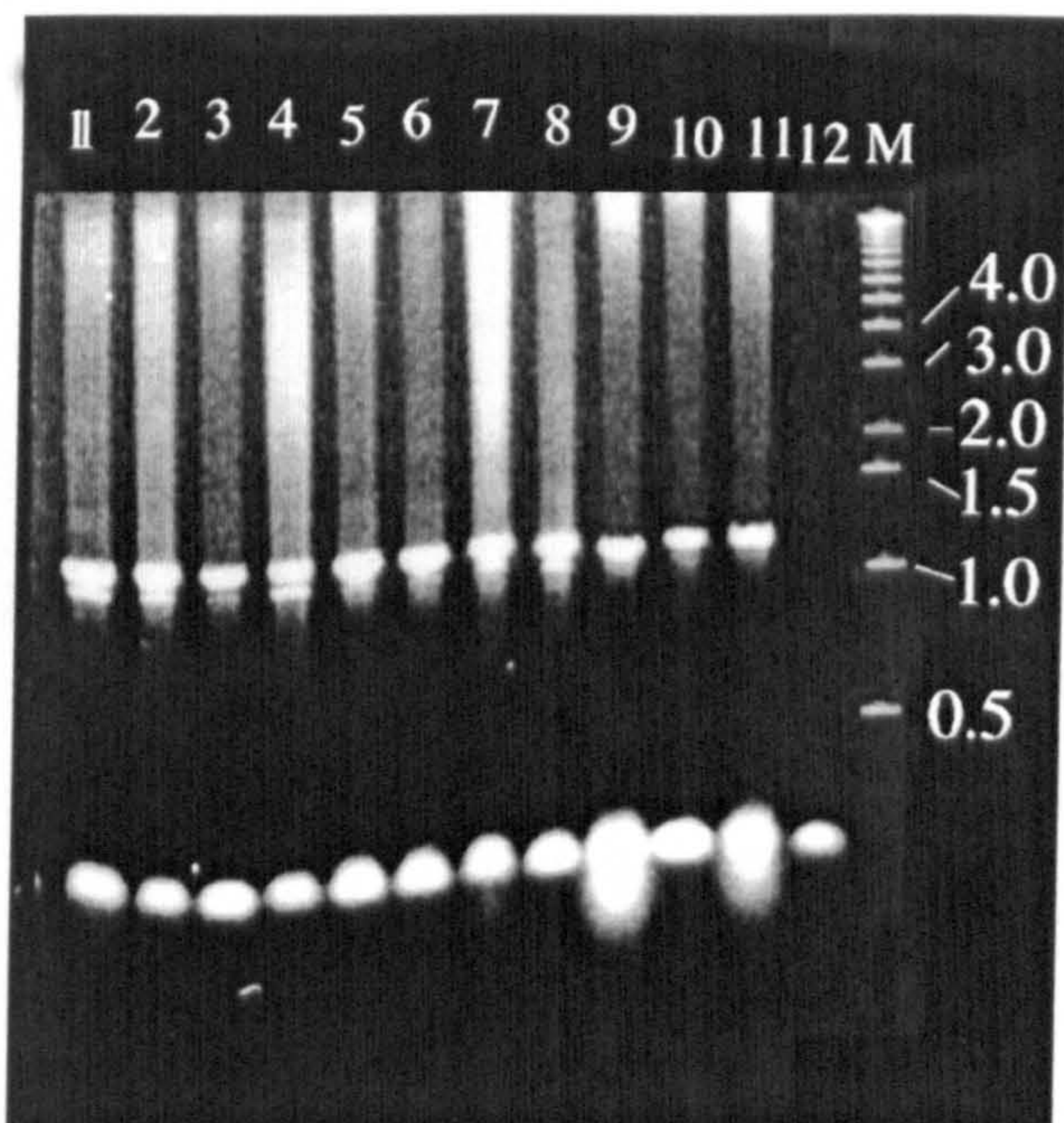
Lane 10: *P. multocida* JRMT1 (positive control)

Lane 11: negative control

Lane M: 1 kb DNA marker

Fig 52:

A.



B.

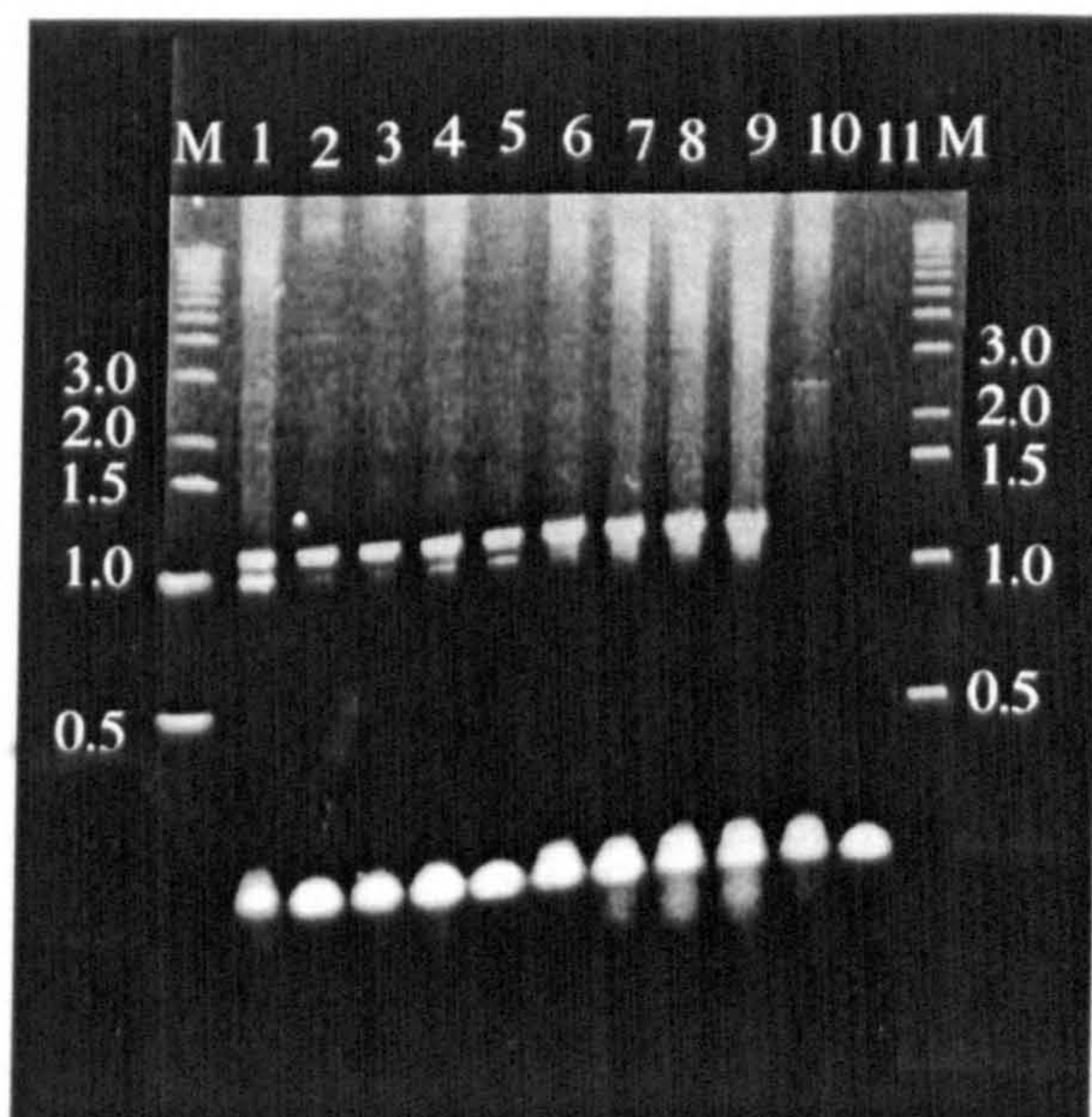


Fig 53: Analysis of plasmids pJRMT41 and pJRMT42 (size as kb)

A: Undigested plasmid DNA

Lanes 1-5: plasmid pJRMT41 (different clones)

Lane 6: plasmid pJRMT15

Lanes 7- 10: plasmid pJRMT42 (different clones)

Lane 11: plasmid pJRMT16

Lane M: supercoiled DNA marker

B:

Left: PCR analysis with *galE* primers

Lane 1: plasmid pJRMT15

Lane 2: plasmid pJRMT41

Lane 3: plasmid pJRMT16

Lane 4: plasmid pJRMT42

Lane M: 1 kb DNA marker

Right:

Lanes 1-2: plasmid pJRMT41 digested with *EcoRI* (different clones)

Lane 3: plasmid pJRMT15 digested with *EcoRI*

Lane 4: plasmid pJRMT42 digested with *EcoRI*

Lane 5: plasmid pJRMT16 digested with *EcoRI*

Lane M: 1 kb DNA marker

Fig 53:

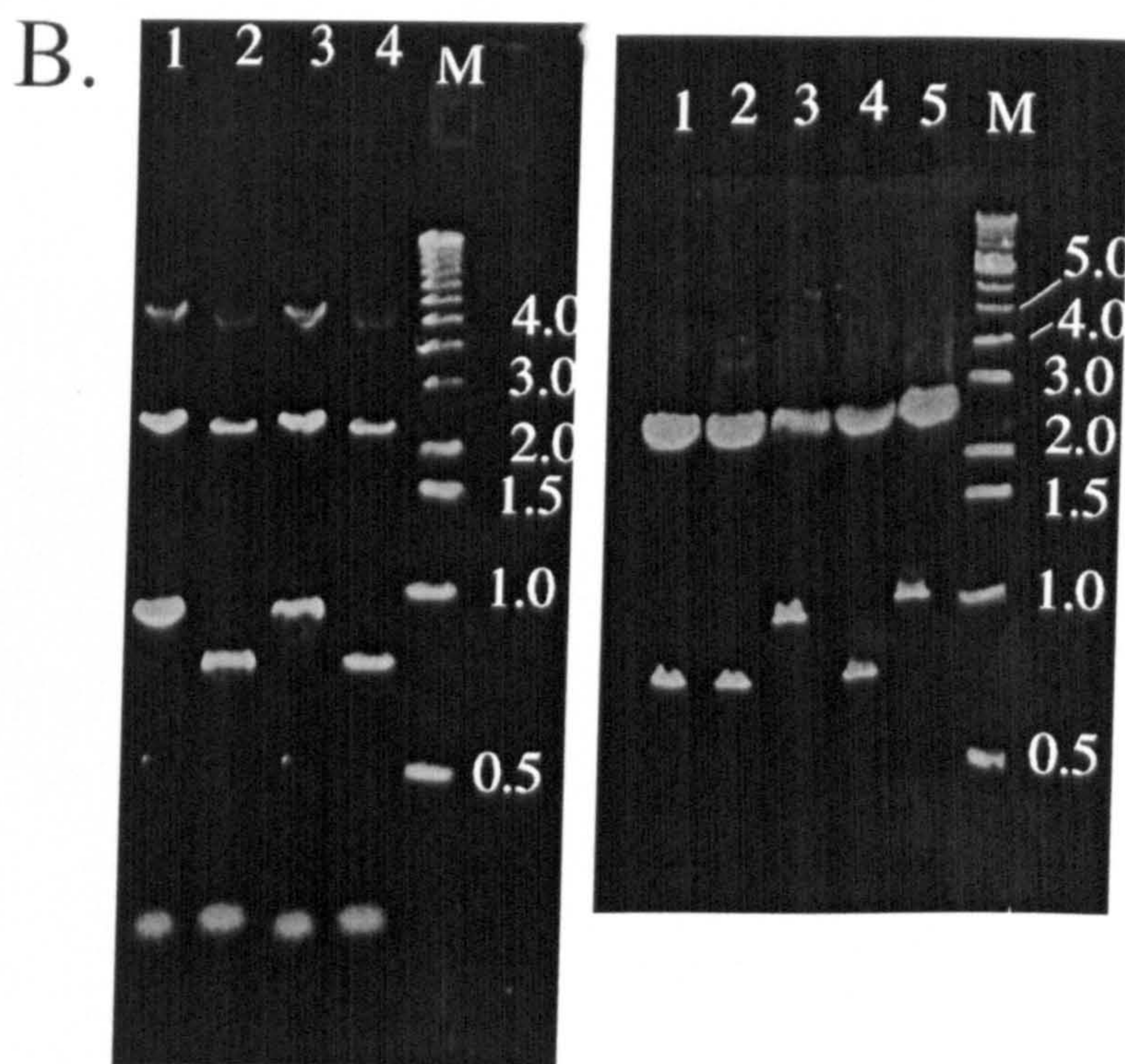
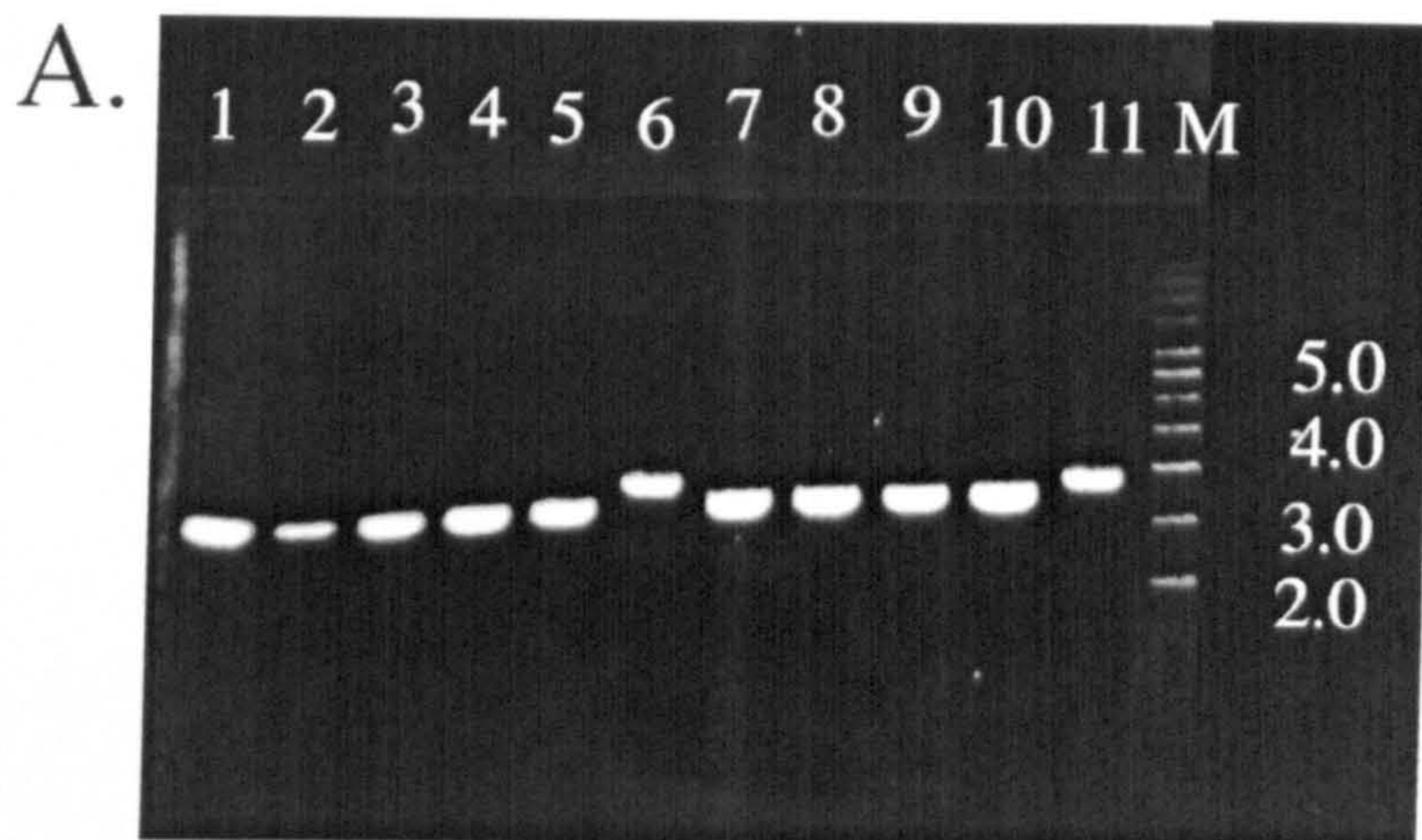
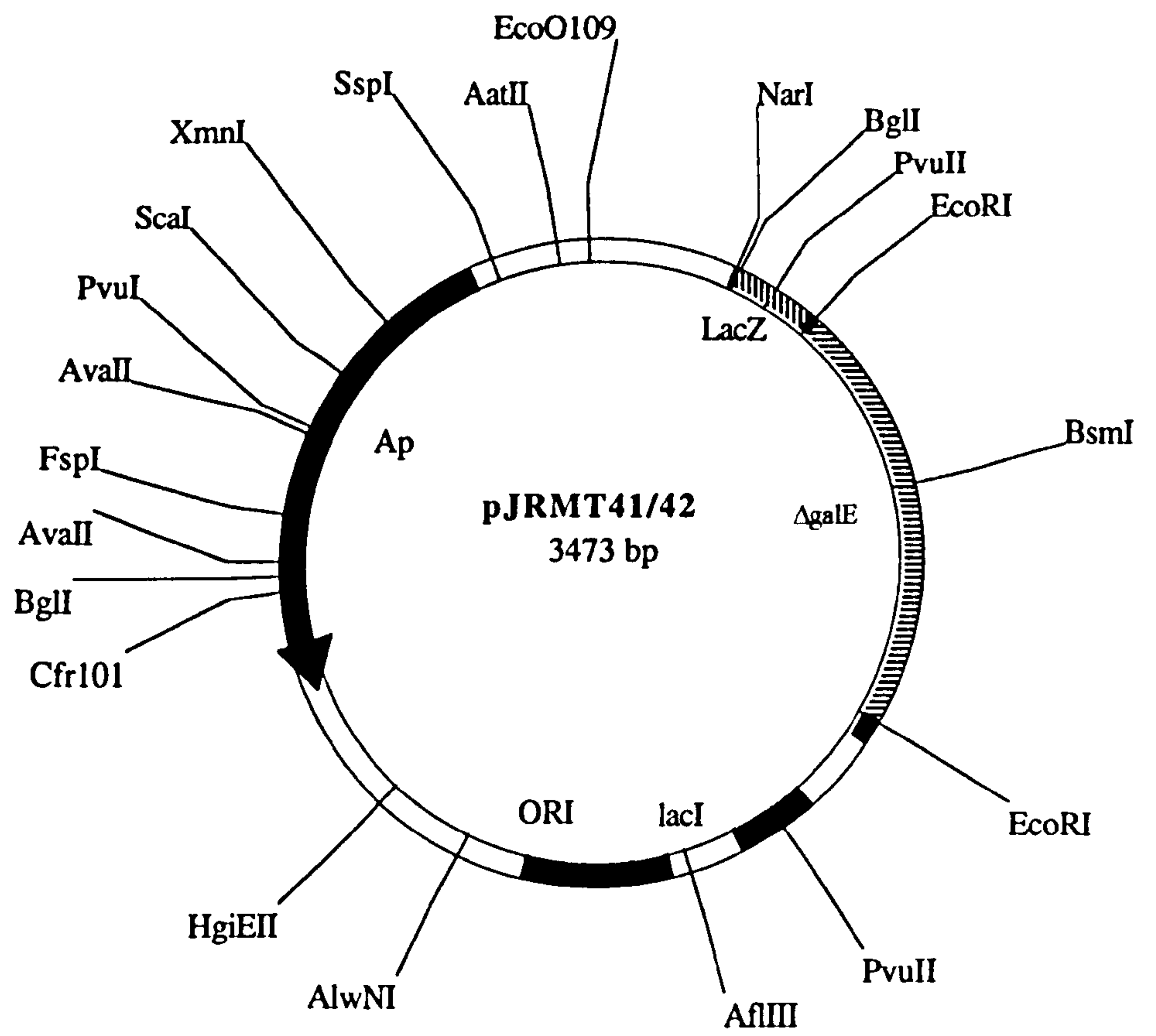


Fig 54: The map of plasmids pJRMT41 and pJRMT42

The plasmids pJRMT15 and pJRMT16 (see Fig. 38) were digested with *NsiI/EcoRV* to remove a 250 bp middle part of the *galE* gene.

Fig 54:



suicide vector pUC19, two fragments of ≈ 1.0 and ≈ 2.7 kb were produced for plasmids pJRMT15 and pJRMT16 but for plasmids pJRMT41 and pJRMT42 two fragments of ≈ 0.75 and ≈ 2.7 kb were produced (Fig. 53B right).

The plasmids pJRMT41 and pJRMT42 were transformed into *P. multocida* 85020 and Quetta strains by electroporation and bacteria plated on to BHI agar supplemented with 5% (v/v) sheep blood containing ampicillin. Ap^R clones were picked and subcultured five times without any antibiotic. Some Ap^S clones were obtained but, by PCR analysis, none of them showed mutated bands.

3. 3. 2. 2. Construction of deleted *galE* gene (98 bp deletion)

Because of unsuccessful results in the last experiment, it was again decided to increase the size of the flanking regions, by deleting a small fragment of the *galE* gene.

The plasmids pJRMT15 and pJRMT16 were digested with *EcoRV* and *BsmI* (at 55 °C for 8 hrs) to remove a 98 bp middle part of *galE* gene (from nt. 865-963) (Fig. 38A) and self-ligated as plasmids pJRMT27 and pJRMT28. The recombinants were confirmed with restriction analysis and PCR. Restriction analysis of plasmids pJRMT15 and pJRMT16 with *EcoRI* produced two fragments of ≈ 1.0 and ≈ 2.7 kb while for plasmids pJRMT27 and pJRMT28 two fragments of ≈ 0.9 and ≈ 2.7 kb were produced (Fig. 55A). Also, PCR analysis of plasmid DNA with the *GalE* primers amplified fragments at predicted size, ≈ 1.0 and ≈ 0.9 kb for plasmids pJRMT15, pJRMT16 and plasmids pJRMT27 and pJRMT28 respectively (Fig. 55B).

The plasmids pJRMT27 and pJRMT28 were transformed into *P. multocida* 85020 and Quetta strains by electroporation and plated out on to BHI agar supplemented with 5% (v/v) sheep blood containing ampicillin. Ap^R clones were picked and checked by PCR but none of them showed mutated bands.

Fig 55: Analysis of plasmids pJRMT27 and pJRMT28 (size as kb)

A: Restriction analysis digestion with *EcoRI*

Lane 1: plasmid pJRMT27

Lane 2: plasmid pJRMT15

Lanes 3-4: plasmid pJRMT28 (different clones)

Lane 5: plasmid pJRMT16

Lane M: 1 kb DNA marker

B: PCR analysis with Gale primers

Lane 1: plasmid pJRMT27

Lane 2: plasmid pJRMT15

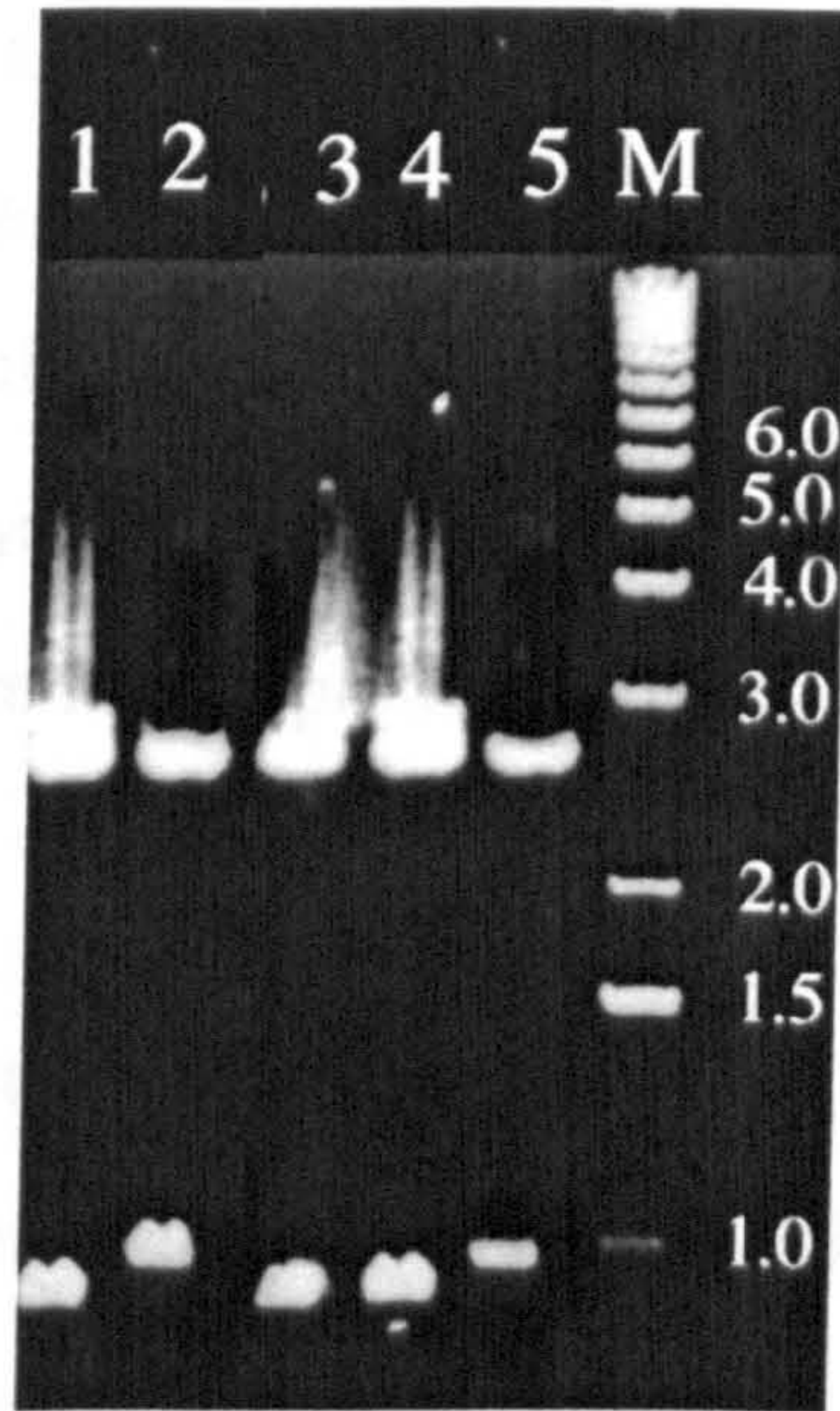
Lane 3: plasmid pJRMT28

Lane 4: plasmid pJRMT16

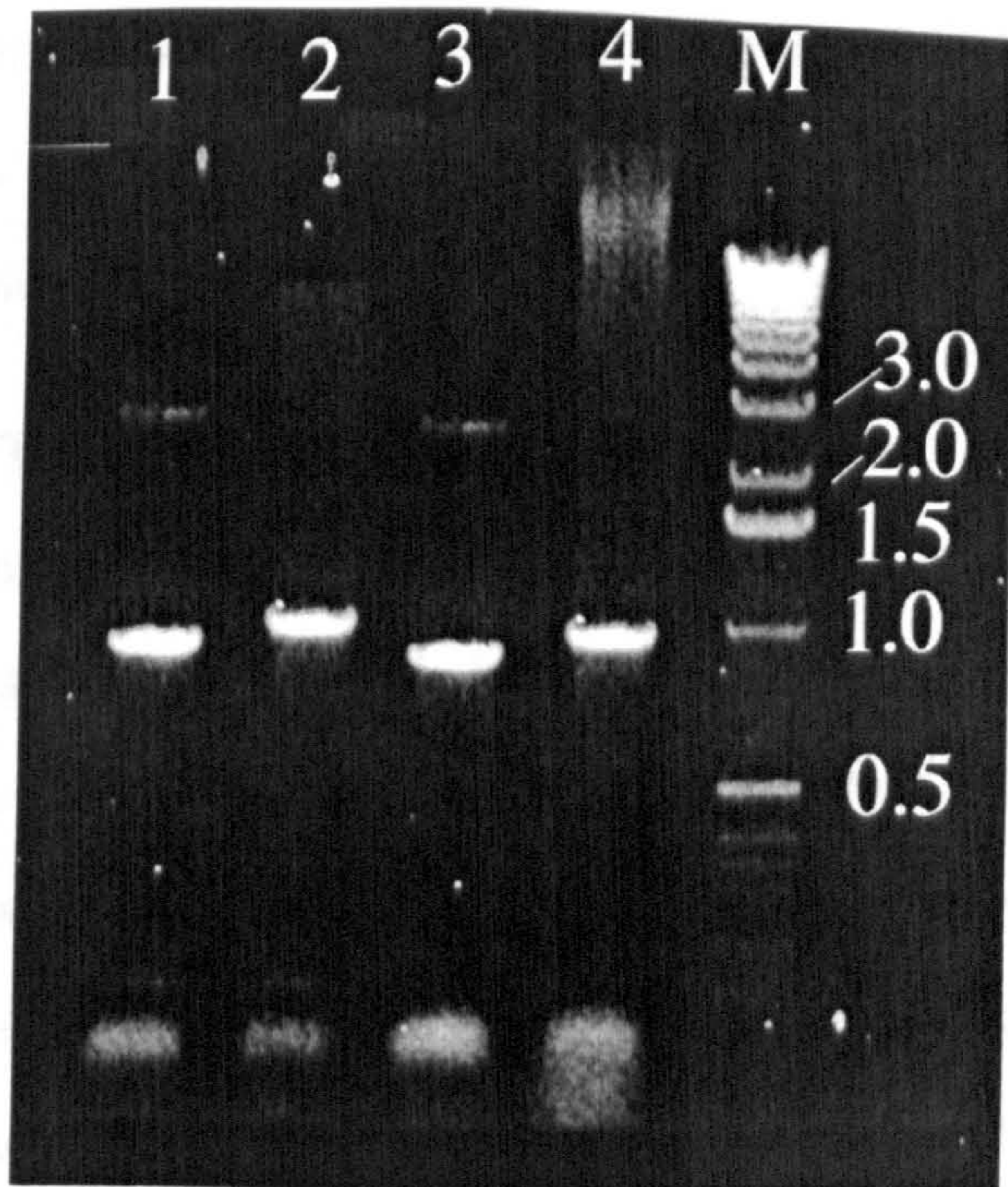
Lane M: 1 kb DNA marker

Fig 55:

A.



