Transposon Mutagenesis Of Virulence Regulatory Genes Of *Bordetella pertussis*

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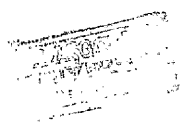

Dedication

I should like to dedicate this thesis to my family and friends, thanking them for their support and encouragement. My special thanks go to Darryl for his patience, advice and humour.

Acknowledgements

Initially I should like to thank my supervisors, Clive Duggleby, John Coote and Roger Parton, for making this PhD thesis a possibility, along with their continued encouragement. At the PHLS, CAMR I should like to thank Peter Greenaway for providing laboratory space and Roy Cowley for advice on a range of topics from microbial genetics to advanced practical jokes. I should also like to thank Andy Robinson and Ted Ashworth for information on the growth and virulence of the pathogen *Bordetella pertussis*. My thanks also go to Ian Livey for his help, both prior to, and during, the period of study for this thesis.

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Out of the cradle

onto dry land

here it is

standing:

atoms with consciousness;

matter with curiosity.

Richard P. Feynman

Publications

The following work has been presented during the period of study for this thesis.

Posters

Ward, M.J., Duggleby, C.J., Parton, R. & Coote, J.G. Delivery of transposons into *Bordetella pertussis*. SGM 111th Ordinary Meeting, 11th-14th April, 1988, Warwick University, UK.

Ward, M.J., Duggleby, C.J., Parton, R. & Coote, J.G. Transposon mutagenesis of the virulence control gene(s) of *Bordetella pertussis*. International Workshop on *Bordetella pertussis*, 18th-20th August, 1988, NIAID, Rocky Mountain Laboratories, Montana, USA.

Oral papers

Ward, M.J., Coote, J.G., Parton, R. & Duggleby, C.J. Transposon mutagenesis of the virulence control gene(s) of *Bordetella pertussis*. SGM 113th Ordinary Meeting, 4th-7th April, 1989, Cambridge University, UK.

Papers

Duggleby, C.J., MacGregor, D. & Ward, M.J. (1990). Transposon mutagenesis in prokaryotes. In, Advances in Gene Technology, vol. 1. (Ed. P.J. Greenaway), pp. 205-239. JAI Press, London.

Abbreviations

AC	Adenylate cyclase
ACDP	Advisory Committee on Dangerous Pathogens
ACT	Adenylate cyclase toxin
Adc	Adenylate cyclase phenotype
ADP	Adenosine diphosphate
AGG	Agglutinogen
AG2A	Monoclonal antibody raised against AGG2
AG3A	Monoclonal antibody raised against AGG3
Amph	Amphotericin
Ap	Ampicillin
ATP	Adenosine triphosphate
bp	Base pair
BG	Bordet-Gengou agar
bvg	*Bordetella* virulence regulatory gene
C	Cytosine
CAA	Casamino acids
cAMP	Cyclic-adenosine monophosphate
CAMR	Centre for Applied Microbiology and Research
Ceph	Cephalexin
cfu	Colony forming units
Ci	Curies
Cm	Chloramphenicol
cm	Centimeter
CR	Congo red dye
Crb	Phenotype for Congo red-binding
CSM	Cyclodextrin solid medium
cyaA	Adenylate cyclase or cyclolysin gene
dATP	Deoxy-adenosine triphosphate
dCTP	Deoxy-cytosine triphosphate
dGTP	Deoxy-guanosine triphosphate
dTTP	Deoxy-thimidine triphosphate
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid

DOC	Deoxycholic acid
drd	Derepressed gene
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FHA	Filamentous haemagglutinin
Fha	Filamentous haemagglutinin phenotype
fha	Filamentous haemagglutinin gene
fim	Fimbrial gene
g	Gram
G_i	Guanine nucleotide binding protein
Gm	Gentamycin
HA	Haemagglutination
His	Phenotype for histidine requirement
HLT	Heat labile toxin
Hlt	Heat labile toxin phenotype
hlt	Heat labile toxin gene
HLY	Haemolysin
Hly	Haemolytic phenotype
HSF	Histamine sensitising factor
IAP	Islets-activating protein
ICR 191	Frameshift mutagen
ic	Intracerebral
in	Intranasal
ip	Intraperitoneal
Inc	Incompatibility group of plasmids
IPTG	Isopropyl β-D-thiogalactopyranoside
IS	Insertion sequence
kb	Kilobase pairs
kD	Kilo Dalton
Km	Kanamycin
lac	Lactose operon gene
Leu	Phenotype for leucine requirement
LOS	Lipooligosaccharide
LPF	Lymphocytosis promoting factor
LPS	Lipopolysaccharide