B.



Also when checked for the presence of plasmid, most of the clones showed that plasmids pJRMT27 and pJRMT28 could replicate in *P. multocida* strains. In a final attempt, the plasmid pAKA19 was tried as a suicide vector. To obtain suitable restriction sites, the deleted *galE* genes were amplified as a ≈0.9 kb fragment by PCR with the GalE primers, using plasmid DNA pJRMT27 and pJRMT28 as templates. The purified fragments from agarose gel were cloned into the pCR®2.1-TOPO cloning vector and transformed into *E. coli* DH5α competent cells. Recombinant plasmids were confirmed by restriction analysis (results not shown) and were named plasmids pJRMT35 and pJRMT36 for *P. multocida* 85020 and Quetta respectively (Fig. 56A).

3. 3. 2. 3. Cloning of deleted *galE* fragment into the vector pAKA19

The *galE* fragments were removed from plasmid pJRMT35 and pJRMT36 by *EcoRI* digestion and cloned into *EcoRI*-digested and dephosphorylated pAKA19 and transformed into *E. coli* DH5α competent cells. Recombinant plasmids were confirmed by restriction analysis (results not shown) and designated as plasmids pJRMT37 and pJRMT38 for *P. multocida* 85020 and Quetta strains respectively (Fig. 56B).

The plasmids pJRMT37 and pJRMT38 were transformed into *P. multocida* 85020 and Quetta strains by both electroporation and conjugation and bacteria plated onto BHI agar supplemented with 5% (v/v) sheep blood containing appropriate antibiotics. Ap^R clones (for transformants) or Ap^R-Sm^R clones (for transconjugants) were analysed by PCR. Some of them showed some differentiation from the wild-type, but, for these transformed cells, the PCR product with GalE primers was bigger than the wild-type size (Fig. 57A). This was not to be expected from the deleted *galE* gene transformed into these cells. When the PCR products were digested with *Apa*LI restriction enzyme (the *Apa*LI site of *galE* gene was removed by digestion with *EcoRV*/*Bsm*I) the wild-type showed two bands around 0.5 kb and some of the transformed

Fig 56:

A: Map of plasmids pJRMT35 and pJRMT36

The deleted *galE* gene (98 bp removed) was amplified by PCR and cloned into plasmid pCR[®]2.1-TOPO vector.

B: The map of plasmids pJRMT37 and pJRMT38

The deleted *galE* gene was cut from plasmids pJRMT35 and pJRMT36 with *EcoRI* and cloned into plasmid pAKA19 which had been digested with *EcoRI* and dephosphorylated.

Fig 56:

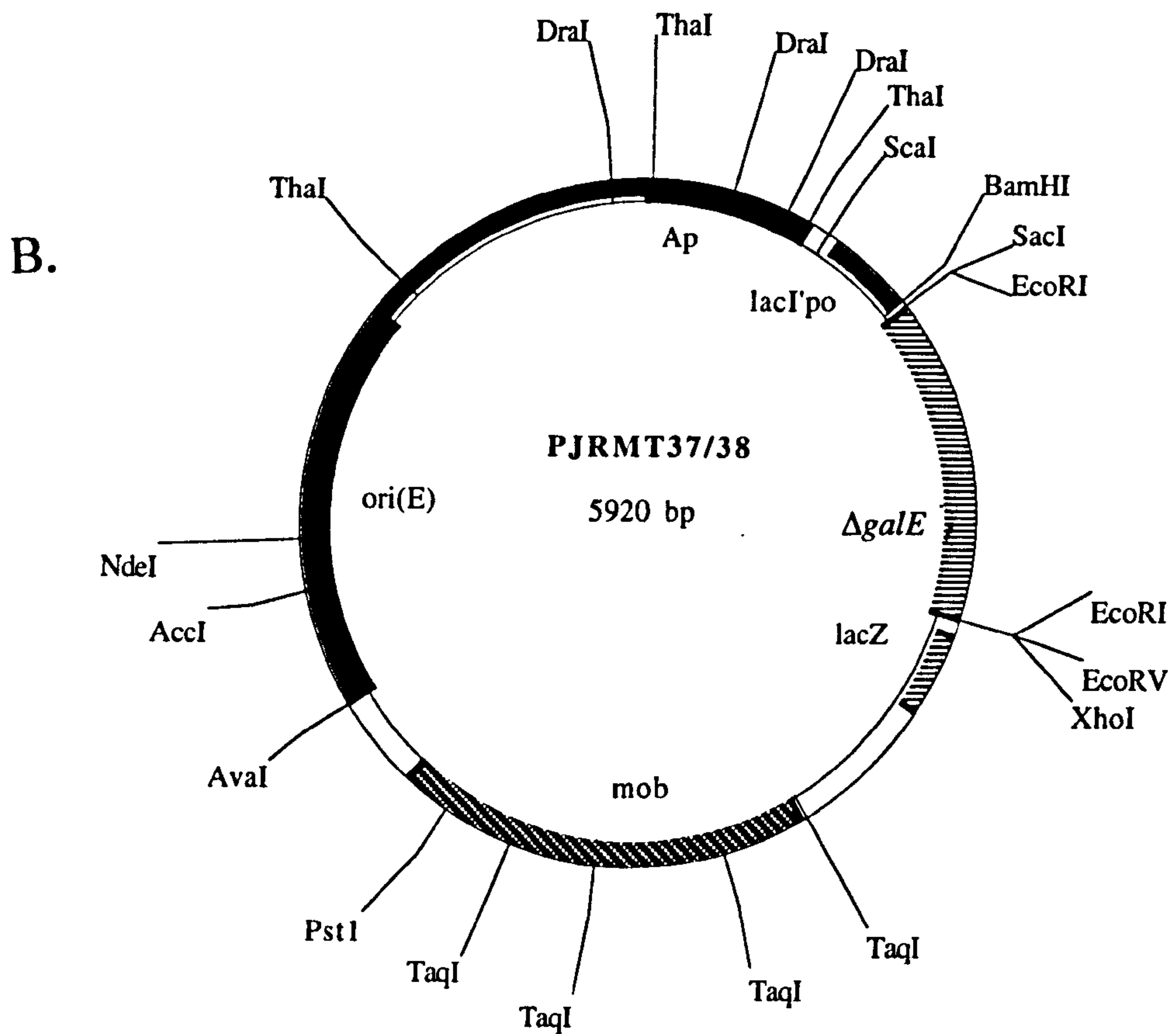
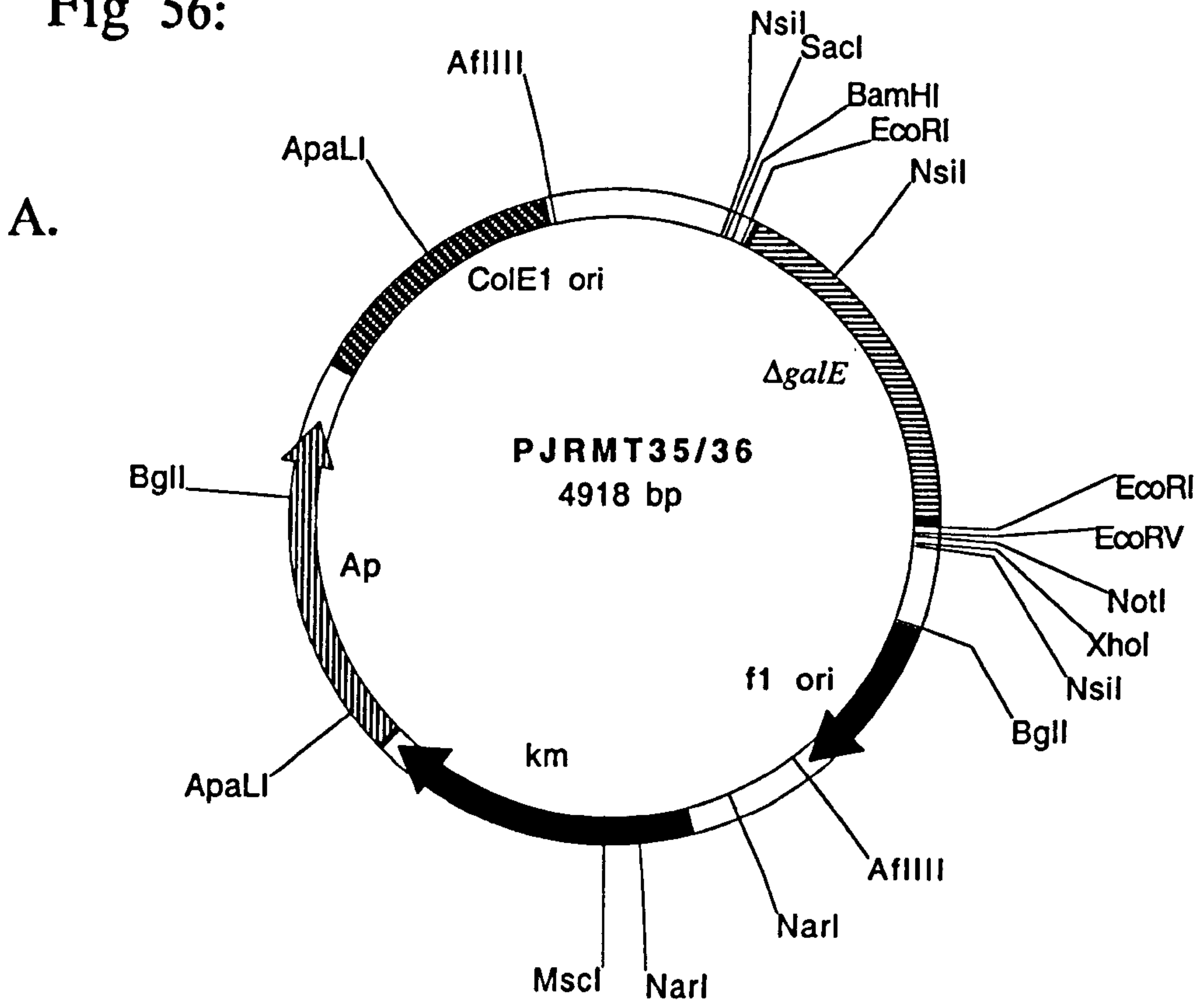


Fig 57: PCR analysis of *P. multocida* strains transformed with plasmids pJRMT37 and pJRMT38 (size as kb)

A: PCR product with GalE primers

Lanes 1-5: *P. multocida* 85020 clones transformed with plasmid pJRMT37

Lanes 6-10: *P. multocida* Quetta clones transformed with plasmid pJRMT38

Lane 11: *P. multocida* 85020 (wild-type)

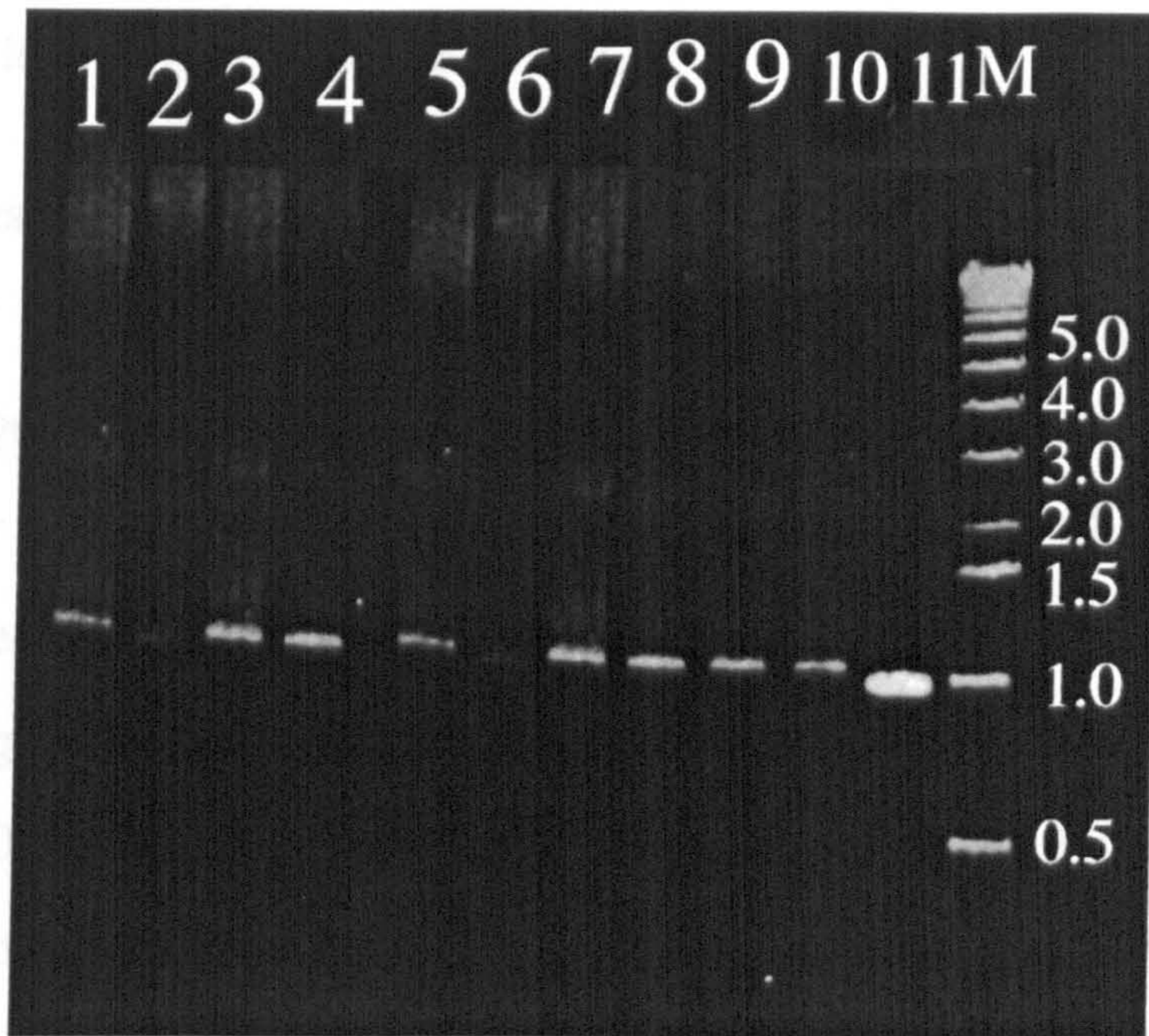
Lane M: 1 kb DNA marker

B: The above PCR products after digestion with *Apa*LI.

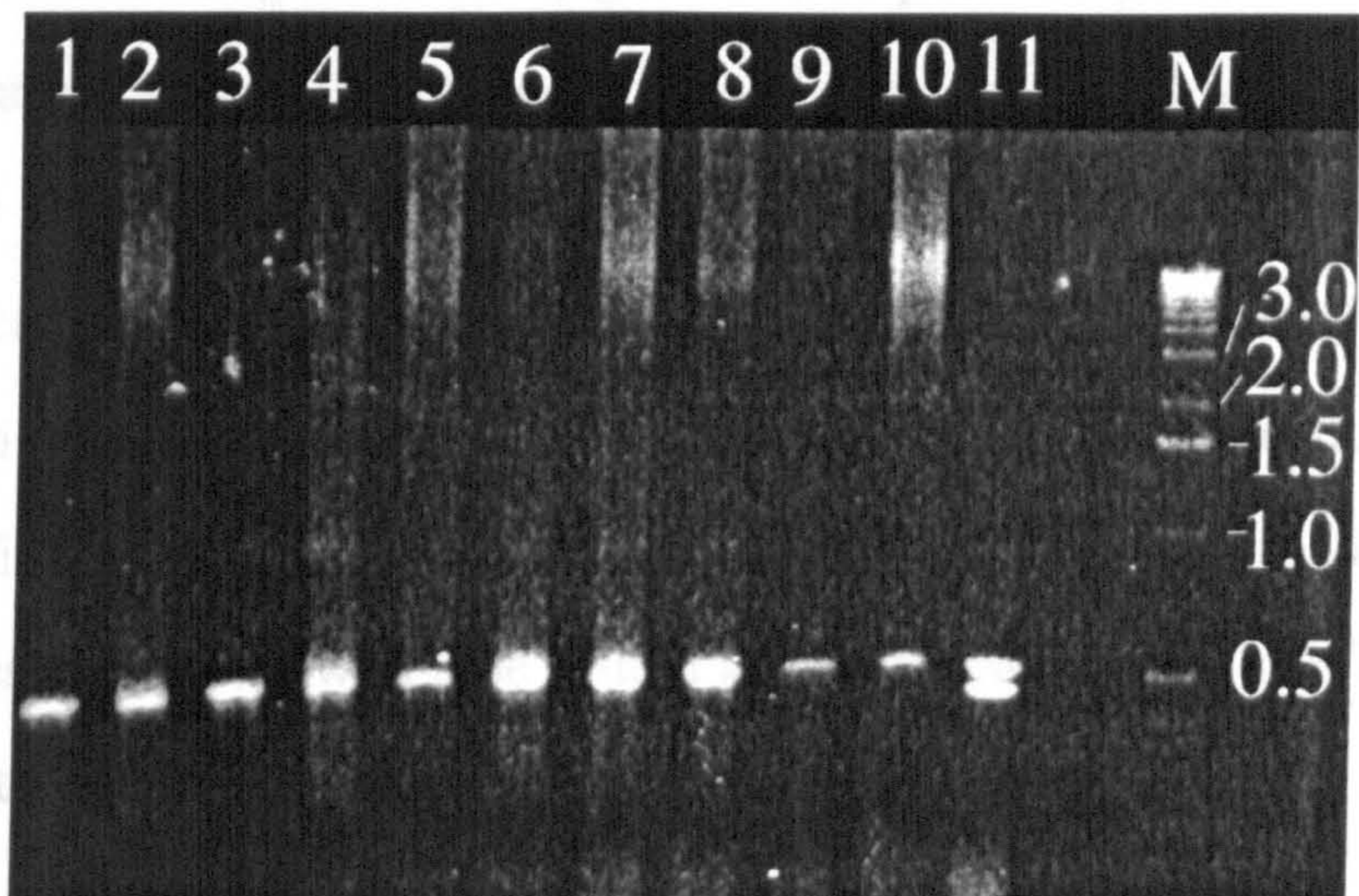
C: The above PCR products after digestion with *Apa*LI, but run for a longer time.

Fig 57:

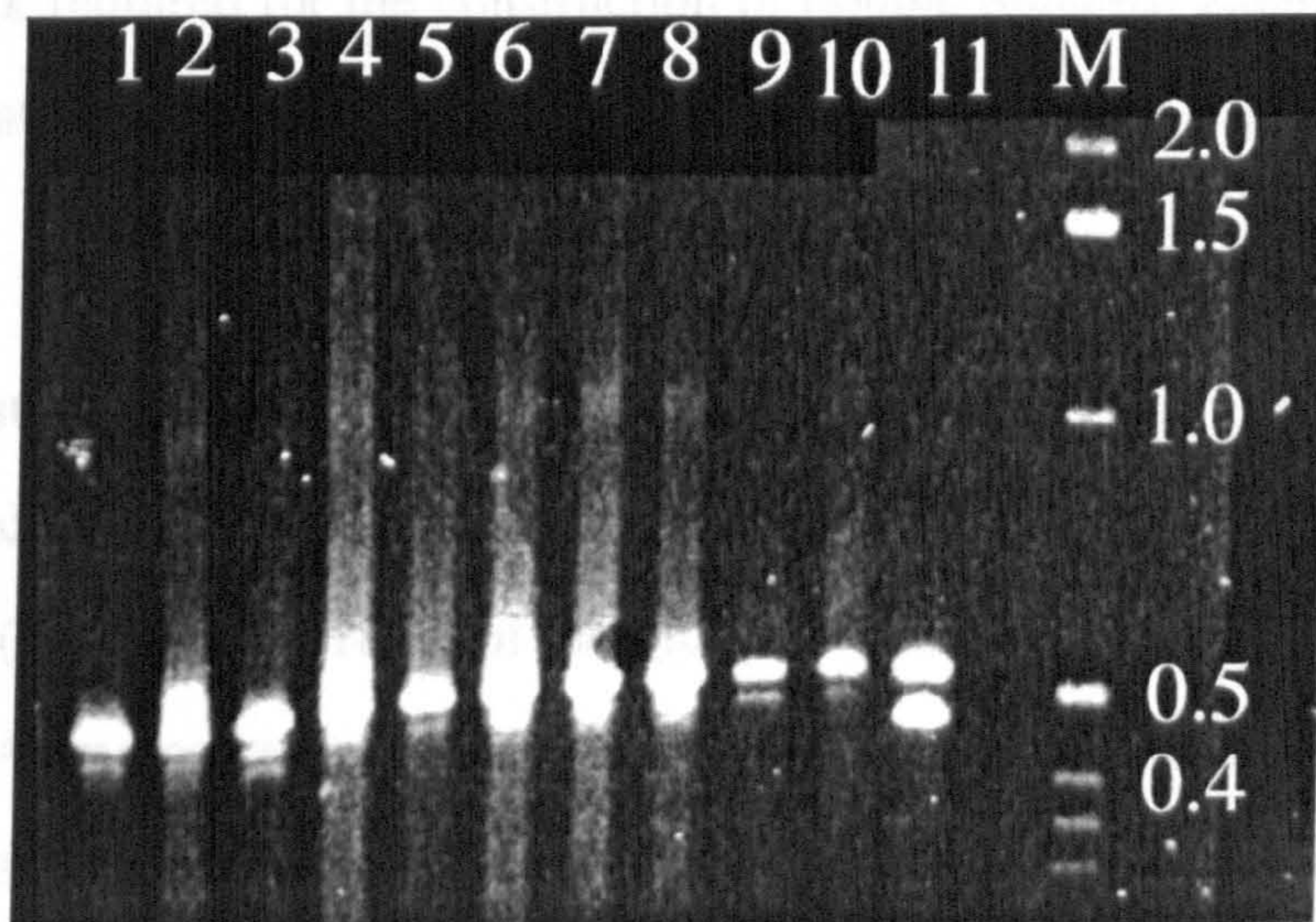
A.



B.



C.



clones showed one band but others showed two bands but with different sizes compared to the wild-type gene size (Fig. 57 B, C).

Compared to *Pasteurella* minimal medium (PMM), the wild-type bacteria grew better in MT minimal medium (Appendix 5. 15). When *P. multocida* strains 85020 and Quetta were inoculated into PMM and MT minimal medium, after incubation for 24 hrs, the OD_{540nm} in PMM was 0.9 and 1.01 whereas in MT minimal medium the values were 1.3 and 1.8 respectively. Also, the wild-type bacteria grew better on MT minimal agar containing glucose (0.2% w/v) and sucrose (0.2% w/v) than PMM agar. When the transformed clones were inoculated into MT minimal medium with glucose and sucrose as carbon source, all were grown in similar values as wild-type parent strain. But, in MT minimal medium containing 0.4% (w/v) galactose in place of glucose and sucrose as sole carbon source, they did not grow, whereas wild-type did grow (Fig. 58). These results therefore indicated that the allelic exchange with pAKA19 was successful in creating putative *galE* deletion derivatives of the *P. multocida* strain 85020. Similar results were obtained for strain Quetta. However, after five subcultures, when checked by PCR analysis with *GalE* primers, all clones showed the wild-type pattern and were able to grow in MT medium just like the wild-type strains.

3. 3. 3. Construction of marker-free *cya* mutants

In further work required for the construction of double mutants, attempts were made to create a deleted *cya* derivative by removing the middle fragment of *cya* gene.

3. 3. 3. 1. Construction of deleted *cya* mutants

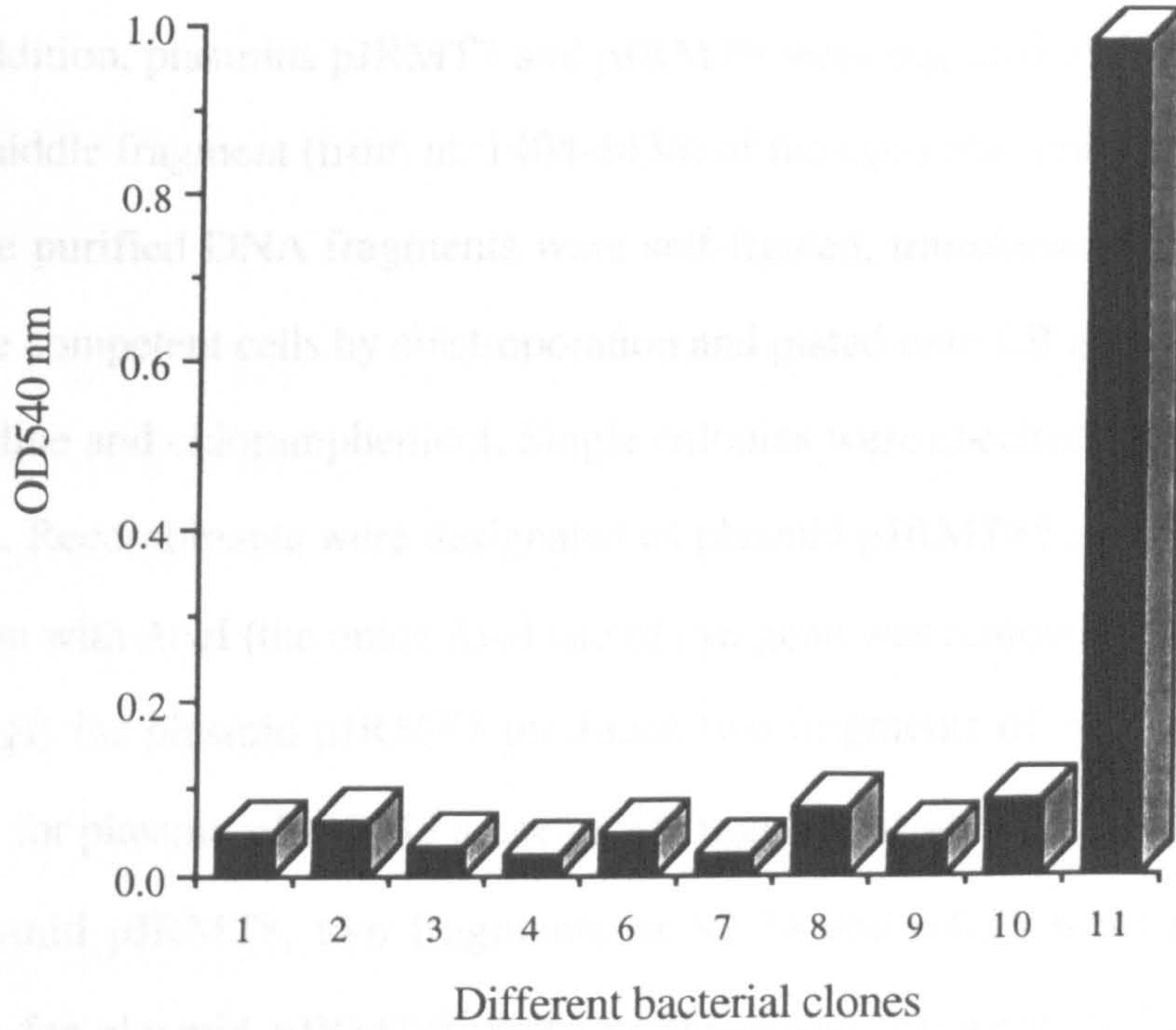
The plasmids pJRMT7 and pJRMT8 were digested with *HpaI* and a 1168 bp middle fragment (from nt. 690-1857) of the *cya* gene removed (see Fig. 25). The purified DNA fragments were self ligated and transformed into *E. coli* XL1Bue competent cells by electroporation and plated onto LB agar containing

Fig 58: Growth of *P. multocida* clones transformed with plasmid pJRMT37 in MT minimal medium containing 0.4% (w/v) galactose as sole carbon source.

Lanes 1-10: different clones (*galE* mutant) of strain 85020

Lane 11: *P. multocida* 85020 (wild-type)

Fig 58:



5.2.3.2. Allelic exchange

The plasmids pRRM143, pRRM144, pRRM145 and pRRM146 were transformed into the *P. aeruginosa* strains by electroporation and plated onto LB agar supplemented with 5% (v/v) sheep blood, gentamicin (20 µg/ml) and chloramphenicol. After incubation for 48 hrs at 37 °C no colonies were found for plasmids pRRM144 and pRRM146 but a few colonies were observed for pRRM143 and pRRM145 derived from the *P. aeruginosa* C600 strain. Some of these were inoculated into BHJ media without antibiotic selection for five times. The new set of strains harbored both plasmids pRRM143 and pRRM145

tetracycline and chloramphenicol. Single colonies were checked by restriction analysis and designated as plasmid pJRMT43 and pJRMT44. Restriction analysis of plasmid pJRMT7 with *EcoRI/SacI* produced three fragments of ≈ 0.6 , ≈ 1.9 and ≈ 3.5 kb but for plasmid pJRMT43 it produced two fragments of ≈ 1.36 and ≈ 3.5 kb. For plasmid pJRMT8 three fragments of ≈ 0.6 , ≈ 1.6 and ≈ 3.5 kb were produced whereas for plasmid pJRMT44 two fragments of ≈ 1.06 and ≈ 3.5 kb were obtained, as predicted (results not shown).

In addition, plasmids pJRMT7 and pJRMT8 were digested with *BcgI* and a 34 bp middle fragment (from nt. 1404-1438) of the *cya* gene removed (see Fig. 25). The purified DNA fragments were self-ligated, transformed into *E. coli* XL1Bue competent cells by electroporation and plated onto LB agar containing tetracycline and chloramphenicol. Single colonies were checked by restriction analysis. Recombinants were designated as plasmid pJRMT45 and pJRMT46. Digestion with *AvaI* (the unique *AvaI* site of *cya* gene was removed by digestion with *BcgI*) for plasmid pJRMT7 produced two fragments of ≈ 1.2 and ≈ 4.66 kb whereas for plasmid pJRMT45 a linearised fragment of ≈ 5.9 kb was produced. For plasmid pJRMT8, two fragments of ≈ 1.24 and ≈ 4.36 kb were produced whereas for plasmid pJRMT46 just one linearised fragment of 5.6 kb was produced (results not shown).

3. 3. 3. 2. Allelic exchange

The plasmids pJRMT43, pJRMT44, pJRMT45 and pJRMT46 were transformed into the *P. multocida* strains by electroporation and plated onto BHI agar supplemented with 5% (v/v) sheep blood containing 5 $\mu\text{g}/\text{ml}$ chloramphenicol. After incubation for 48 hrs at 37°C no colonies were found for plasmids pJRMT44 and pJRMT46, but a few colonies grew for plasmids pJRMT43 and pJRMT45 derived from the *P. multocida* 85020 sequence. Some of these were inoculated into BHI broth without any antibiotic and subcultured five times. The new clones transformed with plasmid pJRMT43 were checked

by PCR with primers Cya4 and Cya5 which amplified two or three bands representing wild type and possible smaller, mutated fragments (Fig. 59A). But, after further subculture for induction of second cross over events in BHI broth without any antibiotic, the transformants lost the mutated bands. The genomic DNA of the clones transformed with plasmid pJRMT45 were digested with *AvaI* and checked by Southern blot hybridisation. The *cya* probe hybridised to two bands for wild-type and three bands for the mutants strains (Fig. 59). It was expected that because of removing the unique *AvaI* site, the *cya* probe should hybridise to one bigger band in mutant strains, but this was not found.

3. 4. Use of a *sacB-sacR* cartridge for constructing directed, unmarked mutations in Gram-negative bacteria by marker exchange-eviction mutagenesis

Previous data indicated that, during the allelic exchange experiments, a first crossing-over to integrate the plasmid DNA into the chromosome apparently occurred but subculture to encourage loss of the plasmid via a second crossing-over event did not occur readily. For this reason a means was sought to select against the plasmid once it had been incorporated into the chromosome via the first recombination event so as to actively encourage loss of the plasmid and promote the second recombination event.

3. 4. 1. Expression of *sacB* gene in *E. coli* DH5 α

The plasmid pEG18.3 (Fig. 60) conferring sensitivity to sucrose, kindly provided by Dr. Peggy Cotter, Department of Microbiology, Immunology and Molecular Genetics, UCLA School of Medicine, University of California, [unpublished data, modified version of plasmid pEG25, Tejada *et al.* (1996)] was transformed into *E. coli* DH5 α by electroporation and plated onto LB agar containing ampicillin and kanamycin. After incubation for 24 hrs at 37°C, 10 single Km^R-Ap^R clones were checked by restriction analysis. Two of the *E.*

Fig 59: Analysis of *P. multocida* clones transformed with plasmids pJRMT43 and pJRMT45 (size as kb)

A: PCR analysis with Cya4 and Cya5 primers of *P. multocida* 85020 transformed with plasmid pJRMT43

Lanes 1-3: transformed clones

Lane 4: *P. multocida* 85020 (wild-type)

Lane 5: negative control

Lane M: 1 kb DNA marker

B: Southern blot hybridisation of *P. multocida* 85020 clones transformed with plasmid pJRMT45

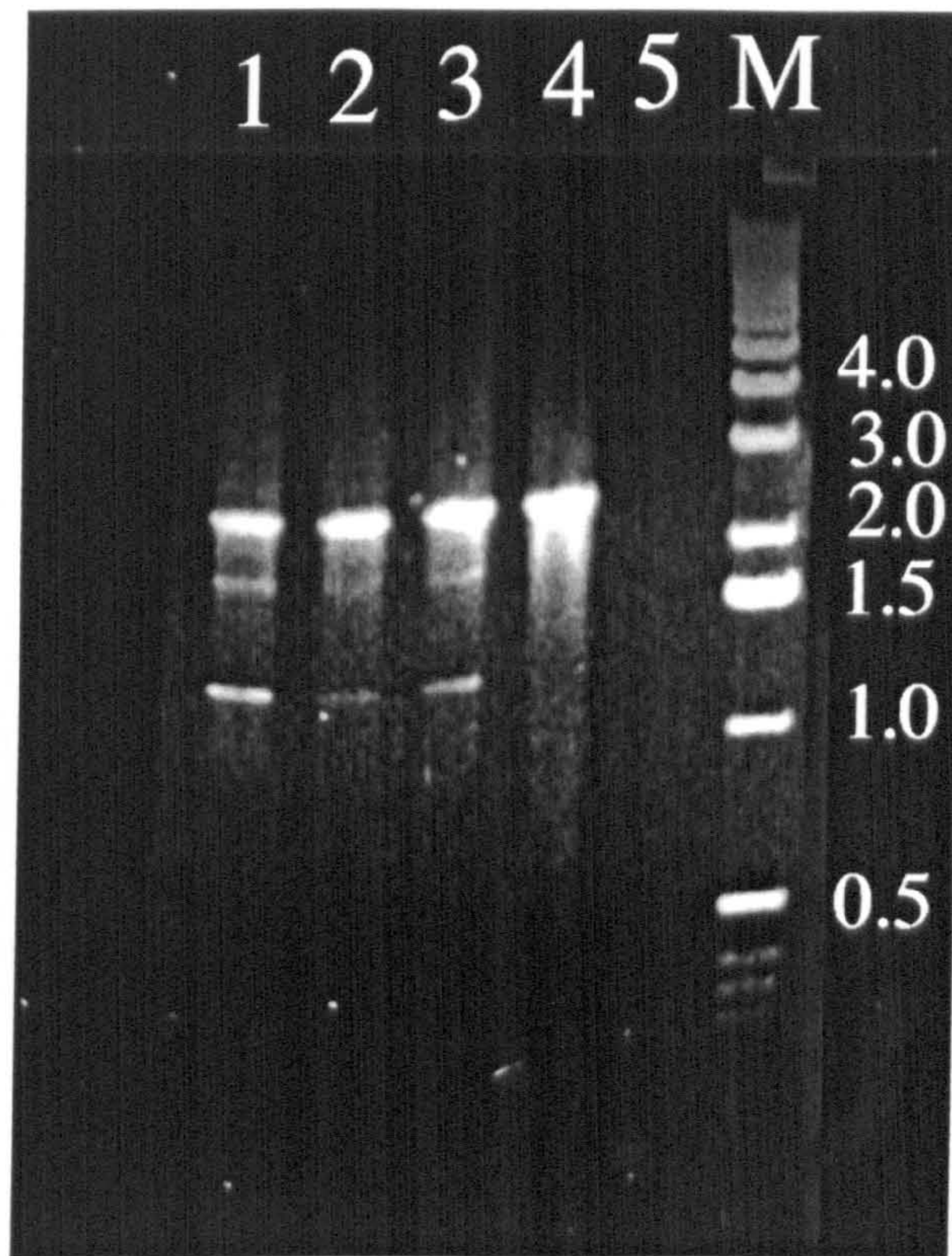
Lanes 1-2: transformed clones

Lane 3: *P. multocida* 85020 (wild-type)

Lane M: 1 kb DNA marker

Fig 59:

A.



B.

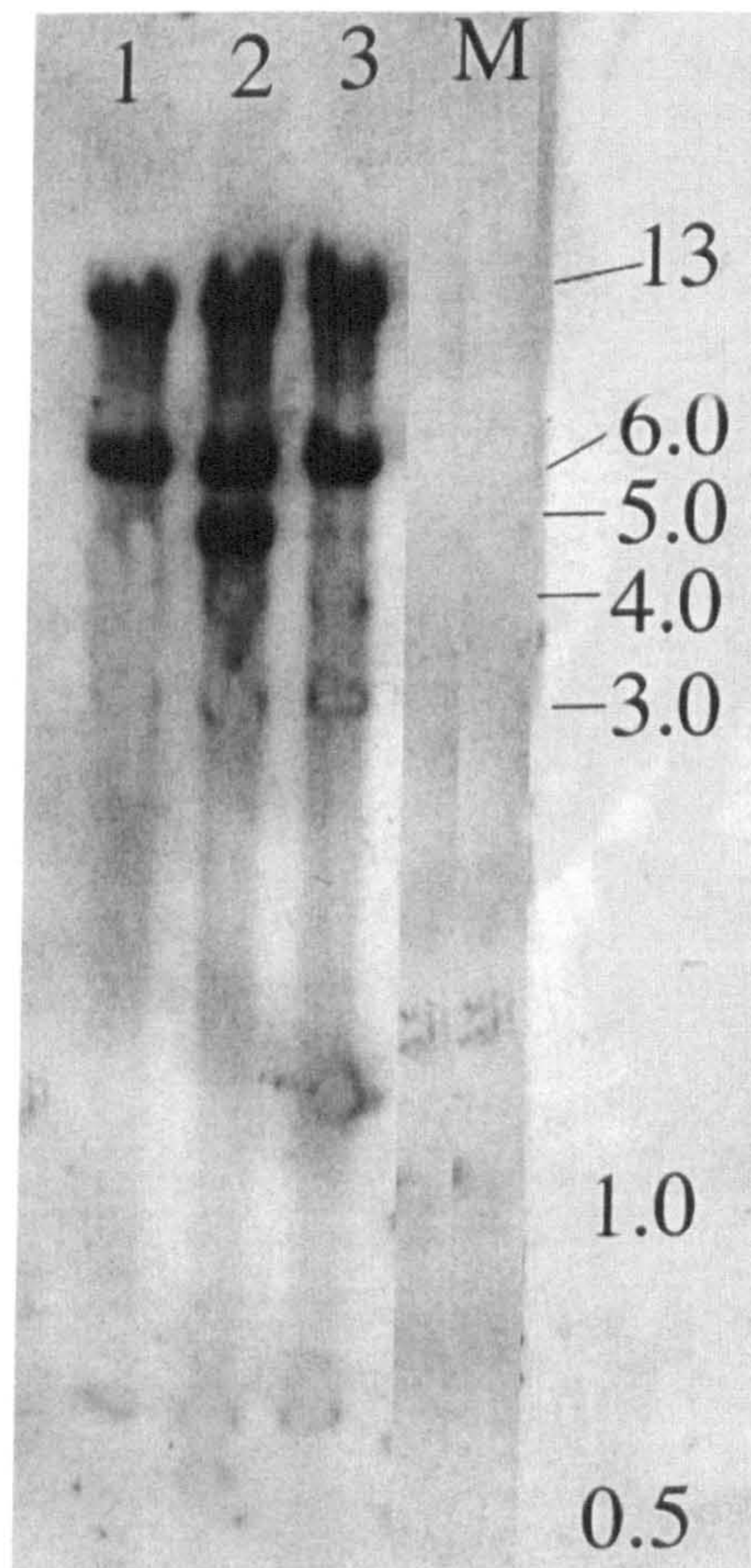
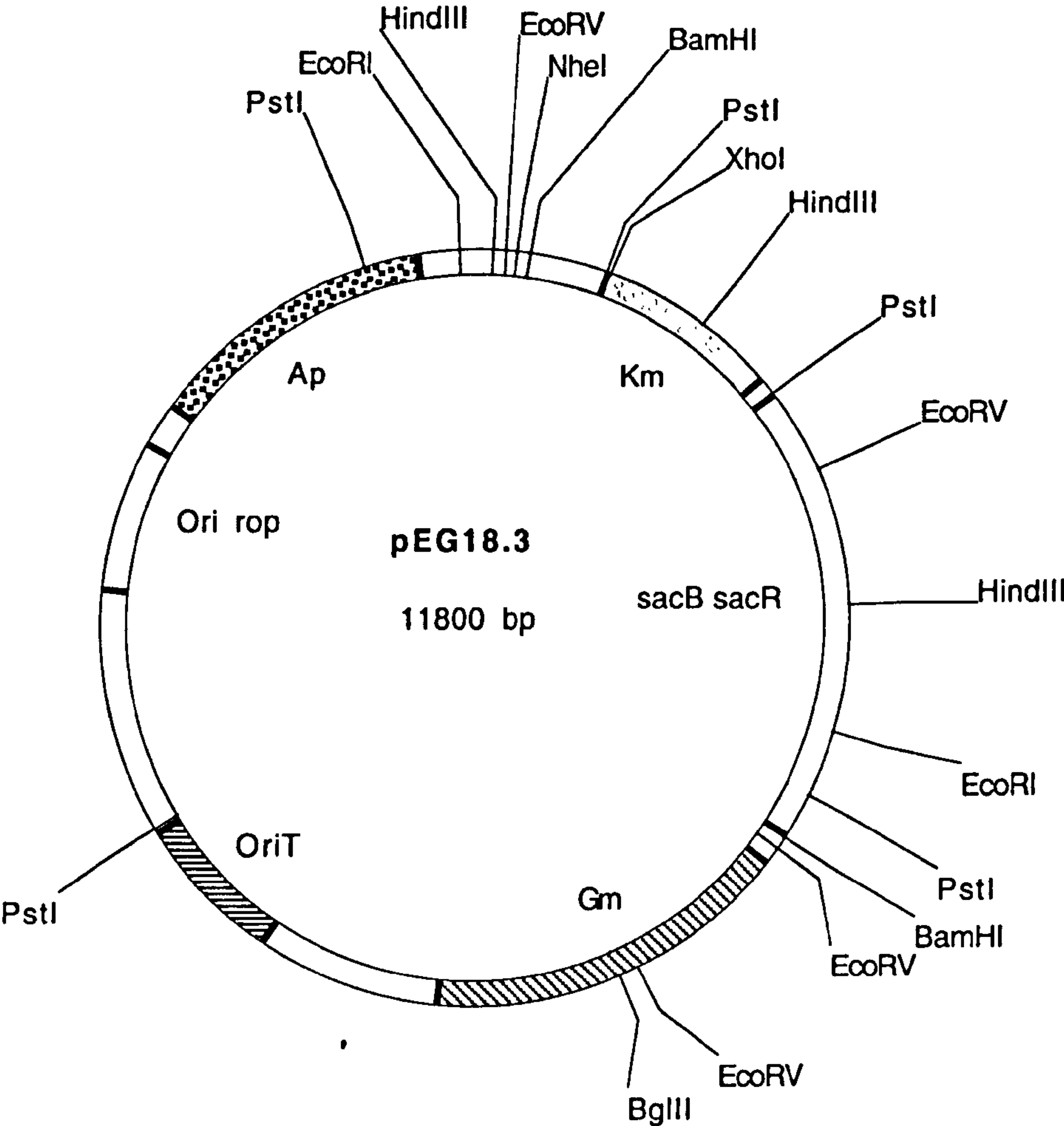


Fig 60: The map of plasmid pEG18.3

This plasmid was used as a source of the *sacB-sacR* genes from *Bacillus subtilis* and contains three antibiotic resistance markers (Ap, Gm and Km).

Fig 60:



coli clones containing plasmid pEG18.3 were inoculated into 5 ml-volumes of LB broth containing appropriate antibiotics and incubated at 37°C overnight with shaking. 10 ml pre-warmed LB broth was inoculated with 0.5 ml of the overnight culture and incubated at 37°C. When the OD_{540nm} reached ≈1.0 (10⁹ C.F.U./ ml), 10× serial dilutions in PBS were prepared and 100 µl aliquots spread onto LB agar containing appropriate antibiotics with and without 5% (w/v) sucrose and incubated at 37°C. After 48 hrs, no colonies grew on LB agar containing 5% (w/v) sucrose, but on control plates (LB agar+ antibiotics without sucrose) the predicted numbers of colonies grew.

3. 4. 2. Expression of *sacB* gene in *P. multocida* strains

3. 4. 2. 1. Cloning of the *sacB* gene into the pAKA16 vector

In marker exchange- eviction mutagenesis, both parts of the *nptI-sacB-sacR* cartridge contributed to the construction on an unmarked, chromosomal mutation through a two-step process. Km^R is used in the first step to select for the exchange of the marked gene for the wild-type allele, as in typical marker-exchange mutagenesis. Sucrose sensitivity is used in the second step to select for the eviction of the marked gene by exchange for an unmarked derivative. The success of the second step is determined initially by growth in the presence of 5% (w/v) sucrose and subsequently confirmed by testing for Km sensitivity.

The plasmid pEG18.3 was digested with *Bam*HI and a ≈3.8 kb fragment containing the Km^R-*sacB-sacR* cartridge removed and cloned into *Bam*HI-digested and dephosphorylated pAKA16. *E. coli* DH5α competent cells were transformed with the ligation mixture and plated onto LB-X-Gal-IPTG agar containing ampicillin and also onto LB agar containing ampicillin and kanamycin. A selection of white colonies from the LB-X-Gal-IPTG agar and also Ap^R-Km^R clones from the LB plates were checked by restriction analysis, and for lack of growth on LB agar containing 5% (w/v) sucrose and the plasmid in one such clone was designated plasmid pJRMT29 (Fig. 61A). Also

Fig 61:

A: Map of plamid pJRMT29

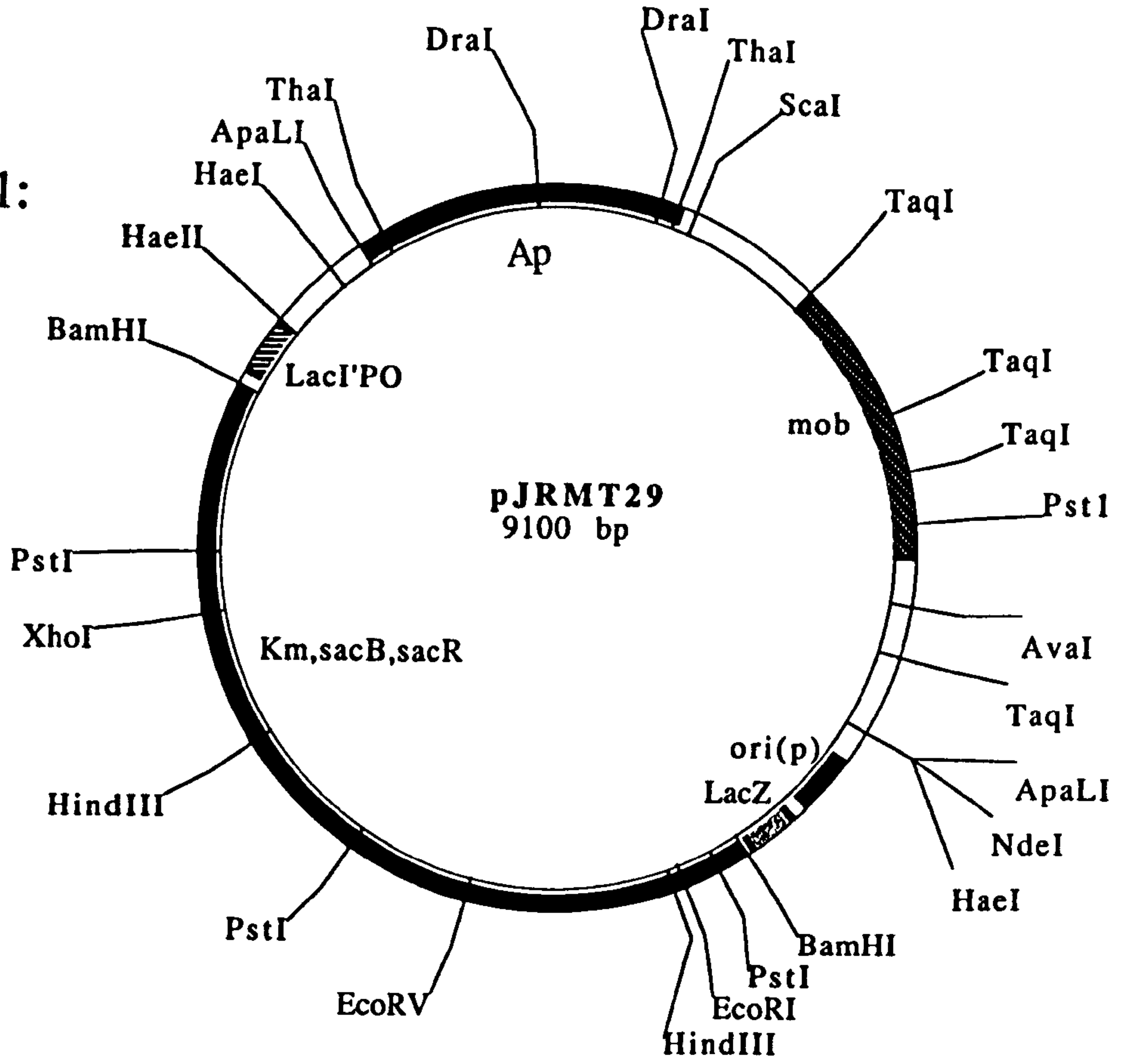
The Km^R-*sacB-sacR* fragment was cut from plasmid pEG18.3 and cloned into pAKA16 which had been digested with *Bam*HI and dephosphorylated.