M	Molar
mA	Milliamps
MeβCD	Heptakis (2, 6-O-dimethyl) β-cyclodextrin
Met	Phenotype for methionine requirement
mg	Milligram
ml	Millilitre
mM	Millimolar
mob	Mobilisation site
mod	Environmental sensor/regulator gene
NA	Nutrient agar
NB	Nutrient broth
NIAID	National Institute of Allergy and Infectious Diseases
Nm	Neomycin phosphotransferase II
nm	Nanometer
OD	Optical density
OLB	Oligo-labelling buffer
OMP	Outer membrane protein
ou	Opacity units
P	Promoter
PBS	Phosphate buffered saline
PHLS	Public Health Laboratory Service
phoA	Phosphatase gene
pmol	Picomole
Pro	Phenotype for proline requirement
psi	Pounds per square inch
PT	Pertussis toxin
PTd	Pertussis toxoid
Ptx	Pertussis toxin phenotype
ptx	Pertussis toxin gene
P.69	69 kilodalton outer membrane protein
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SGM	Society for General Microbiology

Sm	Streptomycin
Sp	Spectinomycin
SS	Stainer and Scholte medium
SSC	Standard saline citrate
TBE	Tris-borate-EDTA buffer
Tc	Tetracycline
TCT	Tracheal cytotoxin
TE	Tris-EDTA buffer
TES	Tris-EDTA-salt buffer
Thi	Phenotype for thiamine requirement (B1)
Thr	Phenotype for threonine requirement
Tn	Transposon
Tp	Trimethoprin
tra	Transfer genes
uv	Ultraviolet
vag	*vir*-activated genes
Vir	Virulence phenotype
vir	Virulence-regulatory gene
vrg	*vir*-repressed genes
WHO	World Health Organisation
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside
::	Novel joint
Δ	Deletion
µg	Microgram
µl	Microlitre
µm	Micrometer
(w/v)	Weight : volume ratio
(v/v)	Volume : volume ratio
°C	Degrees Celsius
^{32}P	Radioisotope of phosphorus

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Summary

A suitable Tn*5* delivery vector for mutagenesis of the pathogen *Bordetella pertussis* was identified as the ColIb-based plasmid pLG221. Insertion mutants, created using this 'suicide' vector, were screened for the loss of virulence-associated properties, or virulence-regulatory functions. No mutants were identified as having lost the heat-labile toxin alone. Additionally, no mutants were identified as being unable to modulate under C-mode growth conditions. However, a number of mutants were identified as being unable to bind the dye Congo red (a property associated with the avirulent phenotype). Several of these strains were subsequently shown to exhibit a pleiotropically negative phenotype for the expression of virulence-associated factors.

The events causing the avirulent phenotype in the Crb$^-$ mutants were initially examined by identification of the Tn*5* insertion sites. Two mutants were shown to have Tn*5* insertions within a region with BamHI, SalI and EcoRI restriction fragments identical to the map of the *bvg* locus. However, no clustering of transposon insertions, indicative of an alternate virulence regulatory locus, were noted between the other mutants.

Complementation studies, introducing the cloned *bvg* locus into the Crb$^-$ mutants, returned virulence-associated properties to all the mutants, suggesting that the avirulent phenotype could only be created by mutations in the *bvg* locus. Hybridisation of radiolabelled pRMB2 DNA (which contains the cloned *bvg* locus) to Southern blots of the Crb$^-$ mutant's DNA identified deletions of this region in two mutants.

Mutants not showing obvious genetic rearrangements in the *bvg* locus were considered to have been caused by frameshift mutations (as previously identified in the Tohama strain). This idea was examined by using the frameshift mutagen ICR 191 to create Crb^+ revertants. However, only a single mutant was shown to revert to virulent characteristics after such treatment. Therefore, the events causing the loss of virulence-associated characteristics in the mutants created in this study were defined in only five of the ten isolates.

A single mutant (strain 51) showing the Crb^- phenotype was shown to be haemolytic, while not producing other virulence-associated factors. This mutant was shown to be avirulent in an infant mouse model. Initial studies have also indicated that a vaccine made from Tn*5* mutant 51 showed some protective activity against intranasal challenge in infant mice, although not against intracerebral challenge.