B: Map of plamid pJRMT30

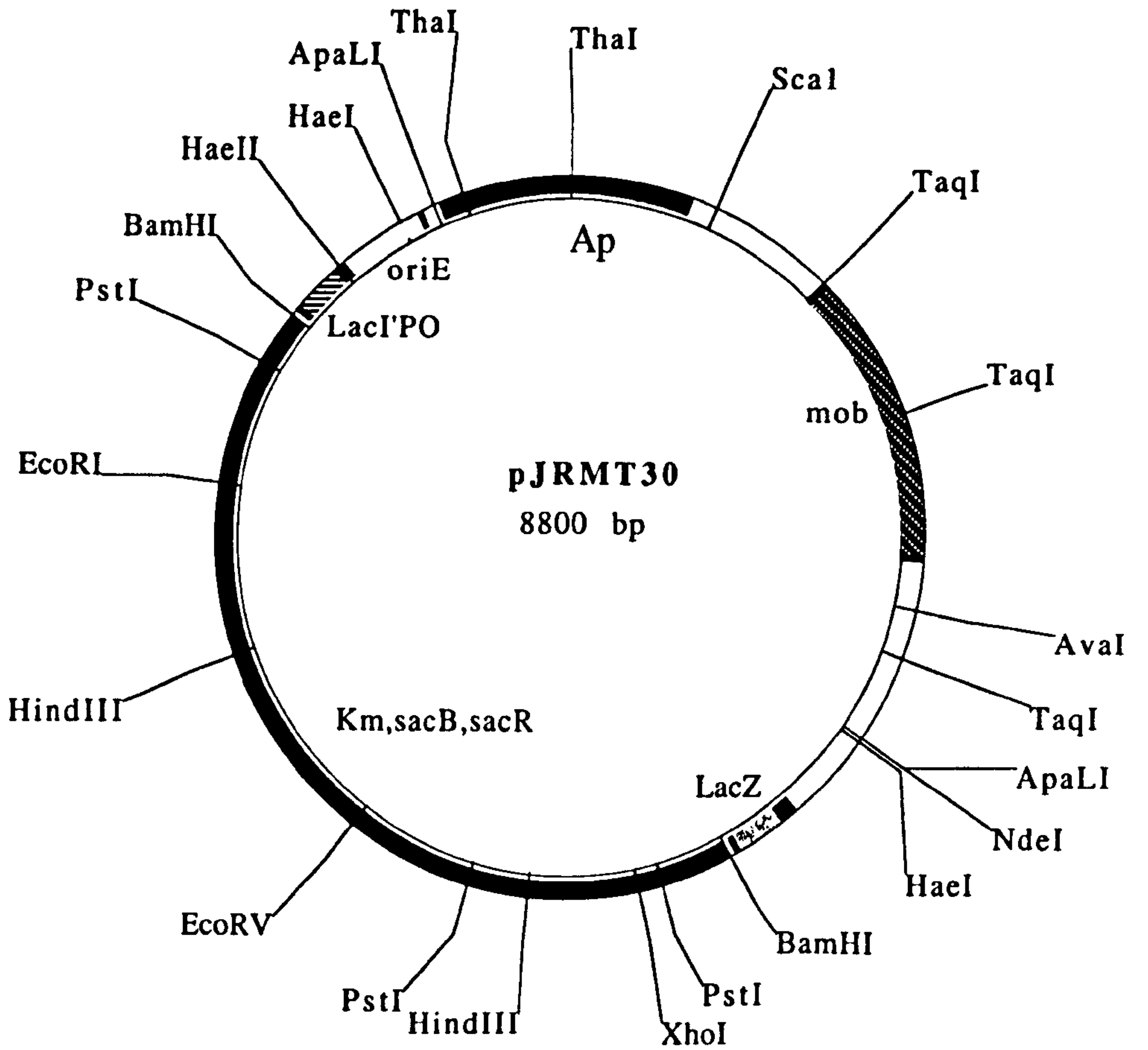
The Km^R-*sacB-sacR* fragment was cut from plasmid pEG18.3 and cloned into pAKA19 which had been digested with *Bam*HI and dephosphorylated.

Fig 61:

A.



B.



the Km^R-*sacR-sacB* cartridge was cloned into plasmid pAKA19 and designated plasmid pJRMT30 (Fig. 61B). *Bam*HI-digestion of plasmids pAKA16 and pAKA19 produced just linearised DNA bands of ≈5.0 and ≈5.3 kb respectively, whereas for plasmids pJRMT29 and pJRMT30 it produced an additional ≈3.8 kb fragment, as expected (results not shown).

3. 4. 2. 2. Introduction of the *sacB* gene into *P. multocida* strains

The plasmid pJRMT29 was transformed into *P. multocida* strains by electroporation and Km^R clones selected. These new clones were analysed for the presence of plasmid DNA and for growth on BHI agar supplemented with 5% (v/v) sheep blood containing 5% (w/v) sucrose and were named *P. multocida* JRMT8 and JRMT9 for *P. multocida* strains 85020 and Quetta respectively. Single clones of these *P. multocida* strains from BHI agar were inoculated into 5 ml-volume of BHI broth and incubated overnight at 37°C with shaking. 10 ml of pre-warmed BHI broth were inoculated with 0.5 ml of overnight culture and incubated at 37°C. When the OD_{540nm} reached ≈1 (≈10⁹ C.F.U./ ml), 10× serial dilutions were prepared in PBS and 100 µl aliquots of each sample spread onto BHI agar supplemented with 5% (v/v) sheep blood and 5% (w/v) sucrose and incubated at 37°C. After 24 hrs, single colonies had grown up but when these were left for about 40 hrs at 37°C all colonies were liquefied because of levansucrase secretion. The *sacB* phenotype was therefore expressed in *P. multocida* caused cell lysis. Also, plasmid pJRMT30 was transformed into *P. multocida* strains and plated on BHI agar supplemented with 5% (v/v) sheep blood. After incubation for 48 hrs at 37°C, a few Km^R colonies grew but these were assumed to be spontaneous Km^R colonies as plasmid DNA could not be detected in them.

3. 4. 3. Construction of marker-free *aroA* mutants with *sacB* gene as a selective marker

3. 4. 3. 1. Insertion of deleted *aroA* gene into pAKA19 vector

Direct cloning of the deleted *aroA* gene into plasmid pJRMT30 was difficult because of incompatible restriction sites in its multiple cloning site with plasmids pJRMT25 and pJRMT26. For this reason, the deleted *aroA* genes were initially cloned into pAKA19 vector. The deleted *aroA* genes were cut from plasmids pJRMT25 and pJRMT26 with *Bam*HI/*Xho*I as a ≈ 1.05 kb fragment and cloned into *Bam*HI/*Xho*I-digested and dephosphorylated pAKA19. The constructs were transformed into *E. coli* DH5 α competent cells by electroporation and plated onto LB-X-Gal-IPTG agar containing ampicillin. White colonies were picked and inoculated into LB broth containing ampicillin. The recombinant plasmids were confirmed by restriction analysis and designated as plasmids pJRMT31 and pJRMT32 for *P. multocida* strains 85020 and Quetta respectively (Fig. 62A). Digestion of plasmid DNA with *Bam*HI/*Xho*I linearised the plasmid pAKA19 as a ≈ 5.0 kb fragment but for plasmids pJRMT31 and pJRMT32 it produced two fragments, of ≈ 1.05 kb as the deleted *aroA* gene and ≈ 5.0 kb as the vector alone (results not shown).

3. 4. 3. 2. Insertion of Km^R-*sacR*-*sacB* cartridge into plasmids pJRMT31 and pJRMT32

The plasmid pEG18.3 was digested with *Bam*HI and the Km^R-*sacB*-*sacR* cartridge removed as ≈ 3.8 kb fragment. This was cloned into the plasmids pJRMT31 and pJRMT32 which had been *Bam*HI-digested and dephosphorylated and these were then transformed into *E. coli* DH5 α competent cells by electroporation. The recombinant plasmids were confirmed by restriction analysis and lack of growth on LB agar containing 5% (w/v) sucrose, and were named plasmids pJRMT33 and pJRMT34 (Fig. 62B). As expected, digestion of plasmids pJRMT31 and pJRMT32 with *Bam*HI

Fig 62:

A: The maps of plasmids pJRMT31 and pJRMT32

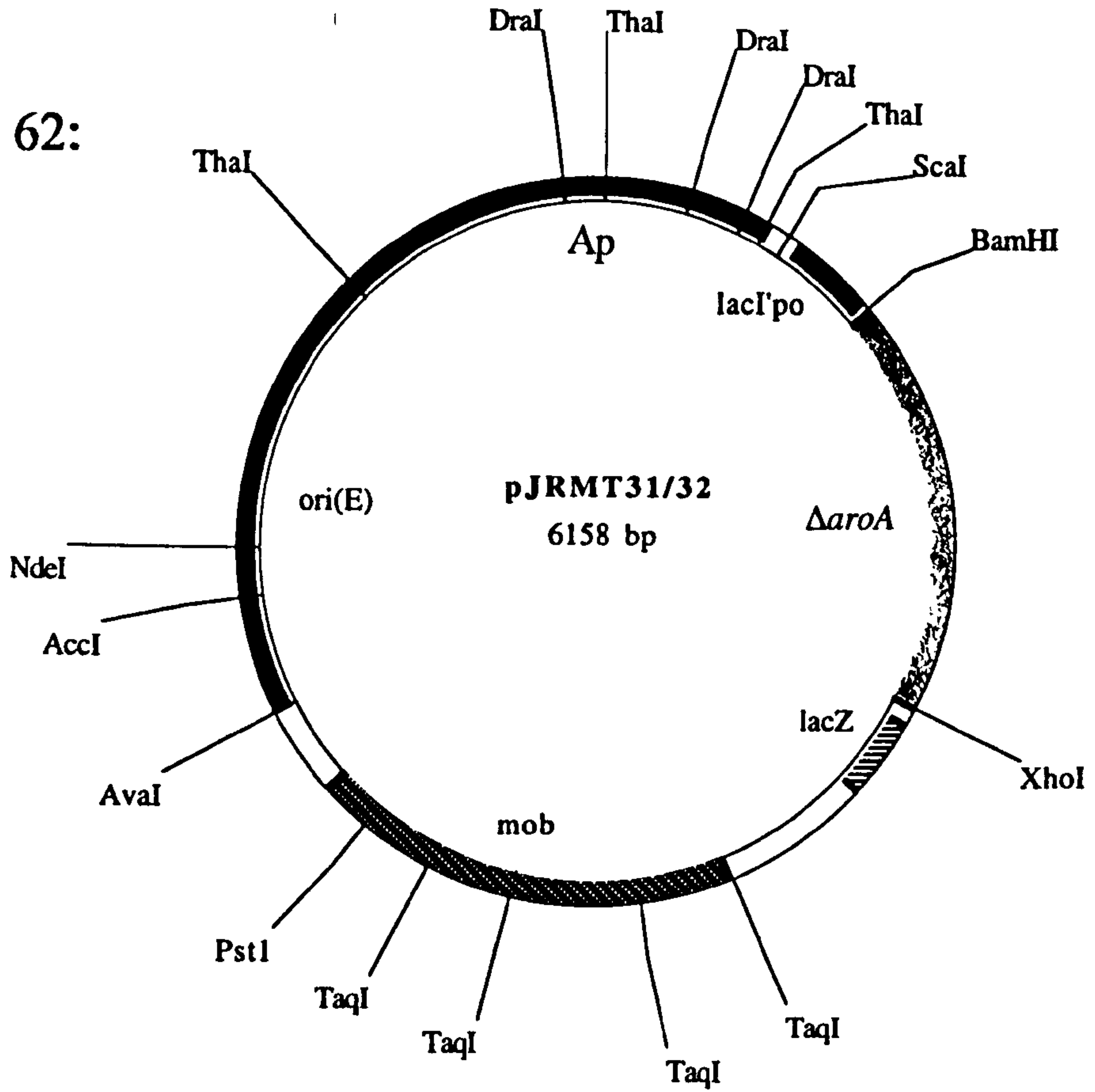
The deleted *aroA* gene was cut from plasmids pJRMT25 and pJRMT26 with *Bam*HI/*Xho*I and cloned into pAKA19 and were named as plasmids pJRMT31 and pJRMT32.

B: The map of plasmid pJRMT33 and pJRMT34

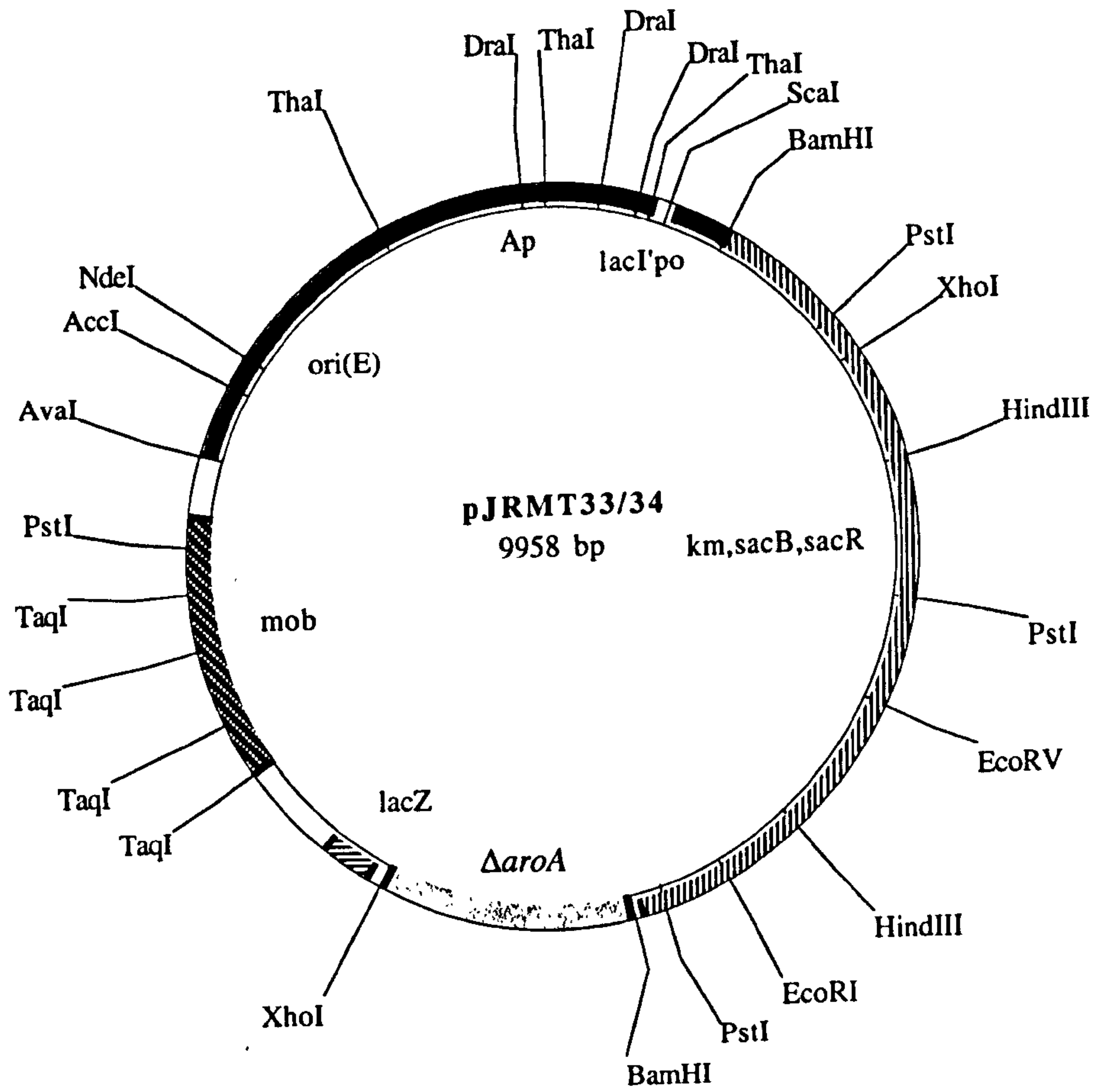
The Km^R-*sacB*-*sacR* fragment was cut from plasmid pEG18.3 and cloned into plasmids pJRMT31 and pJRMT32 and designated as plasmid pJRMT33 and pJRMT34.

Fig 62:

A.



B.



produced just one band of ≈ 6.0 kb as linearised vector whereas another fragment of ≈ 3.8 kb was produced for plasmids pJRMT33 and pJRMT34 representing the inserted Km^R -*sacB*-*sacR* fragment (results not shown). When cells containing these plasmids were spread on LB agar with and without 5% (w/v) sucrose, a few colonies grew on LB agar containing sucrose but confluent growth was obtained on LB agar without sucrose. Some of the colonies that grew on LB agar with 5% (w/v) sucrose were analysed for the presence of plasmid, but the result was negative indicating that they had lost the plasmid and so no longer expressed the *sacB* phenotype with sucrose. The plasmids pJRMT33 and pJRMT34 were then transformed into *E. coli* SM10 λ Pir for conjugation.

3. 4. 3. 3. Allelic exchange

The plasmids pJRMT33 and pJRMT34 were transformed into *P. multocida* strains by electroporation and transconjugation and plated onto BHI agar supplemented with 5% (v/v) sheep blood containing kanamycin or kanamycin and streptomycin (for transconjugants). After incubation for 48 hrs at 37°C, some single colonies were picked from more than 500 colonies and patched onto BHI agar supplemented with 5% (v/v) sheep blood without any antibiotics. They were then plated onto BHI agar supplemented with 5% (v/v) sheep blood with and without kanamycin and also onto plates with 5% (w/v) sucrose. Some sucrose resistant and Km^S samples were selected, inoculated into BHI broth and checked by PCR. Most of them showed two bands, one wild-type in size and one with the correct mutated size and some showed just one band of wild-type size (Fig. 63). However, a few samples showed just one band of the mutated gene size (Fig. 63A lane 7, B lane 5 and C lane 11) and these were selected and transferred to BA plates individually. Representative clones of each new mutant were designated as *P. multocida* JRMT12 and JRMT13 for *P. multocida* 85020 and Quetta strains respectively.

Fig 63: PCR analysis of *P. multocida* clones transformed with plasmids pJRMT33 and pJRMT34 (size as kb)

A and B: *P. multocida* 85020 transformed with plasmid pJRMT33

A:

Lanes 1-7: transformed clones after subculture (Km^S and sucrose^R)

Lane 8: *P. multocida* 85020 (wild-type)

Lane M: 1 kb DNA marker

B:

Lanes 1-12: transformed clones after subculture (Km^S and sucrose^R)

Lane 13: *P. multocida* 85020 (wild-type)

Lane M: 1 kb DNA marker

C: *P. multocida* Quetta transconjugated with plasmid pJRMT34

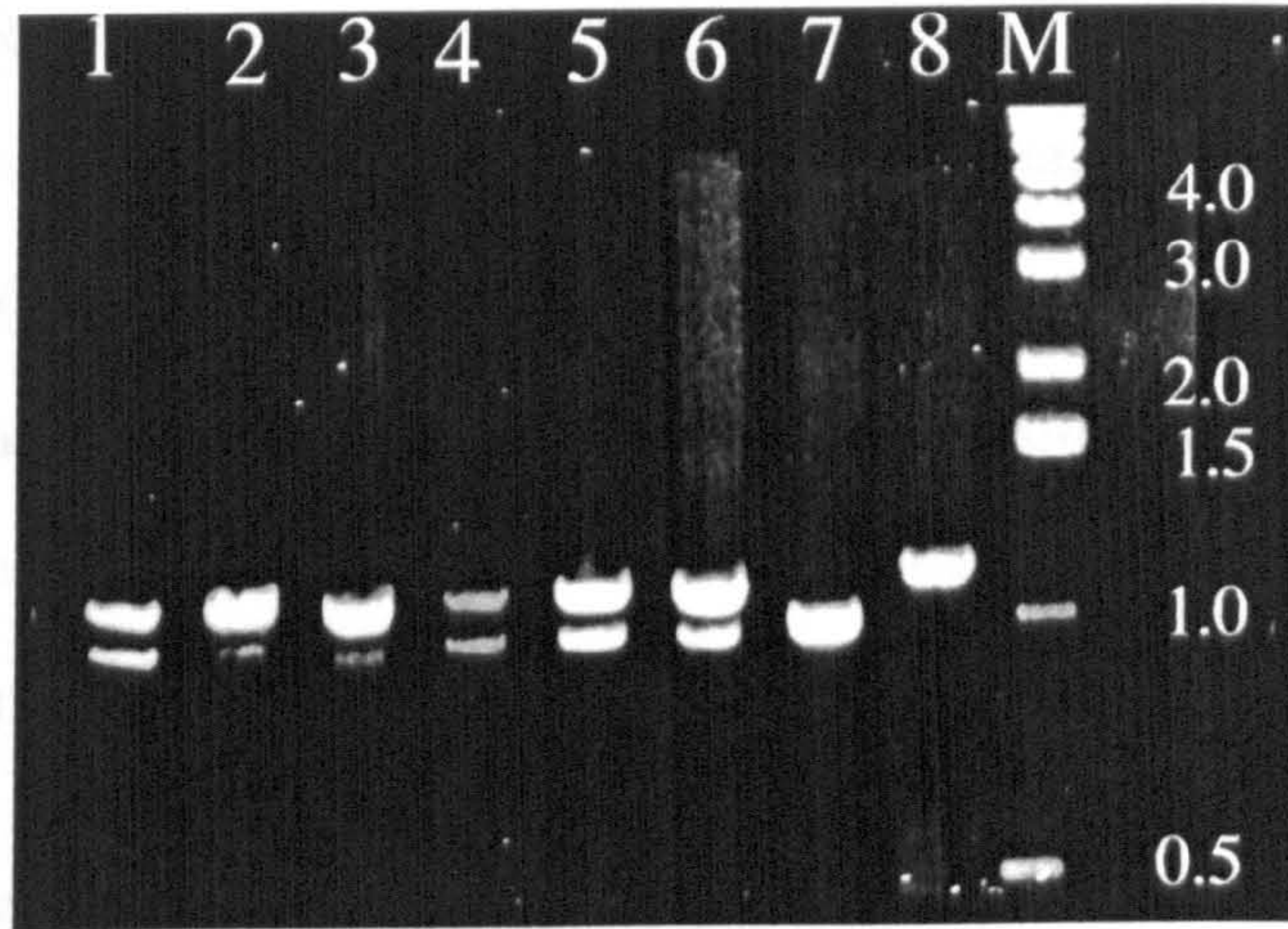
Lanes 1-11: transformed clones after subculture (Km^S and sucrose^R)

Lane 12: *P. multocida* Quetta (wild-type)

Lane M: 1 kb DNA marker

Fig 63:

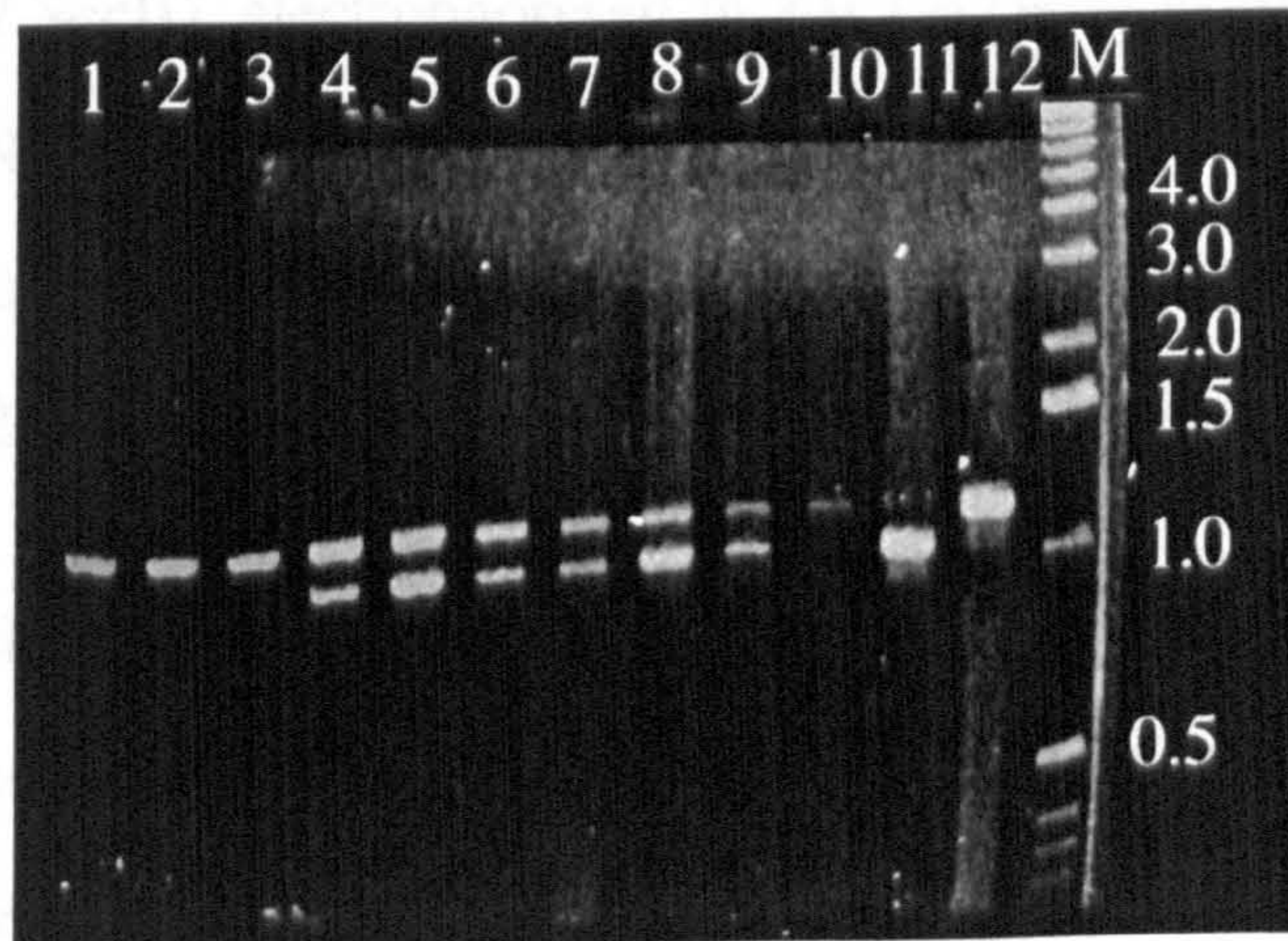
A.



B.



C.



3. 4. 3. 4. Confirmation of deleted *aroA* mutants

The deleted *aroA* mutants were confirmed by PCR and restriction enzyme analysis, growth in PMM and also Southern blot hybridization.

3. 4. 3. 4. 1. PCR

The AroA primers produced a single band (≈ 1.0 kb), smaller than the wild-type band (1.2 kb) (Fig. 64A). When the PCR products were digested with *EarI* (the *EarI* site had been removed from the *aroA* gene sequence by digestion with *AflIII/SacII*), the wild-type amplicon showed two bands (≈ 0.45 and ≈ 0.75 kb) but the mutant amplicon showed only one band (≈ 1.05 kb) (Fig. 64B).

3. 4. 3. 4. 2. Growth in Pasteurella Minimal Medium

As described previously, *aroA* mutants and wild-type strains were checked for growth in PMM. Fresh overnight cultures of the *P. multocida* strains were inoculated into 10 ml-volumes of PMM and incubated at 37 °C with shaking. After 24 and 48 hrs, the OD_{540nm} was measured for each strain (Fig. 65). Only the wild-type strains were able to grow in the minimal medium, confirming the phenotype of the *aroA* mutants.

3. 4. 3. 4. 3. Southern blot hybridisation

Genomic DNA of *P. multocida* wild type and the deleted *aroA* mutant strains was digested with *EarI* (the *EarI* site of *aroA* gene had been removed by digestion with *AflIII/SacII*), electrophoresed in a 0.7% (w/v) agarose gel and blotted to Hybond N⁺ membrane. The blot was hybridised with the *aroA* probe which hybridised to two bands of ≈ 1.3 and ≈ 1.6 kb for wild type strains and only one band of ≈ 2.8 kb for the deleted *aroA* mutant strains (Fig. 66).

The results of this experiment showed that use of the *sacB* selection procedure was successful in selecting for a double cross-over more readily to produce deleted *aroA* derivatives.

Fig 64: PCR analysis of the deleted *aroA* mutation in *P. multocida* strains

(size as kb)

A: PCR products with AroA primers

Lane 1: *P. multocida* JRMT12 (Δ *aroA* mutant)

Lane 2: *P. multocida* 85020 (wild-type)

Lane 3: *P. multocida* JRMT13 (Δ *aroA* mutant)

Lane 4: *P. multocida* Quetta (wild-type)

Lane M: 1 kb DNA marker

B: Amplified *aroA* gene digested with *EcoRI*

Lane 1: *P. multocida* JRMT12 (*aroA* mutant)

Lane 2: *P. multocida* 85020

Lane 3: *P. multocida* JRMT13 (*aroA* mutant)

Lane 4: *P. multocida* Quetta

Lane M: 1 kb DNA marker

Fig 64:

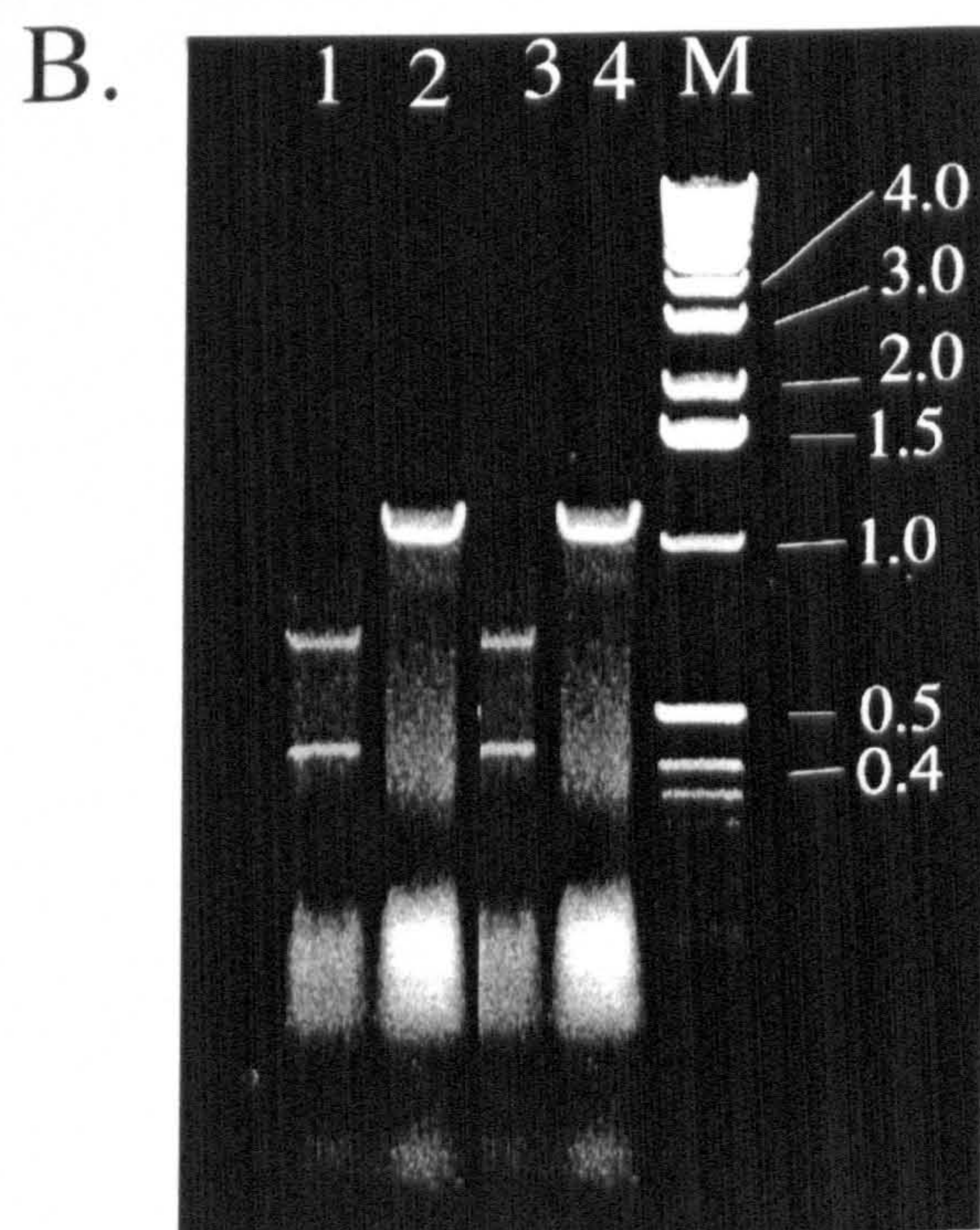


Fig 65: Growth of *P. multocida* strains in Pasteurella Minimal Medium (PMM)

P. multocida strains 85020, JRMT12 ($\Delta aroA$ mutant of 85020), Quetta and JRMT13 ($\Delta aroA$ mutant of Quetta) were inoculated into 10 ml of PMM and, after shaking for 24 hrs and 48 hrs at 37°C the OD_{540nm} was measured.

Fig 65:

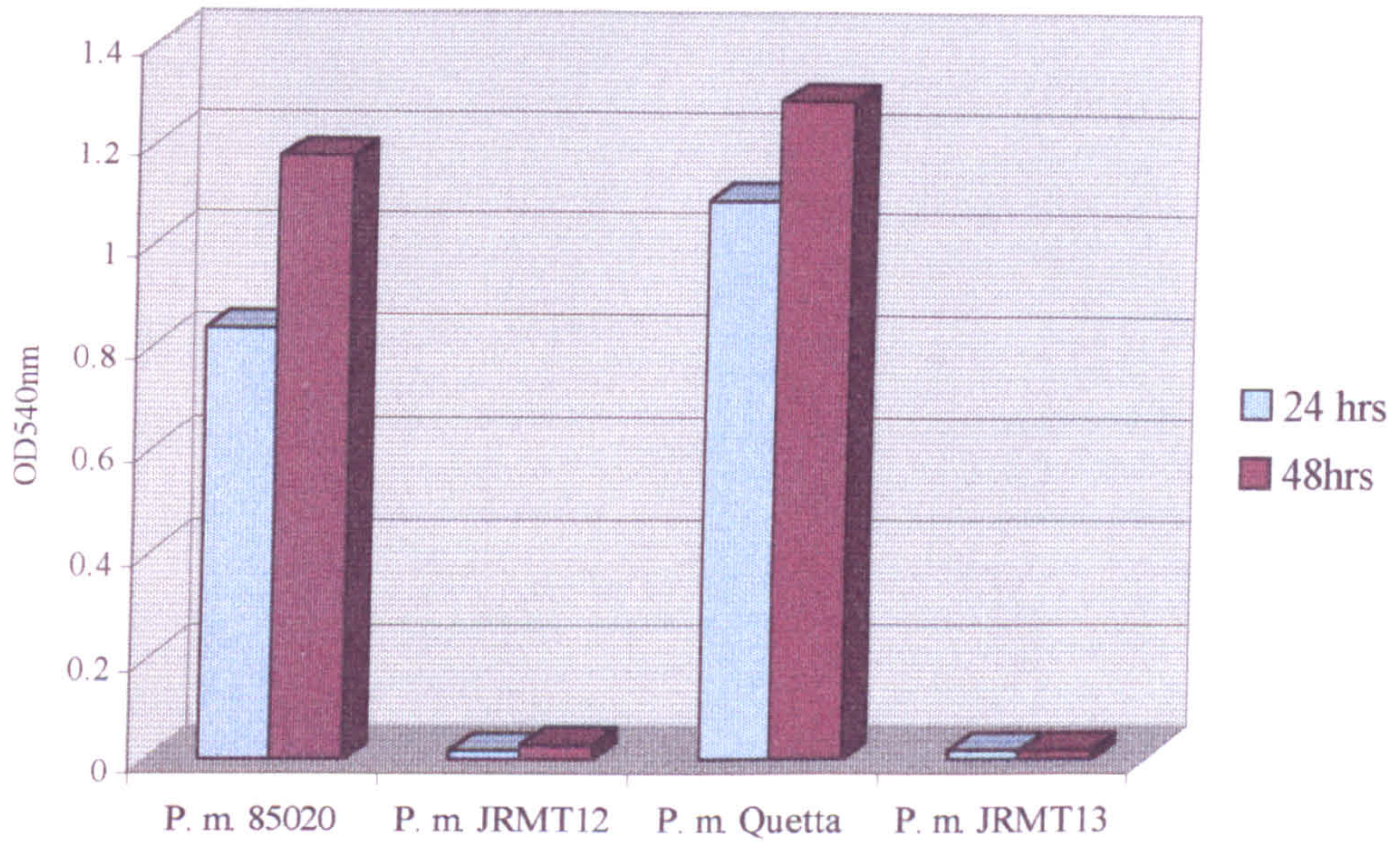


Fig 66: Southern blot hybridisation of deleted *aroA* mutants of *P. multocida* strains (genomic DNA digested with *EarI*) and hybridised with *aroA* probe (size as kb).

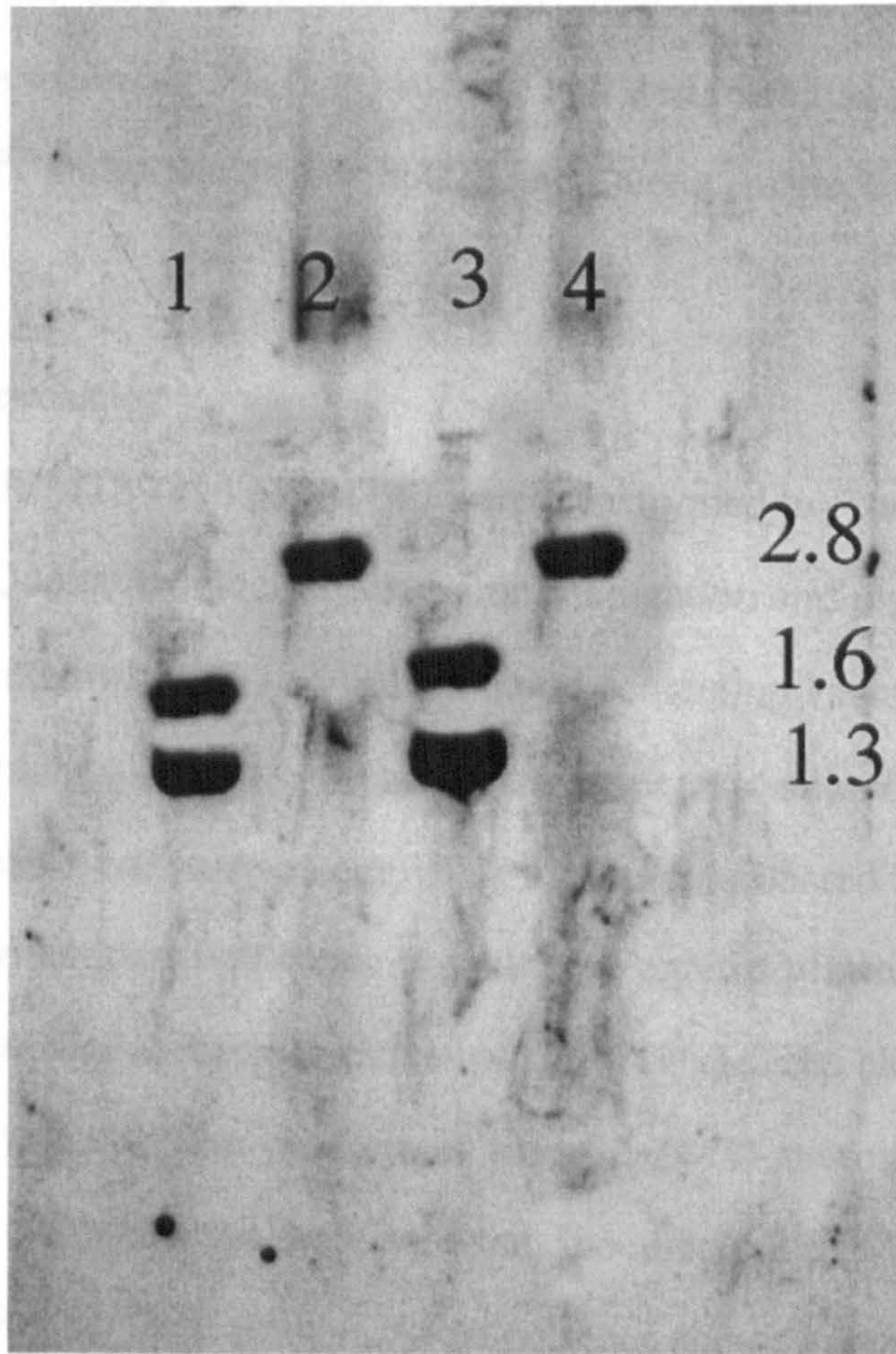
Lane 1: *P. multocida* 85020 (wild-type)

Lane 2: *P. multocida* JRMT12 (Δ *aroA* mutant)

Lane 3: *P. multocida* Quetta (wild-type)

Lane 4: *P. multocida* JRMT13 (Δ *aroA* mutant)

Fig 66:



3. 4. 4. Construction of marker-free *galE* mutants with *sacB* gene as a selective marker

3. 4. 4. 1. Insertion of Km^R -*sacB*-*sacR* cartridge into plasmids pJRMT37 and pJRMT38

The plasmid pGE18.3 was digested with *Bam*HI to remove the Km^R -*sacB*-*sacR* cartridge as a ≈ 3.8 kb fragment and this was cloned into *Bam*HI-digested and dephosphorylated plasmids pJRMT37 and pJRMT38 (Fig. 56B). The recombinant plasmids were confirmed by restriction analysis and lack of growth on LB agar containing 5% (w/v) sucrose and designated as pJRMT39 and pJRMT40 for *P. multocida* strains 85020 and Quetta respectively (Fig. 67).

3. 4. 4. 2. Allelic exchange

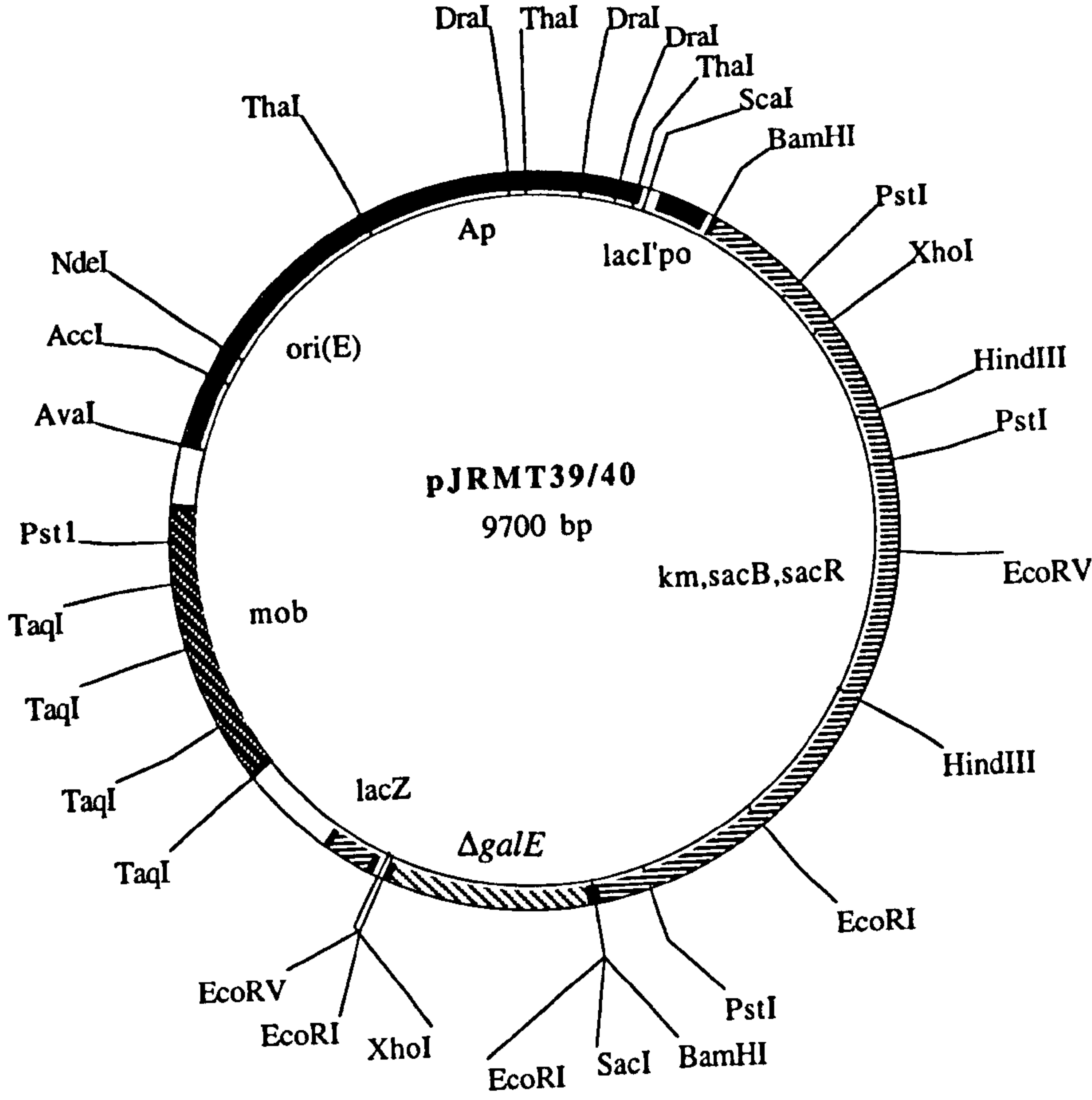
The plasmids pJRMT39 and pJRMT40 were transformed to *P. multocida* strains 85020 and Quetta by electroporation or conjugation and plated onto BHI agar supplemented with 5% (v/v) sheep blood containing kanamycin or kanamycin and streptomycin (for transconjugants). After incubation for 48 hrs at 37°C, 150 colonies were patched onto BHI agar supplemented with 5% (v/v) sheep blood without any antibiotic for selection against plasmid. They were plated then onto BHI agar supplemented with 5% (v/v) sheep blood with and without kanamycin and also onto plates with 5% (w/v) sucrose. Some sucrose resistant and Km^S clones were selected, inoculated into BHI broth and checked by PCR with *GalE* primers. Most colonies showed one band of wild-type size, but some showed one band larger than the wild-type gene, as seen before (see Fig. 57). Digestion of the PCR product with *Apa*LI (the *Apa*LI site had been removed from the *galE* gene by digestion with *Eco*RV/*Bsm*I) showed two bands of ≈ 0.5 kb for the wild-type, as expected, and the mutants showed two bands but at different size from the wild-type (Fig. 57), as seen previously. However, after 10 subcultures into BHI broth,

Fig 67:

A: The map of plasmids pJRMT39 and pJRMT40

The Km^R -*sacB*-*sacR* fragment was cut from plasmid pEG18.3 and cloned into plasmids pJRMT37 and pJRMT38 (see Fig 56).

Fig 67:



all clones showed wild-type pattern. This experiment was repeated more than 30 times by electroporation and transconjugation, but on no occasion was a clone found with the correct predicted PCR amplifier. Thus for this locus, *sacB* selection was not successful.

3. 5. Mouse virulence tests

3. 5. 1. Preparation of inoculum

P. multocida strains, both the wild-type parent strains and mutated strains, grew readily in BHI broth. A maximum viable population of $3-6 \times 10^9$ viable cells per ml was achieved in the shaken cultures in 4-6 hrs at 37°C . The culture went into an early decline phase so that less than 10^6 viable bacteria per ml could be detected after 18 hrs. For this reason, overnight cultures were not used to prepare challenge inocula. Single colonies from a fresh BA plate were inoculated into 5 ml-volumes of BHI broth and incubated overnight at 37°C with shaking, after which 10 ml of pre-warmed BHI broth was inoculated with 0.5 ml of overnight culture. When the $\text{OD}_{540\text{nm}}$ reached ≈ 1 (after 4-6 hrs, exponential phase culture containing $\approx 3-6 \times 10^9$ C.F.U./ ml), $10\times$ serial dilutions in PBS were prepared.

3. 5. 2. Virulence test of *P. multocida* 85020 and its *aroA* mutant, JRMT1

(Experiment 1)

Ten groups of three mice were injected intraperitoneally (i.p.) with 0.5 ml of graded doses of these two strains. All mice were weighed and checked for illness every day for 5 days and the weight gain noted. According to the results of this experiment (Table 11) LD_{50} values for *P. multocida* 85020 of <20 C.F.U./ mouse was estimated and $\geq 1.7 \times 10^5$ C.F.U./ mouse for the JRMT1 strain. Thus, the wild-type strain was very virulent by this route of inoculation and could kill mice in less than 24 hrs from a very low inoculum. The *aroA* mutant strain JRMT1 appeared to be greatly attenuated.

Table 11: The virulence properties of *P. multocida* strain 85020 and its *aroA* mutant JRMT1 by i.p. route (Experiment 1)

Treatment	C.F.U./ mouse	<u>No. of survivors</u> No. challenged
<i>P. m.</i> 85020 (wild-type)	2000	0/3
" "	200	0/3
" "	20	0/3
" "	2.0	2/3
<i>P. m.</i> JRMT1 (85020 <i>aroA</i> mutant)	1.75×10^5	3/3
" "	1.75×10^4	3/3
" "	1.75×10^3	3/3
" "	1.75×10^2	3/3
PBS	0.5 ml	3/3
BHI	0.5 ml	3/3

In the next experiment (Experiment 2) 9 groups of 5 mice were injected with higher doses of *P. multocida* JRMT1 and lower doses of the wild-type *P. multocida* 85020 strain and the weight gain checked over the following six days (Table 12; Fig. 68). Many of the mice given the wild-type strain died on the first day post injection, whereas, mice injected with the top dose of JRMT1 ($\approx 2.85 \times 10^9$ C.F.U.) died on the second day. The mice given $\approx 2.85 \times 10^8$ C.F.U. of *P. multocida* JRMT1 showed some toxicity and lost weight during the first day post inoculation (PI) but by the second day all of them had recovered. The results of this experiment showed that the LD₅₀ of *P. multocida* 85020 was <32 C.F.U./ mouse but for *P. multocida* JRMT1 was more than 2.85×10^8 C.F.U./ mouse (Table 12). As a preliminary protection test, the surviving vaccinated mice were challenged i.p. with ≈ 100 LD₅₀ of *P. multocida* 85020 wild-type parent strain on 7 day post inoculation. In this experiment, the mice given a dose as low as 10^5 C.F.U. of JRMT1 showed some protection (data not shown). The results of this experiment indicated that a dose of *P. multocida* JRMT1 between 10^5 - 10^8 C.F.U./mouse would confer optimal protection. Due to some adverse side effects resulting from a dosage of 2.85×10^8 C.F.U., immunisation with a lower dosage was considered. It was decided that a dosage of 10^7 C.F.U./ mouse would be used, as it offered good protection and no side effects.

Finally for confirmation of these results (Exp. 3), 13 groups of 5 mice were challenged with graded doses of *P. multocida* strains 85020, JRMT1, Quetta and JRMT2 (*aroA* mutant of Quetta) (Table 13). The results of this experiment confirmed that *P. multocida* JRMT1 was highly attenuated with LD₅₀ $\geq 10^8$ C.F.U./ mouse. Similar results were obtained with the Quetta strains and its *aroA* derivative JRMT2 (Table 13). At 48 hrs PI, two mice from the groups given the top dose of JRMT1 and JRMT2 were sacrificed and heart blood plated onto BA and incubated at 37°C for 48 hrs. No colonies were detected

Table 12: The virulence properties of *P. multocida* 85020 strain and its *aroA* mutant JRMT1 by i.p. route (Experiment 2)

Treatments	C.F.U./ mouse	<u>No.of survivors</u> No. challenged
P. m. 85020 (wild-type)	320	0/5
" "	32.0	0/5
" "	3.2	2/5
P. m. JRMT1 (85020 <i>aroA</i> mutant)	2.85×10^9	0/5
" "	2.85×10^8	5/5
" "	2.85×10^7	5/5
" "	2.85×10^6	5/5
PBS	0.5 ml	5/5

Fig 68: Mean weight gain of mice inoculated i.p. with of *P. multocida* 85020 and its *aroA* derivative JRMT1.