INTRODUCTION

1.1 The Bordetellae

The Gram-negative cocco-bacillus *Bordetella pertussis* is the main cause of the severe respiratory tract infection "pertussis" in human infants. Pertussis, or whooping cough, has been recognised as a clinical entity for several centuries with the causative bacterium first cultured by Bordet and Gengou (1906). The organism was originally classified in the genus *Haemophilus* because of a requirement for blood in the culture medium. However, *B. pertussis* has been shown to be able to grow on relatively simple media, with either blood, charcoal, soluble starch or anionic-exchange resins added to overcome the effects of growth inhibitors. Stainer and Scholte (1971) identified a simple, chemically-defined medium suitable for the large-scale culture of *B. pertussis*, which, with the addition of heptakis (2,6-O-dimethyl) β-cyclodextrin (Imaizumi *et al.*, 1983), allows growth from small inocula.

The genus *Bordetella* includes three other species which are also respiratory tract pathogens. *B. parapertussis* (Linneman and Perry, 1977) has been implicated as the cause of a mild form of pertussis in humans. *B. bronchiseptica* (Goodnow, 1980) causes respiratory tract infections in certain other mammals, including kennel cough in dogs and atrophic rhinitis in swine. The recently reclassified *B. avium* (Kersters *et al.*, 1984) is the agent of coryza in turkey poults.

Hybridisation analysis of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* chromosomal DNAs has indicated a close genetic relatedness of these species (Kloos *et al.*, 1981). Studies on the electrophoretic mobilities of 15 metabolic enzymes of these

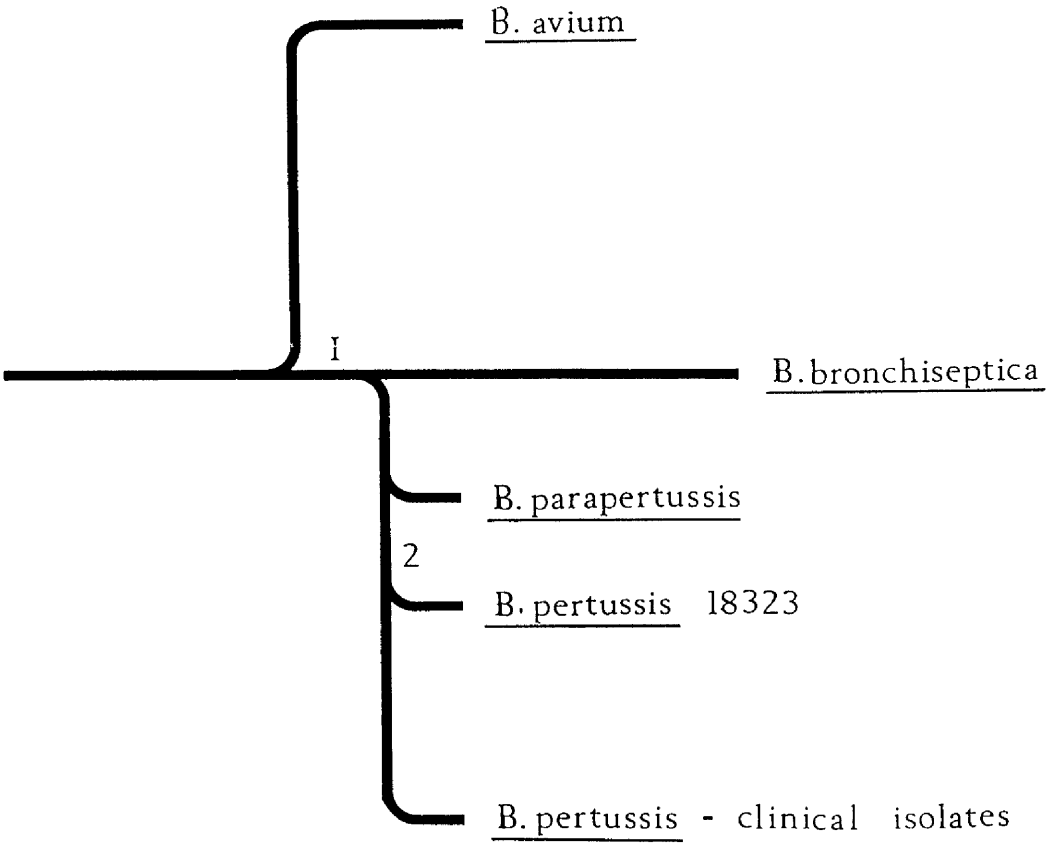
bordetellae (Musser *et al.*, 1986) divided the genus into three groups. The first group contained all *B. bronchiseptica* strains; the second group was composed of all *B. parapertussis* strains and *B. pertussis* strain 18-323; the third group contained the remaining *B. pertussis* strains. This grouping of *B. pertussis* 18-323 with *B. parapertussis* strains has been disputed by the work of McPheat and McNally (1987a) who identified a repeated DNA sequence of approximately 1kb from the genome of *B. pertussis*. This repeat sequence was shown to be absent in *B. parapertussis* and *B. bronchiseptica*, but was present in *B. pertussis* strain 18-323 (Fig. 1). Aricò *et al.* (1987) and Gross *et al.* (1989a) have discussed the possible evolutionary relatedness of the bordetellae based on the nucleotide sequence of the pertussis toxin operon (Fig. 1). According to this data *B. avium* is the most distant relative of *B. pertussis* since pertussis toxin genes are not present in this species. Both *B. parapertussis* and *B. bronchiseptica* possess mutated toxin genes, but *B. pertussis* strains alone produce pertussis toxin.

1.ii Pertussis

Wardlaw and Parton (1988) in their review of the host-parasite relationship state that "*B. pertussis* must be a very well adapted parasite with highly effective mechanisms for its own transmission and survival". The bacteria attach specifically to the cilia of the epithelial cells of the respiratory tract where they multiply and initially cause symptoms resembling those of the common cold. The subsequent paroxysmal phase of the disease is marked by the onset of the characteristic whoop, caused by the rush of air over a partially

Fig. 1 Phylogenetic tree of the bordetellae

The phylogenetic tree of Gross *et al.* (1989a) based on the nucleotide sequence of the pertussis toxin operon indicates (1) the acquisition of the pertussis toxin operon after *B. avium* has split away from the other bordetellae, and (2) the gain of ability to express pertussis toxin found in *B. pertussis*. *B. pertussis* 18-323 appears to be an evolutionary link between *B. parapertussis* and *B. pertussis* clinical isolates, since it shares properties in common with both species (Musser *et al.*, 1986; McPheat and McNally, 1987a).


B. avium
1
B. bronchiseptica
B. parapertussis
2
B. pertussis 18323
B. pertussis - clinical isolates

closed glottis. Patients may exhibit pathophysiological changes including leukocytosis with a predominance of lymphocytes, weight loss, occasional hypoglycaemia and, rarely, encephalopathy [see Walker (1988) for clinical aspects of pertussis].

During the course of the disease the organisms do not enter the blood stream, and the isolation rate for *B. pertussis* drops progressively after the onset of the paroxysmal phase. The inability to culture the organism during the most severe stage of the disease led to the hypothesis that the majority of clinical symptoms are due to long-acting, systemically disseminated toxin(s) produced by the bacteria (Pittman, 1984). *B. pertussis* produces a wide variety of virulence-associated factors, toxins and adhesins, which have been assigned roles in the disease process, promoting a fuller understanding of pathogenicity of pertussis (Robinson *et al.*, 1985a; Weiss and Hewlett, 1986). A description of these factors is given in the next section.

No generally accepted effective treatment for pertussis is available. Broad-spectrum antibiotics given during the early stages of the disease are effective against the bacterium, but have little or no effect on the clinical course of the established disease. Erythromycin may reduce infectivity (Bass, 1983), and has been used in conjunction with glucocorticoids which have been reported to ease coughing and shorten the duration of the disease (Barrie, 1982; Dianese, 1982).