1. Control group (given 0.5 ml PBS)
2. *P. multocida* 85020 (wild type) 320 C.F.U./ mouse
3. *P. multocida* JRMT1 2.85×10^9 C.F.U./ mouse
4. *P. multocida* JRMT1 2.85×10^8 C.F.U./ mouse
5. *P. multocida* JRMT1 2.85×10^7 C.F.U./ mouse

The mice given of *P. multocida* 85020 by i.p. route (320 C.F.U./ mouse) died after 24 hrs (No. 2). Mice given highest dose of *P. multocida* JRMT1 (2.85×10^9 C.F.U./ mouse) died after 48 hrs (No. 3). Mice given a lower dose JRMT1 (2.85×10^8 C.F.U./ mouse) showed some toxicity and lost weight but had recovered by 48 hrs (No. 4)

Fig 68:

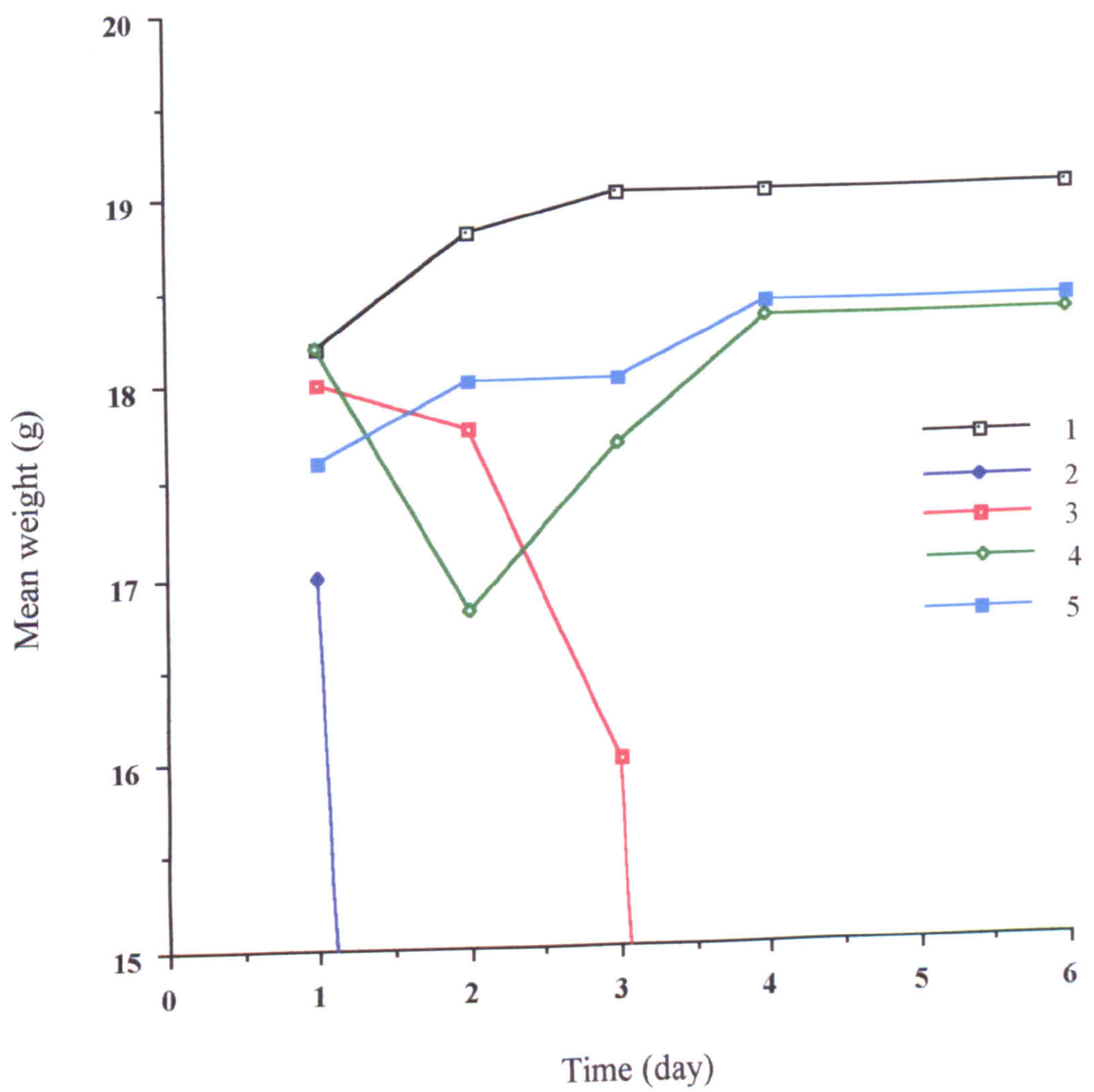


Table 13: The virulence properties of *P. multocida* strains and their *aroA* mutants
(Experiment 3)

Treatment	Challenge dose i.p. (C.F.U./ mouse)	<u>No. of survivors</u> No. challenged
P. m. 85020 (Wild-type)	266	0/5
" "	26.6	0/5
" "	2.66	2/5
P. m. JRMT1 (85020 <i>aroA</i> mutant)	2.11×10^8	5/5
" "	2.11×10^7	5/5
" "	2.11×10^6	5/5
" "	2.11×10^5	5/5
" "	2.11×10^4	5/5
P. m. Quetta (Wild-type)	295	0/5
" "	29.5	0/5
" "	2.95	1/5
P. m. JRMT2 (Quetta <i>aroA</i> mutant)	1.97×10^8	5/5
" "	1.97×10^7	5/5
" "	1.97×10^6	5/5
" "	1.97×10^5	5/5
" "	1.97×10^4	5/5
PBS	0.5 ml	5/5

from these blood samples, indicating that the *aroA* strains had greatly reduced ability to survive and/or multiply *in vivo*.

3. 5. 3. Virulence test of *cya::Km^R* mutant of Quetta strain (Experiment 4)

Six groups of 3 mice were injected with 0.5 ml of graded doses of exponential phase cells of *P. multocida* Quetta and the corresponding *cya* mutant JRMT3 (Table 14). The results showed that the *P. multocida* JRMT3 (*cya::Km^R*) strain was at least 10^3 times more attenuated than the wild-type strain, but not as attenuated for virulence as the *aroA* mutants strains (Tables 11-13).

3. 5. 4. Virulence test of *P. multocida* strains by different routes of inoculation (Experiment 5)

For this experiment, 36 groups of 3 mice were injected with graded doses of *P. multocida* strains by the i.p., i.n. and s.c. routes (Table 15). The results of this experiment indicated that the LD₅₀ of different strains varied by route of inoculation. The LD₅₀ of *P. multocida* 85020 and Quetta strains by the i.p. and s.c. routes was <50 C.F.U./ mouse and by the i.n. route was >200 and >5600 C.F.U./ mouse for 85020 and Quetta strains, respectively. It was also found that the wild type strains could kill the mice by the i.p. challenge at 24 hrs post-inoculation (PI), but by the s.c. route only after 48-72 hrs and by the i.n. route only after 96 hrs (5th day PI). After 5 days PI, samples were taken for culture from all surviving mice that had been inoculated by the i.n. route and from mice that had been inoculated with the top dose of mutant strains by the s.c. and i.p. routes. Aliquots of heart blood and homogenised lungs were plated onto BA plates. A few colonies grew from the blood and lungs of mice that were inoculated by the i.n. route with *P. multocida* 85020 strain, but no colonies grew from the other samples. The mice that inoculated i.n. were showed different stages of hepatisation and adhesion of lungs to the thoracic cavities.

Table 14: The virulence properties of *P. multocida* strain Quetta and its *cya* mutant by i.p. route (JRMT3)

Treatments	C.F.U./ mouse by i.p. route	No. of survivors						
		No. challenged				at time (hrs) PI		
		0	18	24	48	72	96	120
P. m. Quetta (wild-type)	1330	3/3	2/3	0/3	----	----	----	----
" "	133	3/3	1/3	0/3	----	----	----	----
" "	13.3	3/3	2/3	0/3	----	----	----	----
P. m. JRMT3 (<i>cya</i> mutant)	1.2×10^5	3/3	3/3	3/3	3/3	2/3	1/3	0/3
" "	1.2×10^4	3/3	3/3	3/3	3/3	2/3	2/3	1/3
" "	1200	3/3	3/3	3/3	3/3	3/3	3/3	3/3

---- mice dead after 24 hrs

Table 15: Virulence test of *P. multocida* strains by different routes of inoculation

Treatments	Challenge dose (C.F.U./ mouse)	Route of inoculation	<u>No. of survivors</u> No. challenged
<i>P. m.</i> 85020 (wild-type)	2100	i.p.	0/3 (18 hrs PI)
" " "	210	"	0/3 (18 hrs PI)
" " "	21	"	0/3 (24 hrs PI)
" " "	2100	s.c.	0/3 (48 hrs PI)
" " "	210	"	0/3 (48 hrs PI)
" " "	21	"	0/3 (48 hrs PI)
" " "	2100	i.n.	1/3 (96 hrs PI)
" " "	210	"	2/3 (96 hrs PI)
" " "	21	"	3/3 (96 hrs PI)
<i>P. m.</i> JRMT1 (85020 <i>aroA</i> mutant)	2.1×10^9	i.p.	0/3 (24 hrs PI)
" " "	2.1×10^8	"	3/3 (96 hrs PI)
" " "	2.1×10^7	"	3/3 (96 hrs PI)
" " "	2.1×10^9	s.c.	3/3 (96 hrs PI)
" " "	2.1×10^8	"	3/3 (96 hrs PI)
" " "	2.1×10^7	"	3/3 (96 hrs PI)
" " "	2.1×10^9	i.n.	3/3 (96 hrs PI)
" " "	2.1×10^8	"	3/3 (96 hrs PI)
" " "	2.1×10^7	"	3/3 (96 hrs PI)
<i>P. m.</i> Quetta (wild-type)	5600	i.p.	0/3 (18 hrs PI)
" " "	560	"	0/3 (24 hrs PI)
" " "	56	"	0/3 (24 hrs PI)
" " "	5600	s.c.	0/3 (48 hrs PI)
" " "	560	"	0/3 (48 hrs PI)
" " "	56	"	0/3 (48 hrs PI)
" " "	5600	i.n.	3/3 (96 hrs PI)
" " "	560	"	3/3 (96 hrs PI)
" " "	56	"	3/3 (96 hrs PI)

Table 15: (continued)

P. m. JRMT2 (Quetta <i>aroA</i> mutant)	3.12×10^9	i.p.	0/3 (48 hrs PI)
" " "	3.12×10^8	"	3/3 (96 hrs PI)
" " "	3.12×10^7	"	3/3 (96 hrs PI)
" " "	3.12×10^9	i.p.	3/3 (96 hrs PI)
" " "	3.12×10^8	"	3/3 (96 hrs PI)
" " "	3.12×10^7	"	3/3 (96 hrs PI)
" " "	3.12×10^9	i.p.	3/3 (96 hrs PI)
" " "	3.12×10^8	"	3/3 (96 hrs PI)
" " "	3.12×10^7	"	3/3 (96 hrs PI)

The results of this experiment indicated that for *P. multocida* 85020 the LD₅₀ by the i.n. route was ≈ 200 C.F.U. and for Quetta strain was >5600 C.F.U./ mouse whereas for the *aroA* mutant strains the LD₅₀ by the s.c. and i.n. routes was more than 10^9 C.F.U./ mouse. In the next experiment (Experiment 6), 7 groups of 5 mice were inoculated intranasally with graded doses of *P. multocida* 85020 and Quetta strains and observed for 10 days PI (Table 16). This experiment gave a clear indication of the LD₅₀ by the i.n. route. Again the 85020 strain appeared to be more virulent by this route (LD₅₀ ≈ 1000 C.F.U./ mouse) than the Quetta strain (LD₅₀ $\approx 10^5$ C.F.U./ mouse).

3. 6. Mouse protection tests

3. 6. 1. Vaccination with *P. multocida* strains JRMT1, JRMT2 and JRMT3 (Experiment 7)

Eight groups of 5 mice were inoculated with 0.5 ml of culture diluted in PBS and containing $\approx 2-3 \times 10^7$ C.F.U. of *P. multocida* JRMT1 and JRMT2 and $\approx 3.65 \times 10^3$ C.F.U. of JRMT3 strains by the i.p. route. Two weeks later all mice were challenged with different doses of *P. multocida* 85020 or Quetta wild type strains. The uninoculated control groups were challenged with 100 LD₅₀ of the wild-type strains (Table 17).

The results of this test showed that the *P. multocida* JRMT1 and JRMT2 strains completely protected the mice against all the challenge doses with the wild-type strains, but *P. multocida* JRMT3 did not show any protective effects at the vaccination dose used.

3. 6. 2. Effects of different doses and different time intervals between vaccination and challenge (Experiment 8)

Groups of 6 mice were inoculated with 10^7 C.F.U. of *P. multocida* JRMT1 or JRMT2 strains by i.p. route. Two weeks later all groups were inoculated again as before. After two further weeks all mice were challenged with 1000

Table16: Virulence properties of *P. multocida* strains by i.n. inoculation

Treatments	C.F.U./ mouse	Route of inoculation	<u>No.of survivors</u> No. challenged
P. m. 85020 (wild-type)	1.23×10^4	i.n.	1/5 (after 10 days)
" " "	1.23×10^3	i.n.	3/5 (after 10 days)
" " "	1.23×10^2	i.n.	5/5 (after 10 days)
P. m. Quetta (wild-type)	2.23×10^6	i.n.	1/5 (after 10 days)
" " "	2.23×10^5	i.n.	3/5 (after 10 days)
" " "	2.23×10^4	i.n.	4/5 (after 10 days)
Control	PBS 50 μ l	i.n.	5/5 (after 10 days)

Table 17: Protection properties of *P. multocida* strains

Treatments	C.F.U./ mouse	Vaccination route	Challenge dose i.p. (strain)	<u>No. of survivors</u> No. challenged
P. m .JRMT1 (85020 <i>aroA</i> mutant)	2.12×10^7	i.p.	1000 LD50 (wild-type 85020)	5/5 (by 6 days)
" "	2.12×10^7	i.p.	10,000 LD50 (wild-type 85020)	5/5 (by 6 days)
P. m .JRMT2 (85020 <i>aroA</i> mutant)	3.24×10^7	i.p.	1000 LD50 (wild-type Quetta)	5/5 (by 6 days)
" "	3.24×10^7	i.p.	10,000 LD50 (wild-type Quetta)	5/5 (by 6 days)
P. m. JRMT3 (Quetta <i>cya</i> mutant)	3.65×10^3	i.p.	100 LD50 (wild-type 85020)	0/5 (by 2 days)
" "	3.65×10^3	i.p.	100 LD50 (wild-type Quetta)	0/5 (by 3 days)
Control	PBS	i.p.	100 LD50 (wild-type 85020)	0/5 (by 36 hrs)
Control	PBS	i.p.	100 LD50 (wild-type Quetta)	0/5 (by 36 hrs)

and 10,000 LD₅₀ of wild type strains (Table 18). The results of this experiment again showed that the *P. multocida* JRMT1 and JRMT2 strains could protect the mice against challenge with either wild-type strain and no differences were observed between one or two vaccine doses.

3. 6. 3. Effect of different routes of vaccination (Experiment 9)

Since the presumed route of natural infection by *P. multocida* is via the respiratory tract, it is assumed that local defence mechanisms are important in preventing establishment of infection. To determine whether *P. multocida aroA* mutant could function as an effective live respiratory vaccine, we attempted to see which route of immunisation would provide better protection.

Groups of 4 mice were inoculated with graded doses of *P. multocida* JRMT1 or JRMT2 by the i.n. or s.c. routes and JRMT3 via the i.p. route. After two weeks all mice were inoculated again with the same doses of the same strains, except the control groups. Two weeks later mice were challenged with different doses of the wild-type strains by the i.p. route. The mice that were given two doses of *P. multocida* JRMT1 or JRMT2 strains i.n. were completely protected against i.p. challenge but this was not the case for a single dose. The mice given two doses of *P. multocida* JRMT1 or JRMT2 strains s.c. showed some protection, with JRMT1 performing better than JRMT2, but protection was not as good as via the i.n. route. Mice that were given *P. multocida* JRMT3 strain i.p. again did not show any protection (Table 19). The mice given two doses of the strains JRMT1 or JRMT2 by the i.n. route, were protected against heterologous challenge with strains Quetta or 85020 by the i.p. route.

Table 18: Protective properties of *aroA* mutants of *P. multocida* strains 85020 and Quetta by the i.p. route

1st immunization dose (C.F.U./ mouse)	2nd immunization dose (C.F.U./ mouse)	Challenge dose (strain)	No. of survivors No. challenged
2.3×10^7 P.m. JRMT1 (85020 <i>aroA</i> mutant)	1.1×10^7 P.m. JRMT1 (85020 <i>aroA</i> mutant)	1000 LD50 (wild-type 85020)	6/ 6
""	""	1000 LD50 (wild-type Quetta)	6/ 6
—————	""	1000 LD50 (wild-type 85020)	6/ 6
2.3×10^7 P.m. JRMT1 (85020 <i>aroA</i> mutant)	""	10,000 LD50 (wild-type 85020)	6/ 6
—————	""	""	6/ 6
3.0×10^7 P.m. JRMT2 (Quetta <i>aroA</i> mutant)	2.4×10^7 P.m. JRMT2 (Quetta <i>aroA</i> mutant)	1000 LD50 (wild-type 85020)	6/ 6
""	""	1000 LD50 (wild-type 85020)	6/ 6
—————	""	1000 LD50 (wild-type Quetta)	6/ 6
3.0×10^7 P.m. JRMT2 (Quetta <i>aroA</i> mutant)	""	10,000 LD50 (wild-type Quetta)	6/ 6
—————	""	""	6/ 6
Control	—————	100 LD50 (wild-type 85020)	0/ 6 (by 24 hrs)
Control	—————	100 LD50 (wild-type Quetta)	0/ 6 (by 24 hrs)

Table 19: protective properties of *P. multocida* strains JRMT1, JRMT2 and JRMT3 by different routes

1st immunisation dose (C.F.U./ mouse)	2nd immunisation dose (C.F.U./ mouse)	Vaccination route	Challenge doses i.p. (strain)	No. of survivors No. challenged
2.35×10^7 JRMT1 (85020 <i>aroA</i> mutant)	2.15×10^7 JRMR1 (85020 <i>aroA</i> mutant)	i.n.	1000 LD50 (wild-type 85020)	4/ 4 (by day 6 PI)
" "	" "	"	10000 LD50 (wild-type 85020)	4/ 4 (by day 6 PI)
-----	" "	"	1000 LD50 (wild-type 85020)	1/ 4 (by day 6 PI)
-----	" "	"	10000 LD50 (wild-type 85020)	0/ 4 (by day 6 PI)
2.35×10^7 JRMT1 (85020 <i>aroA</i> mutant)	2.15×10^7 JRMT1 (85020 <i>aroA</i> mutant)	"	1000 LD50 (wild-type Quetta)	3/ 4 (by day 6 PI)
2.35×10^7 JRMT1 (85020 <i>aroA</i> mutant)	2.35×10^7 JRMT1 (85020 <i>aroA</i> mutant)	s.c.	1000 LD50 (wild-type 85020)	2/ 4 (by day 6 PI)
" "	" "	"	10000 LD50 (wild-type 85020)	1/ 4 (by day 6 PI)
-----	" "	"	1000 LD50 (wild-type 85020)	0/ 4 (by day 3 PI)
-----	" "	"	10000 LD50 (wild-type 85020)	0/ 4 (by day 2 PI)
2.35×10^7 JRMT1 (85020 <i>aroA</i> mutant)	2.15×10^7 JRMT1 (85020 <i>aroA</i> mutant)	"	1000 LD50 (wild-type Quetta)	1/ 4 (by day 4 PI)
2.35×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	2.42×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	i.n.	1000 LD50 (wild-type Quetta)	4/ 4 (by day 6 PI)
" "	" "	"	10000 LD50 (wild-type Quetta)	4/ 4 (by day 6 PI)
-----	" "	"	1000 LD50 (wild-type Quetta)	2/ 4 (by day 6 PI)
-----	" "	"	10000 LD50 (wild-type Quetta)	1/4 (by day 6 PI)
2.35×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	2.42×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	"	1000 LD50 (wild-type 85020)	4/ 4 (by day 6 PI)
2.35×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	2.42×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	s.c.	1000 LD50 (wild-type Quetta)	0/ 4 (by day 3 PI)
" "	" "	"	10000 LD50 (wild-type Quetta)	1/ 4 (by day 3 PI)
-----	" "	"	1000 LD50 (wild-type Quetta)	0/ 4 (by day 3 PI)
-----	" "	"	10000 LD50 (wild-type Quetta)	0/ 4 (by day 2 PI)
2.35×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	2.42×10^7 JRMT2 (Quetta <i>aroA</i> mutant)	"	1000 LD50 (wild-type 85020)	0/ 4 (by day 3 PI)

Table 19: (continued)

2.67×10^7 JRMT3 (Quetta <i>cya</i> mutant)	2.67×10^7 JRMT3 (Quetta <i>cya</i> mutant)	i.p.	100 LD50 (wild-type Quetta)	0/ 4 (by day 3 PI)
" "	" "	"	1000 LD50 (wild-type Quetta)	0/ 4 (by day 2 PI)
-----	" "	"	100 LD50 (wild-type Quetta)	0/ 4 (by day 2 PI)
-----	" "	"	1000 LD50 (wild-type Quetta)	0/ 4 (by day 2 PI)
2.67×10^7 JRMT3 (Quetta <i>cya</i> mutant)	2.67×10^7 JRMT3 (Quetta <i>cya</i> mutant)	"	100 LD50 (wild-type 85020)	0/ 4 (by day 2 PI)
PBS	PBS	i.p.	100 LD50 (wild-type 85020)	0/ 4 (by day 2 PI)
PBS	PBS	i.p.	100 LD50 (wild-type Quetta)	0/ 4 (by day 2 PI)

3. 6. 4. Effect of different vaccination intervals and vaccination route

(Experiment 10)

Eight groups of 5 mice were vaccinated with *P. multocida* JRMT1 by the i.p., i.n. and s.c. routes. After 2 weeks, 4 groups were challenged i.p. with 1000 LD₅₀ of *P. multocida* 85020 and rest of them were challenged 2 weeks later by the i.p. route.

The results of this experiment (Table 20) showed that the mice given the vaccine i.p. were protected against i.p. challenge, but mice vaccinated by the i.n. route were not fully protected against challenge at either 2 or 4 weeks after vaccination. Vaccination by the s.c. route gave little or no protection.

3. 6. 5. Effect of different vaccination and different challenge routes

(Experiment 11)

Twelve groups of 3 mice were vaccinated with a single dose of *P. multocida* JRMT1 by different routes (i.p., i.n. and s.c.)

After 2 weeks, 1 group vaccinated by each route was challenged with 100 LD₅₀ of *P. multocida* 85020 either by the i.p., i.n. or s.c. routes. The strategy for this experiment is shown below.

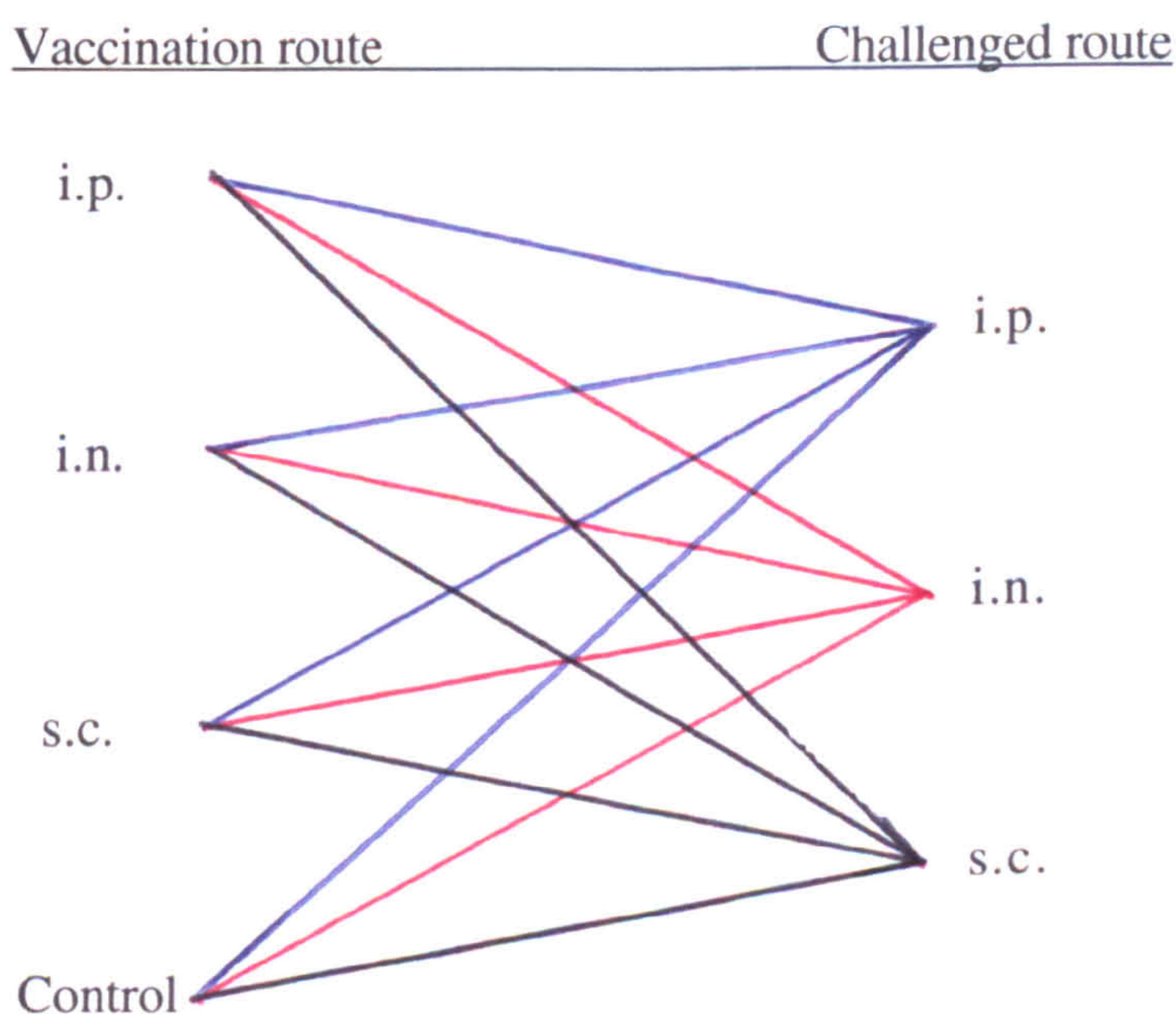


Table 20: Effect of different vaccination intervals and vaccination routes

Treatments	Vaccination route	C.F.U./ mouse	Challenge date i.p. (PI)*	No.of survivors No. challenged
P.m. JRMT1 (85020 <i>aroA</i> mutant)	i.p.	3.2×10^7	2 weeks	5/5
P. m. JRMT1 (85020 <i>aroA</i> mutant)	i.n.	3.2×10^7	2 weeks	3/5
P. m. JRMT1 (85020 <i>aroA</i> mutant)	s.c.	3.2×10^7	2 weeks	0/5
Control	-----	-----	2 weeks	0/5
P. m. JRMT1 (85020 <i>aroA</i> mutant)	i.p.	3.2×10^7	4 weeks	5/5
P. m .JRMT1 (85020 <i>aroA</i> mutant)	i.n.	3.2×10^7	4weeks	2/5
P. m. JRMT1 (85020 <i>aroA</i> mutant)	s.c.	3.2×10^7	4 weeks	1/5
Control	-----	-----	4 weeks	0/5

* All mice challenged with 100 LD₅₀ of *P. multocida* 85020 wild-type strain and checked for 6 days

The results of this experiment (Table 21) showed that the mice given vaccine i.p. were completely protected against challenge by all routes, but the mice given vaccine i.n. showed complete protection against i.n. challenge but only some protection against i.p. and s.c. challenge, whereas the mice vaccinated s.c. did not show any protection against challenge by any route. These results suggested that the best way for vaccination with these live attenuated strains was i.p. or i.n. routes.