1.iii Virulence Factors of *B. pertussis*

1.iii.a. Adenylate cyclase and haemolysin

B. pertussis produces a mainly extracytoplasmic adenylate cyclase

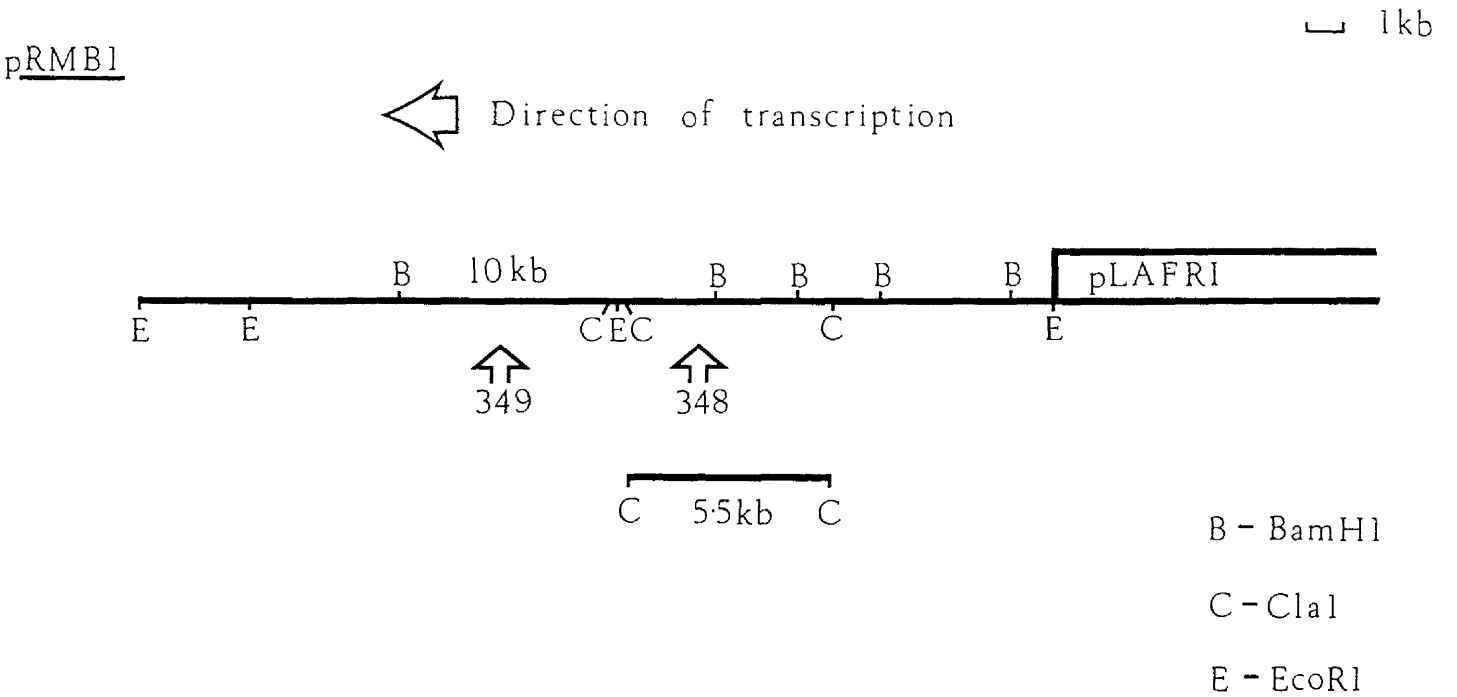
(AC) (Hewlett and Wolff, 1976), which is stimulated by the eukaryotic calcium-binding protein calmodulin (Wolff *et al.*, 1980). Two forms of the enzyme have been identified; i. the adenylate cyclase enzyme, which converts ATP → cAMP (Hewlett *et al.*, 1976), and ii. the adenylate cyclase toxin (ACT), which can enter eukaryotic cells to catalyse the above reaction, thereby impairing cellular function (including the activities of phagocytes) (Confer and Eaton, 1982). Hewlett and Gordon (1988) have suggested that ACT may conform to the A-B subunit model of toxins (Gill, 1978).

B. pertussis also produces a haemolysin (HLY). Weiss *et al.* (1983) suggested that the genes which express HLY and AC are linked on an operon, since a single insertion of Tn*5* in BP348 [see Section 1.viii.d.] caused the simultaneous loss of both factors. The close association of these genes was confirmed by Brownlie *et al.* (1986), using a recombinant plasmid (pRMB1) which restored AC and HLY activity to BP348. The LAFR1-derived cosmid pRMB1 has been mapped (Fig. 2) and subcloned (Brownlie *et al.*, 1988). Glaser *et al.* (1988a) cloned, expressed and sequenced the adenylate cyclase operon. The 1250 carboxy-terminal amino-acids of the protein were found to have significant homology to both *E. coli* α-haemolysin and *Pasteurella haemolytica* leucotoxin (Glaser *et al.*, 1988b), suggesting that AC and HLY activities are conferred by the same protein, the adenylate cyclase toxin or cyclolysin, in *B. pertussis*.