3. 7. Spread of the bacteria into different organs

This part of the work was designed to compare the spread of the microorganisms to different organs when inoculated by different routes.

For this experiment, 8 groups of 8 mice were inoculated with *P. multocida* strains (85020, Quetta, JRMT1 and JRMT2) by i.p. and i.n. routes. All mice were weighed and identified individually. Every day, 2 mice from each group were sacrificed and the liver, lungs and spleen of each mouse were removed aseptically and transferred to pre-weighed Universal bottles. Also 0.1 ml of heart blood from each mouse was collected aseptically and diluted into 5 ml sterile PBS. The samples, 0.1 ml of neat and 10× serial dilutions for each organ were plated out onto BA and incubated overnight at 37°C. The colonies on each plate were counted and final C.F.U. of microorganism per gram of each organ and per ml of blood were calculated (Tables 22-25).

The results of this experiment showed that i.p. inoculation allowed proliferation of the wild-type parent strains in all of the tissues by 24 hrs, and mice developed a fatal peritonitis and septicaemia and died by 36 hrs. Inoculation i.n. also led to proliferation of the parent strains in all tissues, but to a lower level. By 48 hrs the parent strain were apparently beginning to be cleared, but proliferation had increased again by 72 hrs. An initial challenge dose of $\approx 10^4$ C.F.U. of *P. multocida* 85020 increased to more than 10^{13} organisms per gram of lung in 72 hrs in one instance. Interestingly, the Quetta

Table 21: Effect of different vaccination and different challenge routes

Treatments	Vaccination route	C.F.U./ mouse	Challenge route*	No.of survivors No. challenged
P. m. JRMT1 (85020 <i>aroA</i> mutant)	i.p.	2.6×10^7	i.p.	3/3 (by day 6 PI)
" " "	"	2.6×10^7	i.n.	3/3 (by day 6 PI)
" " "	"	2.6×10^7	s.c.	3/3 (by day 6 PI)
P. m. JRMT1 (85020 <i>aroA</i> mutant)	i.n.	2.6×10^7	i.p.	1/3 (by day 6 PI)
" " "	"	2.6×10^7	i.n.	3/3 (by day 6 PI)
" " "	"	2.6×10^7	s.c.	0/3 (by day 3 PI)
P. m. JRMT1 (85020 <i>aroA</i> mutant)	s.c.	2.6×10^7	i.p.	0/3 (by day 3 PI)
" " "	"	2.6×10^7	i.n.	0/3 (by day 3 PI)
" " "	"	2.6×10^7	s.c.	0/3 (by day 3 PI)
Control	----	-----	i.p.	0/3 (by 36 hrs PI)
Control	----	-----	i.n.	0/3 (by day 4 PI)
Control	----	-----	s.c.	0/3 (by day 2 PI)

* Two weeks after vaccination all mice were challenged with the appropriate dose of *P. multocida* 85020 wild-type strain (100 LD50)

Table 22: Viable counts of bacteria in different organs after 24 hrs

Mouse No.	Challenge Strain	Challenge dose C.F.U./mouse	Route of inoculation	Hrs P I	C.F.U. per Gram of:			
					Liver	Lung	Spleen	Blood
1	P. m. 85020	723	i.p.	24 hrs	8.52×10^{10}	1.54×10^{11}	4.74×10^{12}	1.03×10^9
2	" "	" "	"	"	1.65×10^{10}	7.33×10^{10}	6.9×10^{11}	1.33×10^9
3	P. m. JRMT1	4.1×10^7	"	"	120	Neg	7000	1.55×10^4
4	" "	" "	"	"	80	"	1.3×10^4	500
5	P. m. Quetta	337	"	"	1.2×10^{11}	1.49×10^{10}	1.45×10^{13}	2.0×10^{10}
6	" "	" "	"	"	2.15×10^{10}	3.85×10^{11}	1.62×10^{13}	1.0×10^{10}
7	P. m. JRMT2	6.68×10^7	"	"	360	Neg	1200	550
8	" "	" "	"	"	120	"	3000	400
9	P. m. 85020	7.23×10^4	i.n.	24 hrs	180	9900	5.2×10^5	1500
10	" "	" "	"	"	2.76×10^4	7.26×10^6	4.2×10^4	1500
11	P. m. JRMT1	4.1×10^9	"	"	Neg	7.92×10^4	Neg	50
12	" "	" "	"	"	"	3.63×10^4	"	30
13	P. m. Quetta	3.37×10^6	"	"	1200	3.47×10^4	1.9×10^5	5.0×10^4
14	" "	" "	"	"	1140	1650	1000	500
15	P. m. JRMT2	6.68×10^9	"	"	Neg	8.35×10^4	Neg	1000
16	" "	" "	"	"	240	7.69×10^5	"	500

Neg= No colonies detected

Table 23: Viable counts of bacteria in different organs after 48 hrs

Mouse No.	Challenge Strain	Challenge dose C.F.U/ mouse	Route of inoculation	Hrs PI	C.F.U. per Gram of:			
					Liver	Lung	Spleen	Blood
1	P. m. 85020	723	i.p.	48	-----	-----	-----	-----
2	" "	" "	"	"	-----	-----	-----	-----
3	P. m. JRMT1	4.1×10^7	"	"	Neg	Neg	Neg	Neg
4	" "	" "	"	"	"	"	"	"
5	P. m. Quetta	337	"	"	-----	-----	-----	-----
6	" "	" "	"	"	-----	-----	-----	-----
7	P. m. JRMT2	6.68×10^7	"	"	Neg	Neg	1000	Neg
8	" "	" "	"	"	60	"	Neg	"
9	P. m. 85020	7.23×10^4	i.n.	"	Neg	6.2×10^8	1000	Neg
10	" "	" "	"	"	"	9.9×10^5	Neg	100
11	P. m. JRMT1	4.1×10^9	"	"	Neg	2.64×10^4	"	Neg
12	" "	" "	"	"	"	9900	"	"
13	P. m. Quetta	3.37×10^6	"	"	Neg	3.48×10^6	"	"
14	" "	" "	"	"	"	2.18×10^4	"	"
15	P. m. JRMT2	6.68×10^9	"	"	Neg	2.64×10^4	"	"
16	" "	" "	"	"	"	2.31×10^4	"	"

Neg= No colonies detected

---- = Mouse died before 48 hrs

Table 24: Viable counts of bacteria in different organs after 72 hrs

Mouse No	Challenge Strain	Challenge dose C.F.U./mouse	Route of inoculation	Hrs PI	C.F.U. per Gram of:			
					Liver	Lung	Spleen	Blood
1	P. m. 85020	723	i.p.	72 hrs	-----	-----	-----	-----
2	" "	" "	"	"	-----	-----	-----	-----
3	P. m. JRMT1	4.1×10^7	"	"	Neg	Neg	Neg	Neg
4	" "	" "	"	"	"	"	"	"
5	P. m. Quetta	337	"	"	-----	-----	-----	-----
6	" "	" "	"	"	-----	-----	-----	-----
7	P. m. JRMT2	6.68×10^7	"	"	Neg	Neg	Neg	Neg
8	" "	" "	"	"	"	"	"	"
9	P. m. 85020	7.23×10^4	i.n.	72 hrs	2.77×10^{11}	2.58×10^{13}	6.28×10^{10}	2.23×10^7
10	"	" "	"	"	7200	9.9×10^{10}	1000	163
11	P. m. JRMT1	4.1×10^9	"	"	Neg	4.39×10^4	Neg	Neg
12	" "	" "	"	"	"	4.85×10^4	"	"
13	P. m. Quetta	3.37×10^6	"	"	4.32×10^4	1.18×10^5	4.3×10^3	210
14	" "	" "	"	"	7.68×10^3	2.67×10^5	2.2×10^4	3.1×10^4
15	P. m. JRMT2	6.68×10^9	"	"	Neg	330	Neg	Neg
16	" "	" "	"	"	"	2970	"	"

Neg= No colonies detected

---- = Mouse died before 72 hrs

Table 25: Viable counts of bacteria in different organs after 96 hrs

Mouse No.	Challenge strain	Challenge dose C.F.U/mouse	Route of inoculation	Hrs PI	C.F.U. per Gram of:			
					Liver	Lung	Spleen	Blood
1	P. m. 85020	723	i.p.	96 hrs	-----	-----	-----	-----
2	" "	" "	"	"	-----	-----	-----	-----
3	P. m. JRMT1	4.1×10^7	"	"	Neg	Neg	Neg	Neg
4	" "	" "	"	"	"	"	"	"
5	P. m. Quetta	337	"	"	-----	-----	-----	-----
6	" "	" "	"	"	-----	-----	-----	-----
7	P. m. JRMT2	6.68×10^7	"	"	Neg	Neg	Neg	Neg
8	" "	" "	"	"	"	"	"	"
9	P. m. 85020	7.23×10^4	i.p.	96 hrs	9.6×10^9	8.28×10^8	5.0×10^9	4.78×10^4
10	" "	" "	"	"	7.2×10^7	5.81×10^9	2.0×10^6	1.23×10^4
11	P. m. JRMT1	4.1×10^9	"	"	Neg	Neg	Neg	Neg
12	" "	" "	"	"	"	"	"	"
13	P. m. Quetta	3.37×10^6	"	"	Neg	Neg	Neg	Neg
14	" "	" "	"	"	"	"	"	"
15	P. m. JRMT2	6.68×10^9	"	"	Neg	99	Neg	Neg
16	" "	" "	"	"	"	165	"	"

Neg= No colonies detected

---- = Mouse died before 96 hrs

strain was cleared by 96 hrs, but strain 85020 persisted. For the *aroA* mutants, inoculation i.p. allowed proliferation into all tissues except the lungs by 24 hrs, although at a much reduced level compared to the wild-type parent strains. The bacteria were essentially cleared by 48 hrs and the mice survived. Inoculation i.n. allowed some proliferation in the lungs, which still persisted at 96 hrs for the Quetta strain. Low numbers of bacteria were also detected in blood and liver at 24 hrs post-inoculation but not at later times.

3. 8. Phagocytosis assay

The capacity of the B:2 *P. multocida* strains to invade and survive within macrophage-like cells was studied. Prolonged survival of the organism within macrophage, a feature exhibited by pathogens such as *Salmonella typhimurium*, would indicate that cell-mediated immune response would be needed to eliminate the organism from this intracellular niche.

Table 26 and 27 shows the assay protocol and results. From the digitonin-treated RAW cells, 10 \times serial dilutions were prepared from each sample in PBS and 100 μ l portions spread on BA and incubated at 37 $^{\circ}$ C for 48 hrs. The viable count results are shown in Table 27. Taking the data from the control plates first, exposure of the RAW cells mixed with *P. multocida* to digitonin without first removing the antibiotic, resulted in no colonies detected (wells 11 and 12). This indicates that no bacteria survived exposure to the antibiotic. Only bacteria which were able to take up an intracellular location could survive the antibiotic treatment, as long as the antibiotic was removed by washing before lysis of the mammalian cells with digitonin. No antibiotic treatment gave surviving colonies at 10 $^{-4}$ dilution (well 9 and 10) indicating that washing cells did not remove all the bacteria. The remainder of the wells gave results for wells where the RAW cells had been exposed to the *P. multocida* strain for 2 hrs, followed by washing and antibiotic treatment to remove extracellular bacteria. A further 3 washes was followed by exposure to digitonin at time intervals to release

Table 26: Phagocytosis assay procedure using RAW mouse macrophage-like cells

1. P.m. 85020	+	cells*	for 2 hrs	2x wash	added antibiotics	left for 2 hrs	3x wash	-----	added digitonin	left for 30 min	plated out
2. "	"	"	"	"	"	"	"	-----	"	"	"
3. "	"	"	"	"	"	"	"	left for 60 min	added digitonon	left for 30 min	plated out
4. "	"	"	"	"	"	"	"	" 120 "	"	"	"
5. P.m. JRM T1	+	"	"	"	"	"	"	-----	added digitonin	left for 30 min	plated out
6. "	"	"	"	"	"	"	"	-----	"	"	"
7. "	"	"	"	"	"	"	"	left for 60 min	added digitonin	left for 30 min	plated out
8. "	"	"	"	"	"	"	"	" 120 "	"	"	"
9. P.m. 85020	+	cells	for 2 hrs	2x wash	no antibiotic	left for 2 hrs	3x wash	-----	added digitonin	left for 30 min	plated out
10. P.m. JRM T1	+	"	"	"	"	"	"	-----	"	"	"
11. P.m. 85020	+	cells	for 2 hrs	2x wash	added antibiotic	left for 2 hrs	no wash	-----	added digitonin	left for 30 min	plated out
12. P.m. JRM T1	+	"	"	"	"	"	"	-----	"	"	"
13. P.m. 85020		no cells	"	no wash	added antibiotic	-----	-----	-----	added digitonin	left for 30 min	plated out
14. P.m. JRM T1		"	"	"	"	-----	-----	-----	"	"	"

* RAW mouse macrophage-like cells

Table 27: Viable counts from the phagocytosis assay

No	Treatments	Neat	Dil. 10 ⁻¹	Dil. 10 ⁻²	Dil. 10 ⁻³	Dil.10 ⁻⁴
1	P.m. 85020+ cells	Non countable	2.19 × 10 ⁴	1.11 × 10 ⁴	----	
2	" " "	" "	2.18 × 10 ⁴	2000	----	
3	" " "	7.88 × 10 ⁴	1.14 × 10 ⁴	1700	----	
4	" " "	4.74 × 10 ⁴	7800	800	----	
5	P.m. JRMT1+ cells	Non countable	8.74 × 10 ⁴	1.31 × 10 ⁴	600	
6	" " "	" "	9.24 × 10 ⁴	1.88 × 10 ⁴	1700	
7	" " "	" "	7.28 × 10 ⁴	7500	1000	
8	" " "	" "	5.20 × 10 ⁴	3700	500	
9	P.m. 85020 + cells	Confluent	Non countable	1.08 × 10 ⁴	1400	200
10	P.m. JRMT1+ cells	"	Non countable	4.35 × 10 ⁴	3800	400
11	P.m. 85020 + cells	----	----			
12	P.m. JRMT1+ cells	----	----			
13	P.m. 85020 -----	----	----			
14	P.m. JRMT1 ----	----	----			

---- = No colonies detected

intracellular bacteria. The results indicate that the wild-type parent strain 85020 could enter RAW cells and survive for at least 2 hrs. Interestingly, the *aroA* mutant became intracellular to a greater extent than its parent strain, but its number decreased in a similar manner over a period of 2 hrs. This experiment was repeated and gave essentially similar results.

4. DISCUSSION

4. 1. Construction of defined mutants of *P. multocida* strains

Despite the availability of different vaccines, including killed whole-cell and live-attenuated vaccines, the control of haemorrhagic septicaemia in cattle and buffalo still remains problematic because the current vaccines are not sufficiently effective and require repeated administration. The ideal and more preferred live vaccine for HS should mimic the natural infection and be capable of surviving for a period of time within the host sufficient to stimulate protective immunity. It should contain a genetically-defined mutation(s) which renders the vaccine strain unable to establish continued growth *in vivo* and which minimises the risk of reversion to the wild-type. Rationally-attenuated *aro* mutants of several bacterial species provide some of these criteria and have been demonstrated to be strongly immunogenic and to confer solid protection against experimental challenge at least in the mouse model, but also in some instances in cattle (Ramos *et al.* 1998) (Table 3).

In this section we have reported the molecular cloning of the *aroA* gene of *P. multocida* B:2 as well as the construction of mutant strains of *P. multocida* strains 85020 and Quetta, by insertional inactivation of the *aroA* gene with a Km^R cassette. Additional data indicated that the *aroA* strains have potential as effective live vaccines.

The *aroA* mutant strains were functionally evaluated by EPSP synthase assay and animal tests. In comparison to *P. multocida* 85020 and Quetta, the wild-type parent strains, JRMT1 and JRMT2, the *aroA* mutant strains, did not show any detectable activity for EPSP synthase. EPSP synthase occurs at relatively low levels in bacteria; for example, for *Escherichia coli* it must be purified over 2000-fold to achieve homogeneity (Lewendon and Coggins, 1983). As reported by Lewendon and Coggins (1983) it was very difficult to

estimate the amount of enzyme in crude extracts of *E. coli* (ATCC 14948), but after $(\text{NH}_4)_2\text{SO}_4$ fractionation EPSP synthase activity was located in the 50-70% saturation fraction as 0.021 units/ mg. Also in a report by Duncan *et al.* (1994) the specific activity of *E. coli* K12 (ATCC14948) was cited as 0.004 units/ mg.

Survival of mice and the LD₅₀ values, demonstrated that the *P. multocida aroA* mutants are highly attenuated for prolonged colonisation and infection of internal organs relative to the wild-type parent strains. The persistence of the mutant strains in mouse tissues, as determined by the recovery of viable bacteria was shorter than that described by Dougan *et al.* (1989) for a genetically attenuated *Salmonella typhimurium aroA* mutant. The more rapid clearance of *P. multocida aroA* mutants may be related to possible different colonisation sites in the tissues and to difference between the species in their ability to survive intracellularly. Clearance of *P. multocida aroA* mutants from the lung was similar to that described for a *Bordetella pertussis aroA* mutant where Roberts *et al.* (1990) found that the number of *Bordetella pertussis aroA* bacteria decreased daily until they were cleared from the lungs between days 5 and 8.

As noted by Tacket *et al.* (1992), pathogenic *Salmonella typhimurium*, *Salmonella dublin* and *Salmonella typhi* have been successfully attenuated by insertional inactivation or deletion of *aroA* genes. Such *aroA* strains of *S. typhimurium* used as live oral vaccines are safe in mice and calves and protect these animals against lethal challenge with virulent parent strains. Homchampa *et al.* (1992) constructed an *aroA* mutant of *P. multocida* serotype A:1, a fowl cholera agent, and they found that it was highly attenuated and protected mice against wild-type challenge.

The behaviour of *aroA* mutants is not necessarily the same in all bacterial species. Oyston *et al.* (1996) found that the *aroA* mutant of *Yersinia pestis* was

attenuated in the guinea-pig but still remained virulent in mice. In guinea-pigs, immunity was induced with a single low dose of the *aroA* mutant and protected them against challenge with wild-type virulent strain. The *aroA* mutant was still virulent in mice although there was an increased time to death. In comparison to the *aroA* mutant of *Yersinia enterocolitica* that is attenuated in mice, *Y. pestis* produces a toxin, which is active in mice. This may go some way towards explaining the observed host-dependent nature of the attenuation.

The present data confirm the use of rational attenuation as a means of constructing non-reverting, live vaccine strains of *P. multocida*. When injected into mice, the *aroA* mutant strains described here showed considerable loss of virulence and no illness was observed following injection of 10^7 or 10^9 C.F.U./ mouse by the i.p. or i.n. routes respectively. This compares with an LD₅₀ of less than 50 and 10^3 C.F.U./ mouse for the parent strains by the i.p. or i.n. routes, respectively. Inability to isolate the *aroA* mutants from peripheral blood of mice at 48 hrs after i.p. injection indicated that these strains had greatly reduced ability to survive *in vivo*.

Live fowl cholera vaccine administered in drinking water was reported to stimulate cross-immunity to a heterologous strain in chickens (Heddleston *et al.*, 1975), but Adler *et al.* (1996) reported that the *aroA* mutant of *P. multocida* A:1 strain could not protect mice against heterologous challenge by i.p. route and they suggested that this may be depend either an excessive attenuation or that host-specific cross-protective antigens induced in the chicken were not expressed in mice due to differing environmental conditions such as body temperature. Finally they suggested that the cross-immunity is manifested only when vaccination and/or challenge occurs by alternative oral or i.n. routes. In the experiments reported here i.p. immunisation with one dose of *P. multocida* JRMT1 (*aroA* mutant of strain 85020) and JRMT2 (*aroA* mutant of strain Quetta) completely protected mice against homologous and

heterologous challenge with 1000 LD₅₀ of the wild-type strains (Tables 18 and 19). As reported by Adler *et al.* (1999) it has been speculated that the *in vivo* expression of cross-protective antigens may be induced by the low concentration of iron in the host tissues. Iron, essential for bacterial growth, is largely unavailable *in vivo* as a result of binding by host proteins such as transferrin and lactoferrin. Pathogenic bacteria, such as *P. multocida*, overcome these iron limitations by expressing of iron regulatory proteins. The mechanism of *in vivo* iron acquisition by *P. multocida* is not clear, but some high molecular weight outer membrane proteins (OMPs) (Snipes *et al.*, 1988; Choi *et al.*, 1991) were identified when *P. multocida* was grown under iron limiting conditions. Adler *et al.* (1999) found that these outer membrane proteins appear to be expressed *in vivo* and it has been speculated that these iron-regulated OMPs may be involved in cross-protective immunity. Glisson *et al.* (1993) showed that whole-cell bacterins grown under iron limiting conditions could stimulate cross-immunity in mouse model. Ruffolo *et al.* (1998) demonstrated that OMPs of *P. multocida* produced under iron-limiting conditions, which mimic the concentration of iron *in vivo*, could induce cross-protective immunity in mice whereas OMPs produced under iron replete conditions stimulated only homologous protection.

Since the route of natural infection by *P. multocida* is probably via the respiratory tract, we evaluated the effect of i.n. vaccination. One dose of i.n. live *aroA* vaccine of either strain induced relatively poor levels of protection against an i.p. challenge with the homologous wild-type *P. multocida* strains. However, after a second dose of live cells, there was a sharp increase in the number of survivors of a lethal homologous or heterologous challenge (Table 19). As was found by Collins (1973), one dose of formalin killed *P. multocida* strain when used by the i.n. route produced partial protection in the mouse model against i.p. challenge whereas two doses of i.n. vaccine produced strong immunity against the wild-type strains. Smith *et al.* (1981) reported that

i.n. immunisation with a *P. multocida* bacterin protected the mice from an i.p. *P. multocida* challenge. In the work of Collins and Woolcock (1976), mice were protected from an i.p. challenge if immunised i.p. with formalin-killed *P. multocida*, but i.p. immunised mice responded poorly to an i.n. challenge and some mice died as a result of the challenge. Thus, according to the results obtained on Table 18, the local defence mechanisms may have a significant role in preventing establishment of infection.

In *aro* mutants, the attenuation might be either as a result of unavailability of metabolites derived from the biosynthetic pathway due to inhibition of a key enzymatic step (downstream effect) or because of accumulation of toxic substrate or related metabolite due to enzyme inhibition (upstream effect). As mentioned by Gunel-Ozcan *et al.* (1997), shikimate pathway inhibition studies on plants and the original studies on attenuation of *Salmonella typhimurium aroA* mutant suggest that downstream effects are probably responsible for the attenuation of *aroA* mutants. For the *Bordetella pertussis aroA* mutant, Roberts *et al.* (1990) proposed that attenuation was probably due to starvation *in vivo* of an essential aromatic metabolite rather than indirect effects on the expression of virulence factors. Our finding confirmed these suggestions, because when *P. multocida* strains JRMT1 and JRMT2 (the *aroA* mutants of strains 85020 and Quetta respectively) were inoculated into aromix supplemented PMM, they grew to an $OD_{450nm} \approx 0.5-0.6$, in comparison to $OD \approx 0.03-0.04$ in PMM without aromix supplementation (Fig. 22)

4. 2. Construction of double mutants

As cited by Curtiss (1990), a difficulty with auxotrophs is the potential to reverse the attenuating mutation phenotypically by inclusion of the required nutrient in the diet of animals inoculated with the auxotrophic mutant. Thus ideally the mutated vaccine strains should have two or more attenuating deletion mutations to preclude loss of the attenuation traits by reversion, gene

transfer or growth supplements. This is critical for the safety of live vaccine strains and is an essential requirement, because live vaccine strains used for protection of animals might sometimes be transmitted through the food chain to humans. Also, Curtiss (1990) reported that phenotypic reversion occurred readily for *pab* and *pur* mutants of *Salmonella typhimurium*. Thus strains with two or more mutations conferring auxotrophy have been constructed. However, as noted by Levine *et al.* (1987), a construct of *Salmonella typhi* with two mutations in the *aroA* and *purA* genes was not immunogenic in humans because of the severe attenuation imposed by this combination of mutations. A similar observation was made by O'Callaghan *et al.* (1988) for Δ *aroA*, Δ *purA* mutations of *Salmonella typhimurium* which were not immunogenic in mice. On the other hand, Hone *et al.* (1991) constructed a double *aroD aroC* mutant of *Salmonella typhi* and found that the mutant strains were highly attenuated and were protective in a mouse model against wild-type challenge. Tacket *et al.* (1992) constructed double mutation strains such as *aroA*, *aroD* and *cya*, *crp* of *Salmonella typhi* that were highly attenuated and yet immunogenic in human. Thus, double mutations affecting the same broad physiological function retain immunogenicity, but retain the potential problem of phenotypic reversion.

An attempt was made, therefore, to construct double mutations in *P. multocida* strains. For this purpose the *cya* gene was chosen and inactivated by deletion and insertion of a Km^R cassette, and also by deletion and insertion of an Ap^R cassette, because the recipient strains contained an *aroA* mutation with a Km^R insertion. No reports were found in which a defined *P. multocida cya* mutant was constructed and tested for virulence and protection properties. This locus was chosen for study because Tacket *et al.* (1992) reported that a strain of *Salmonella typhi* carrying a deletion in the *cya* gene had reduced virulence, but was immunogenic in humans. Unfortunately, allelic exchange in the *cya* locus proved to be very difficult. Using this strategy, it was possible to

introduce plasmids pJRMT10, containing the Km^R inactivated *cya* gene of *P. multocida* strain 85020, and pJRMT11, containing the Km^R inactivated *cya* gene of *P. multocida* strain Quetta, into wild-type *P. multocida* strains. The results of subsequent Southern blot analysis were consistent with integration of the vector into the *cya* gene, but the size of the resulting restriction fragments which hybridised to the *cya* gene probe suggested duplication in this region. Such an event could occur through homologous recombination of daughter genomes at the intact and mutated alleles of the *cya* locus during DNA replication under kanamycin selection pressure. Donnenberg and Kaper (1991) reported duplication of the *eae* gene of *E. coli* under ampicillin selection pressure. The Southern blot results with the *P. multocida* transformants, showed that Km^R clones had a different pattern to the wild-type parent strains, and indicated that the first crossing-over event happened with high frequency and the plasmid containing the Km^R-interrupted *cya* gene integrated into the genome, but for unknown reasons the second crossing-over event never happened except in one clone. This *cya*::Km^R strain (JRMT3), when tested in mice for virulence and protection properties by the i.p. route, showed some attenuation with a LD₅₀ of about 1000 C.F.U./ mouse in comparison to the wild-type parent strain with a LD₅₀ less than 50 C.F.U./ mouse, but it did not induce protection. The possible reason for this may be that the low number of organisms inoculated to avoid toxicity was not enough for stimulation of the immune system. Curtiss and Kelly (1987) reported that a *Salmonella typhimurium cya* mutant was highly attenuated in the mouse model by oral administration with more than 10⁹ C.F.U. and protected the mice against challenge with 10000 LD₅₀ of the wild-type parent strain by the oral route.

Because the *cya* construct JRMT3 was only slightly attenuated it was considered that introduction of *cya* and *aroA* mutations into the same *P. multocida* strain might create a vaccine candidate that retained good immunogenicity but which might have lost the problem of toxicity. Data

presented here indicated that, at high doses, the *aroA* mutant strains exhibited some toxicity (Table 12) which might be reduced by the introduction of a second attenuating *cya* mutation.

For construction of double mutant strains, *P. multocida* strains JRMT1 and JRMT2 (*aroA* mutants) were used as recipients and repeated attempts were made to introduce the Ap^R-interrupted *cya* gene by electroporation. Again the *cya* locus showed problems for allelic exchange with just the first cross-over event apparently occurring, and when Ap^R clones were checked by Southern blotting, they showed both wild-type and mutated hybridisation patterns at the *cya* locus. Harman and Dobrogosz (1983) noted that a *cya* mutant of *E. coli* was difficult to manipulate because it had a tendency to revert to a wild-type phenotype at high frequency in liquid or on agar culture. They proposed that, in *E. coli*, one mechanism for *cya* suppression is the formation of compensatory mutations in the *crp* gene.

Another possibility that was explored was the construction of a second mutation site in the *P. multocida* chromosome by targeting the *galE* gene. As demonstrated by Germanier and Furer (1975) the *galE* mutants of *Salmonella typhimurium* and *Salmonella typhi*, which are unable to synthesise the enzyme UDP-galactose-4-epimerase, were avirulent and immunogenic in the mouse model by the i.p. route. It is not clear, however, whether it is the sensitivity to galactose (resulting in cell lysis) or the inability to synthesise complete LPS which renders the cells susceptible to non-specific host defense mechanisms, leads to avirulence *in vivo*. Unfortunately, the Km^R-interrupted *galE* gene again presented problems for allelic exchange in *P. multocida* strains. One of the main problems was multiplication of suicide vectors, pJRMT17, pJRMT18, pJRMT19 and pJRMT20, in *P. multocida* strains, because the plasmids with *ColE1* origin were maintained in the presence of kanamycin and actually did not appear to act as suicide vectors in *P. multocida* strains. The

same phenomenon has been described in other strains of *P. multocida*. Homchampa *et al.* (1992, 1994) and Henestrosa *et al.* (1997) reported that the suicide vectors pMEC124 and pMEC200 (pUC18-base plasmids) and pUA659 (a pGP704-base plasmid) respectively were able to replicate in *P. multocida* serotypes A:1, *P. haemolytica* serotype A:1 and *P. multocida* serotype D:12 strains respectively. It is possible that the frequency of gene recombination at the *galE* locus in *P. multocida* is relatively low, since only a few transconjugants were obtained with either *P. multocida* 85020 or Quetta strains from the huge number of colonies screened. When these colonies were subcultured for selection of the deleted *galE* mutants, no colonies of the correct genotype were obtained.

The behavior of *galE* mutant strains in different bacterial species is not the same. In contrast to *galE* mutants of *E. coli* and *Haemophilus influenzae*, *galE* mutants of *Neisseria meningitidis* (Jennings *et al.*, 1993) and *Neisseria gonorrhoeae* (Robertson *et al.*, 1993), although they produced rough LPS, were not galactose sensitive, because they did not utilise galactose as a carbon source. In *Yersinia enterocolitica*, mutation in the *galE* gene either did not affect the Leloir pathway or the organism has an alternative pathway for galactose utilisation, because when this mutants was grown in media containing glucose or galactose, neither sugar had any effect on the LPS profile of the mutant (Pierson and Carlson, 1996). In *Bacillus subtilis*, as reported by Krispin and Allmansberger (1998), glucose is able to alleviate the effects of galactose, as it does in *galE* mutants of enterobacteria, but causes cell death during late log phase. Thus, one task of the GalE protein is therefore to protect the cell from the toxic effects of galactose and glucose or derivatives of both sugars which accumulate in its absence. As was found by Henestrosa *et al.* (1997), a *P. multocida galE* mutant of serotype D:12 that they constructed showed some slight attenuation and delay in time to death of

mice and they proposed that the *galE* mutation could be useful as part of double mutant strains for construction of live vaccines. The reasons why the experiments reported here failed to achieve the required *galE* construct are not clear, as the procedures adopted were essentially similar to those reported by Henestrosa *et al.* (1997).

4. 3. Construction of marker-free *P. multocida* strains

Because the properties of the *aroA* derivatives JRMT1 and JRMT2 suggested that attenuation of the *aroA* locus would provide a good vaccine candidate, it was decided to construct a marker-free derivative more suitable for use as a vaccine.

In this part of the work, the construction of *P. multocida* strains with a deletion mutation in *aroA* gene were described and also their virulence and protection properties, using i.p. challenge of mice. An improved procedure for allelic exchange was developed using the *Bacillus subtilis sacB* gene as a positive selective marker.

Most currently available gene cloning vectors for bacteria are based on multicopy plasmids carrying antibiotic resistance markers. Although recombinant plasmids may be readily maintained in bacteria cultivated in culture under controlled environmental conditions, they are frequently unstable within host organisms when grown in the absence of antibiotics. Furthermore, antibiotic resistance markers are generally perceived as undesirable in organisms designed for release in large quantities, such as live vaccines or bacteria developed to degrade chemical pollutants.

Construction of deleted *aroA* mutants according to the procedure of Homchampa *et al.* (1997), by a combination of repeated subculture and auxotrophic enrichment, proved unsuccessful. When selected Km^R clones were checked by PCR, they showed the wild type pattern. In another attempt,

screening for sensitivity to ampicillin or kanamycin carried by the vectors was attempted. When the resulting colonies were checked by PCR, it was found that most clones contained both wild-type and mutated alleles and repeated subculturing in the absence of antibiotic to promote loss of the integrated plasmid via a second crossing-over was not successful. After further subculture, the clones lost the mutated allele and reverted to the wild-type.

Finally, for first time in *Pasteurella* spp., the *sacB* gene of *Bacillus subtilis* was used as a positive selective marker and was found to be more effective in *P. multocida* for induction of the double cross-over event. The *sacB* locus encodes the enzyme levan sucrase, which is toxic for Gram-negative bacteria only in presence of sucrose (Gay *et al.*, 1985). The *sacB* gene was included in to the suicide vector pAKA19 to provide a conditionally lethal phenotype, but was first cloned into plasmid pAKA16 to check expression of the sucrose sensitivity phenotype in *P. multocida* strains. When *P. multocida* strains JRMT8 (85020 containing the *sacB* gene) and JRMT9 (Quetta containing the *sacB* gene) were plated on BHI agar supplemented with 5% (v/v) sheep blood containing 5% (w/v) sucrose, after overnight incubation at 37 °C, the predicted number of colonies had grown up, but when left for about 40 hrs at 37 °C, all colonies were liquified. This indicated that the *sacB* gene phenotype was expressed in *P. multocida* strains and rendered them sensitive to sucrose, and that it could be used as a positive selection marker in these strains of *P. multocida*. Jost *et al.* (1997) used the *Bacillus subtilis sacB* gene as a counter-selectable marker in serogroups A:1 (PBA-100 and X-73), A:3 (P-1059), A:4 (P-1662) and B:2 (M1404) strains of *P. multocida*. But they found that expression of the *sacB* gene failed to render any of the strains sensitive to sucrose, and they reported that the *sacB* gene cannot be used as a positive selection system for allelic exchange in *P. multocida* strains. They suggested that, because fructose is a major component of the capsule of *P. multocida* strains (Rimler and Rhoades, 1989) the lack of cell death as a result of

expression of *sacB* gene may be attributed to the ability of this organism to utilise the activated fructose for inclusion into capsular material. The reason for this discrepancy between the work reported here and that of Jost *et al.* (1997) is unclear. Perhaps Jost *et al.* did not leave their plates long enough to see expression of the sucrose-sensitive phenotype.

The *P. multocida* strains, after transformation of the suicide vector and integration of the mutated version of *aroA* gene into the *aroA* locus, were exposed to sucrose to directly select for organisms that had undergone a second recombination event resulting in the loss of the suicide vector. As reported, different non-antibiotic markers have been used in Gram-negative bacteria for induction of marker-free mutations. Herrero *et al.* (1990) reported successful results with three different markers such as resistance to the herbicide bialphose, to mercuric salts and organomercurial compounds and to arsenite in *Pseudomonas putida* and *Klebsiella pneumoniae*. Also Donnenberg and Kaper (1991) used the *sacB* gene of *Bacillus subtilis* to construct an *eae* deletion mutant in enteropathogenic *E. coli*. In addition to plasmids pJRMT31 and pJRMT32 containing the *aroA* deletion allele, we developed a *sacB*-containing suicide vector for general use and designated this as pJRMT30.

However, for construction of a marker-free *galE* mutant, again there were problems during the allelic replacement event. After transfer of suicide plasmids containing the deleted *galE* allele, with or without the *sacB* gene, into *P. multocida* strains by electroporation or transconjugation, PCR analysis demonstrated an unexpected amplicon, larger than the wild-type *galE* gene (Fig. 57). However, inoculation of the strains into minimal medium containing galactose showed suppression of growth compared to the wild type strains (Fig. 58). These data indicated that the suicide vectors containing the deleted *galE* allele integrated into the *P. multocida* chromosome as a first cross-over event but, in a second cross-over event, part of *galE* locus was

duplicated. After subculture, the *galE* mutation reverted to the wild-type or, as reported by Germanier and Furer (1971), the frequency of secondary mutations in the *galE* locus was sufficiently high *in vitro* to select for the wild-type genotype.

4. 4. Spread of *P. multocida* into different organs of the mouse

The BALB/c mice were highly susceptible to infection by *P. multocida* whether introduced i.p. or i.n.. The growth of the challenge organism in the liver, lung, spleen and blood was quantitated daily for up to 4 days. *P. multocida* parent strains were able to multiply very rapidly *in vivo*, so that the introduction of a small number of viable bacteria into the peritoneal cavity of non-vaccinated mice quickly resulted in an *in vivo* population of more than 10^9 viable organisms per gram of liver, lung, spleen and blood resulting in death of the mice within 24 hrs. As suggested by Collins (1973), it is probable that unrestricted extracellular growth of the unopsonised organisms occurs within the peritoneal cavity. This is due to the virtual absence of phagocytosis and inactivation of the challenge inoculum by the host macrophages, which allows this organism to grow in the tissue at rates normally only achievable *in vitro*. In contrast, for the *P. multocida* JRMT1 and JRMT2 *aroA* mutant strains, after high challenge dose (10^7 C.F.U./ mouse), the bacteria were able to spread into the liver, spleen and blood after 24 hrs, but at greatly reduced numbers of viable bacteria compared to the parent strains. In the lungs, however, no colonies were detected. After 48 hrs, all mice given ≈ 700 C.F.U. of the wild-type parent strains had died but the *aroA* mutant strains even at doses of 10^7 C.F.U. had been cleared and no colonies were detected in any of the internal organs except for a few bacteria detected in the liver of one mouse inoculated with 10^7 C.F.U. of the JRMT2 (Table 23).

When mice were given *P. multocida* strains i.n. at an initial challenge dose of 10^4 C.F.U., after 24 hrs, the wild-type strain 85020 decreased but after 48 hrs had increased more than 100 times in the lungs and also the infection had spread to the liver, spleen and blood. In contrast, the initial challenge dose of strain Quetta had decreased by 48 hrs. A similar situation was reported by Collins (1973) where wild-type *P. multocida* strains serotype 5:A isolated from turkey when inoculated i.n. replicated rapidly in unvaccinated mice, and after 24 hrs, the bacteria had spread into the internal organs. However, in the present work, the number of the *aroA* mutant strains in the lungs was reduced by about 10^5 times after 24 hrs and only a few colonies were detected in blood and in the liver. Interestingly, the *aroA* mutants did persist in the lungs for up to 96 hrs, but appeared to be largely confined to this location. It was clear, however, that this degree of persistence was sufficient to engender a protective immune response because i.n. vaccination of JRMT1 and JRMT2 fully protected mice against challenge with the virulent parent strains if a two dose vaccination schedule was used (Table 19).

4. 5. Phagocytosis assay

Until recently, the *Pasteurellae* were generally considered to be extracellular pathogens associated predominantly with the respiratory tract of animals and birds, and accordingly, it was assumed that humoral immunity played the principal role in control of *P. multocida* infections. However, some investigations have shown that avian strains of *P. multocida* serotypes A:3 and 3,4:A can enter and survive within eukaryotic cells (Collins, 1973; Lee *et al.*, 1994; Rabier *et al.*, 1997). Collins (1973) found that mouse alveolar and peritoneal macrophages killed 80-90% of those *P. multocida* taken up within about 60 min in vitro, but the surviving organisms multiplied extensively and complete sterilisation was never achieved in their experiments.

When we exposed *P. multocida* 85020 and its *aroA* mutant, strain JRMT1 strains to macrophage-like cells, both strains entered RAW cells and survived for at least 2 hrs and the number of intracellular bacteria decreased over 2 hrs. According to the Table 25, the number of phagocytosed *aroA* mutant bacteria was more than that of its wild-type parent strain but the reason for this is not clear. Truscott and Hirsh (1988) proposed that *P. multocida* produces a substance(s) that interferes with the function of phagocytic cells. Toxins with similar activity have been demonstrated in *P.(Mannheimia) haemolytica* isolated from cattle (Kaehler *et al.*, 1980; Baluyut *et al.*, 1981; Chang *et al.*, 1985) and in *P. multocida* isolated from rabbits (Anderson *et al.*, 1984; Chang *et al.*, 1985; Chanter *et al.*, 1986). Also Lee *et al.*. (1994) noted that the virulent strain of *P. multocida* serotype 3,4:A consistently attained invasion levels in turkey epithelial monolayer at least one log greater than avirulent strains. Intracellular survival of recombinant, bioluminescent *Bordetella bronchiseptica* was evaluated by Forde *et al.* (1998). They found that *B. bronchiseptica* could survive for at least 4 days in mouse macrophage-like cells and that a critical population size appeared to be for intracellular persistence. The potential for cell invasion and intracellular survival of *P. multocida* B:2 strains, in macrophage and other cell types, deserves further study, perhaps with the use of bioluminescent derivatives.

4. 6. Conclusion

The results presented here have shown that *aroA* derivatives of the *P. multocida* B:2 strains may represent excellent candidates for an attenuated live vaccine against HS. Attenuated strains of *P. multocida* B:2 have been constructed by interruption or deletion of the *aroA* and *cya* genes. The safety and efficacy of these strains has been demonstrated in a mouse model of infection. The next requirement is to evaluate the kinetics of antibody responses following administration of live *aroA* vaccine and antibody classes after inoculation by different routes, such as i.n. and i.p. Vaccine trials in the

target species, either cattle or buffaloes, will however be needed in order to establish that the safety and protective properties demonstrated in the mouse are reflected in similar properties in cattle or buffaloes.

Although, both Km^R and deletion derivatives of the *aroA* locus were obtained in this work, the other two loci tested, *cya* and *galE*, were remarkably intractable to allelic exchange. The reasons for this are not obvious. One explanation would be that *P. multocida* B:2 has more than one chromosome carrying duplications of the *cya* and *galE* genes. This would explain why both wild-type and mutant alleles were detected in recombinant colonies. Increasing numbers of microorganisms have been shown to contain more than one chromosome (Kolsto, 1997; Yamaichi *et al.*, 1999; Heidelberg *et al.*, 2000), for example, Heidelberg *et al.* found 105 genes with copies on both *Vibrio cholerae* chromosomes, but whether this represents a reason for the problems found with allelic exchange in *P. multocida* must await the sequencing of the genome of this important veterinary pathogen.

5. APPENDICES

5. 1. Brain Heart Infusion (BHI) broth or agar

Brain Heart Infusion, dehydrated (Oxoid)	37.0 g
Distilled water	to 1 litre

The medium dissolved in distilled water and sterilised by autoclaving for 20 min at 121 °C. For solid medium, 1.2% (w/v) agar (Technical No.3, Oxoid) was added before autoclaving. The 5% (v/v) sheep blood (B&E laboratories, Scotland) supplement was added to solid medium after it was allowed to cool to 50 °C.

5. 2. Luria-Bertani (LB) broth or agar

Tryptone (Oxoid)	10.0 g
Yeast extract (Oxoid)	5.0 g
NaCl (Analar, BDH)	10.0 g
Distilled water	to 1 litre

The solutes were dissolved in distilled water by shaking and the pH was adjusted to 7.0 with 1M NaOH. For solid medium, 1.2% (w/v) agar was added before autoclaving.

5. 3. Preparation of LB-X-Gal-IPTG plates

20 mg of X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside, Sigma) was dissolved per ml in dimethyl formamide (Sigma) and stored at -20 °C.

200 mg of IPTG (isopropyl- β -D-thiogalactopyranoside, Sigma) was dissolved per ml in distilled water and stored at -20 °C.

One ml of X-Gal and 100 μ l of IPTG stock solutions (it give 100 μ g ml⁻¹ final concentration of each) were added to 200 ml of melted LB agar, mixed by gentle rotation or inversion of the bottle and poured into petri dishes.

5. 4. Preparation of antibiotic solutions

Stock solutions of antibiotics were made at the following concentrations (%w/v): ampicillin (1%), kanamycin (1%), chloramphenicol (1%), carbenicillin (3%), tetracycline (1.2%). tetracycline and chloramphenicol were dissolved in ethanol. Kanamycin, ampicillin, carbenicillin were dissolved in distilled water and filter sterilised by passing the solution through an Acrodisc of 0.45 μ m pore size (Milipore). All antibiotic solutions were stored at -20°C.

5. 5. Tris-EDTA (TE) buffer, pH 8.0

	Per L of dH ₂ O	Final conc.
Tris	1.212 g	10 mM
EDTA	0.372 g	1.0 mM

Dissolved in H₂O, the pH adjusted to 8 with HCl, sterilised by autoclaving at 121°C for 20 min and stored at 4°C.

5. 6. Tris-Boric acid-EDTA (TBE) buffer (10× stock solution):

	Per L of dH ₂ O	Final conc.
Tris	107.8 g	89 mM
Boric acid	55.0 g	89 mM
EDTA	1.488 g	2 mM

Dissolved in distilled water by gently stirring with a magnetic stirrer and stored at room temperature.

5. 7. SOC medium

A) SOC base

	Per L of dH ₂ O	Final conc.
Tryptone	20.0 g	2% (w/v)
yeast extract	5.0 g	0.5% (w/v)
NaCl	0.58 g	10 mM
KCl	0.18 g	2.5 mM

The solutes were dissolved by shaking and the pH was adjusted to 7.0 with NaOH. The broth was sterilised by autoclaving and stored at 4 °C.

B) SOC Additive (10× concentration):

	Per 100 ml dH ₂ O	Final conc.
glucose:	2.0 g	20 mM
MgCl ₂ , 6H ₂ O	2.03 g	10 mM
MgSO ₄ , 7H ₂ O	2.46 g	10 mM

The solutes were dissolved by using a magnetic stirrer, filter sterilised and stored at 4 °C. The SOC additive was added to SOC base in a ratio of 1:10 just before use.

5. 8. Terrific broth

A) Basal medium

To 900 ml of distilled water were added:

Tryptone	12.0 g
Yeast extract	24.0 g
Glycerol	4.0 ml

B) Additive

To 100 ml of distilled water were added:

KH ₂ PO ₄	2.31 g
K ₂ HPO ₄	12.54 g

Both basal medium and additive were made up separately, sterilised by autoclaving and mixed together before use.

5. 9. Gel-loading buffer

Sucrose	40% (w/v)
Bromophenol blue	0.25% (w/v)

Dissolved and mixed well in sterile distilled water and stored in aliquots at -20°C.

5. 10. Pasteurella Minimal Medium (PMM):

Composition:

<u>Components</u>	<u>mM</u>	<u>g/L</u>
1. Glucose	11.1	1.999
2. Sucrose	7.3	2.4988
3. Na ₂ SO ₃	1.6	0.2016
4. K ₂ HPO ₄	23.0	4.006
5. FeSO ₄ , 7H ₂ O	0.01	0.0028
6. NH ₄ Cl	9.5	0.5082
7. K ₂ SO ₄	0.28	0.0488
8. CaCl ₂ , 2H ₂ O	5×10 ⁻⁴	7.35×10 ⁻⁵
9. MgCl ₂ , 6H ₂ O	0.53	0.1078
10. NaCl	50.0	2.922
11. L-Cysteine	2.9	0.457
12. L-Methionine	4.5	0.6714
13. L-Leucine	5.0	0.656
14. L-Glutamic acid	4.5	0.6621
15. Nicotinamide	24.6	3.0037
16. Ca.Pantothenate.	5.9	1.41
17. Thiamine HCl	4.5	1.5178

Reagents 3-10 were dissolved in distilled water and autoclaved, reagents 1 and 2 were dissolved separately in distilled water and filter sterilised and reagents 11- 17 were dissolved in distilled water and filter sterilised and stored at 4°C. All reagents were mixed together just before use. When required, an "aromix" supplement consisting of L-tryptophan and L-phenylalanine (Sigma), each to a final concentration of 40 µg/ ml, and 2,3 dihydroxybenzoic acid and *p*-hydroxybenzoic acid (Sigma) each at 10 µg/ ml were added.

5. 11. Southern blot hybridisation reagents

a. Maleic acid Buffer:

	Per L of dH ₂ O	Final conc.
Maleic acid	11.6 g	0.1 M
NaCl	8.76 g	0.15 M

The pH was adjusted to 7.0 with concentrated or solid NaOH, the solution autoclaved and stored at room temperature.

b. Blocking reagent (stock solution):

Blocking reagent (Boehringer Mannheim, Germany) was dissolved in maleic acid buffer to a final concentration of 10% (w/v) with shaking and heating.

c. 20× SSC

	Per L of dH ₂ O	Final conc.
NaCl	175.32 g	3.0 M
Tri sodium citrate	88.0 g	0.3 M

The pH was adjusted with HCl to ≈7.0

d. Standard hybridisation solution

In 5× SSC was added:

N-laurylsarcosine (from 10% stock solution)	0.1% (v/v)
SDS (from 10% stock solution)	0.02% (v/v)
Blocking reagent (from 10% stock solution)	1% (v/v)

e. Washing buffer

Add 0.3% (v/v) Tween 20 to the maleic acid buffer.

f. Blocking buffer

Dilute blocking reagent stock solution 1:10 with maleic acid buffer.

g. Detection buffer

	Per L of dH ₂ O	Final conc.
Tris-HCl	12.1 g	100 mM
NaCl	5.85 g	100 mM

h. 2× washing solution

Into 2× SSC add 0.1% (v/v) SDS

i. 0.5× washing solution

Into 0.5× SSC add 0.1% (v/v) SDS

j. Probe stripping solution

Into 0.2 N NaOH was added 0.1% (v/v) SDS

5. 12. Denaturation solution (for agarose gel)

	Per L of dH ₂ O	Final conc.
NaOH	20.0 g	0.5 N
NaCl	87.66 g	1.5 M

Dissolved and stored at room temperature.

5. 13. Neutralisation solution (for agarose gel)

	Per L of dH ₂ O	Final conc.
Tris-HCl	60.57	0.5 M

NaCl	87.66	3.0 M
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Dissolved, adjust pH to 7.5 with concentrated HCl and stored at room temperature.

5. 14. Assay buffers and reagents

A. Reagents for protein assay:

Reagent A:	2% (w/v) Na ₂ CO ₃ in 0.1 N NaOH
Reagent B:	0.5% (w/v) CuSO ₄ , 5H ₂ O in 1% (w/v) sodium tartarate
Reagent C:	Mix 50 ml of reagent A with 1 ml of reagent B, daily prepared fresh
Reagent D:	Commercial Folin- Ciocalteu reagent (Sigma) diluted with distilled water 1: 1

B. 2× EPSP assay buffer

Potassium phosphate buffer 1 M (pH 7.0)	20 ml
ADP 25 mM	20 ml
MgCl ₂ 1 M	0.5 ml
H ₂ O	to 100 ml (≈59.5 ml)

C. One mM stock EPSP

Barium EPSP (white solid)	3.5 mg
Di-potassium sulphate (K ₂ SO ₄) 0.5M	50 μl
H ₂ O	5.0 ml

Stir until a milky suspension results, then spin off precipitate and collect supernatant carefully.

5. 15: Mohammad Tabatabaei (MT) minimal medium

Composition:

<u>Components</u>	<u>g/ L</u>
1. Na ₂ HPO ₄	5.8
2. KH ₂ PO ₄	3.0
3. NaCl	0.5
4. NH ₄ Cl	1.0
5. MgSO ₄ .7H ₂ O	0.247

Vitamin additives:

1. Thiamine HCl	1.5
2. L-Cysteine	0.09
3. L-Methionine	0.13
4. L-Leucine	0.13
5. L-Glutamic acid	0.13
6. Nicotinamide	0.6
7. Ca.pantothenate	0.28

The reagents 1- 4 were dissolved in an appropriate volume of distilled water and autoclaved and before use filter sterilised vitamin additives, MgSO₄ solution (5 ml from 200 mM solution/ L) and sugars (0.2-0.4% w/v) were added. For making solid media, 1.2% (w/v) bacteriological agar plus reagents 1-4 were dissolved in an appropriate volume of distilled water and autoclaved. Then, before pouring, filter sterilised vitamin additives, MgSO₄ solution (5 ml from 200 mM solution / L) and sugars (0.2-0.4% w/v) were added.

5- 16. Bordet-Gengou medium

Bordet-Gengou agar base (Difco):	30.0 g
Bacteriological agar (Oxoid)	15.0 g
Distilled water:	to 1 litre

Boiled to dissolve in distilled water containing 10 g glycerol and sterilised by autoclaving for 15 min at 121 °C. The 20% (v/v) defibrinated horse blood (B&E laboratories, Scotland) supplement was added to solid medium after it was allowed to cool to ≈50 °C.

5. 17: Cyclodextrin liquid (CL) medium:

Compositins:

<u>Components</u>	<u>g/ L</u>
1. Sodium L-glutamate	10.7
2. L-Proline	0.24
3. NaCl	2.5
4. KH ₂ PO ₄	0.5
5. KCl	0.2
6. MgCl ₂ .6H ₂ O	0.1
7. CaCl ₂	0.02
8. Tris	6.1
9. Casamino acids	10.0
10. MeβCD	1.0
11. L-Cysteine	0.04
12. FeSO ₄ . 7H ₂ O	0.01
13. Niacin	0.004
14. Glutathione (reduced)	0.15
15. Ascorbic acid	0.4

Reagents 1-10 were dissolved in 950 ml of distilled H₂O and pH adjust to 7.6 with 3.0 N HCl then autoclaved at 121 °C for 15 min. Reagents 11- 15 dissolved in 50 ml distilled H₂O, filter sterilised and added to the autoclaved media at a 1: 20 (v/v) ratio before use.

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