A similar calmodulin-dependent, extracellular ACT (composed of the oedema factor and protective antigen) has been identified in *Bacillus anthracis* (Leppla, 1982). The enzyme kinetics of this toxin, and requirement for divalent cations, resemble those of the

Fig. 2 Restriction map of the cosmid pRMB1

The recombinant plasmid pRMB1 is composed of the broad-host-range vector pLAFR1 and the cloned adenylate cyclase and haemolysin determinants (Brownlie *et al.*, 1988). Transposon insertions in mutants showing the loss of HLY (BP349), or AC and HLY (BP348), have been mapped to the 10kb BamH1 fragment as indicated (Brownlie *et al.*, 1988; Glaser *et al.*, 1988b). The 5.5kb ClaI fragment present in the subclone pRMB11 is also shown and contains both the start of the *cyaA* open reading frame and, approximately 3kb upstream, the repeat sequence IS481v1 (McPheat *et al.*, 1989).


1kb
pRMB1
Direction of transcription
B
10kb
B
B
B
B
pLAFRI
E
E
C
E
C
C
E
349
348
C
5·5kb
C
B - BamH1
C - Cla1
E - EcoR1

B. pertussis ACT (Leppla, 1984). Additionally, the central region of the *B. anthracis* adenylate cyclase includes an area of good homology with the N-terminal part of the *B. pertussis* enzyme (Escuyer *et al.*, 1988), suggesting that these cyclases have originated from a common ancestor. However, Hewlett and Gordon (1988) have suggested that these analogous toxins are immunologically and functionally distinct.

A transposon mutant of *B. pertussis* unable to express the adenylate cyclase operon [BP348; see Section 1.viii.d.] has been shown to have reduced virulence in mice after intracerebral or intranasal challenge (Weiss *et al.*, 1983; Ölander *et al.*, 1986), indicating that this toxin is an important virulence factor. Brézin *et al.* (1987) incubated a challenge inoculum with a monoclonal anti-AC antibody and showed that this protected mice from respiratory infection, again indicating the ACT to be a major toxin. The ACT has been suggested as a possible candidate for inclusion in acellular vaccines (Novotny *et al.*, 1985).

1.iii.b. Agglutinogens

Phase I strains of *B. pertussis* produce one or more heat-labile agglutinogens (AGG). These surface components have been shown to be involved in the production of agglutinins, antibodies which can cause bacterial cell agglutination. AGG-1 is found in all strains of *B. pertussis*, whilst agglutinogens 2-6 may be present in various combinations. Preston *et al.* (1982) suggested that there are three major agglutinogens (1, 2 and 3), while 4, 5 and 6 are minor. The variable combinations of agglutinogens differentiate the main *B. pertussis* serotypes.

The fimbrial agglutinogens (2 and 3) of *B. pertussis* are possible

candidates for the initial attachment of the bacteria to the ciliated epithelial cells of the respiratory tract during the disease process, thus enabling the organism to resist the normal host-clearance mechanisms (Beachley, 1981). Gorringe *et al.* (1985) showed that monoclonal antibodies to agglutinogens 2 and 3 inhibit the binding of *B. pertussis* to Vero cells in a serotype-specific manner. In addition, mice immunised with purified fimbriae are protected against subsequent intranasal challenge with virulent *B. pertussis* (Robinson *et al.*, 1985b; Zhang *et al.*, 1985). However, Tuomanen and Weiss (1985) have shown that non-fimbriate *B. pertussis* adhere to ciliated human cells, and Tuomanen (1988) has suggested that there is little evidence for the role of agglutinogens as adhesins to ciliated cells in humans.

The structural subunits of the serotype 2 and 3 fimbriae (*fim* 2 and *fim* 3 respectively) have been cloned and sequenced (Livey *et al.*, 1987; Mooi *et al.*, 1990). Synthetic oligonucleotides derived from amino-acid sequences of *B. pertussis* fimbrial subunits have been used to identify clones in a *B. pertussis* genomic library (Pedroni *et al.*, 1988). One such clone contains a gene which has been called *fim* X, and shares homology with, but is not identical to, the genes coding for fimbrial agglutinogens 2 and 3.

Charles *et al.* (1989) have cloned and sequenced the gene encoding P.69, a 69-kilodalton non-fimbrial outer-membrane protein which has been identified as an agglutinogen of *B. pertussis* (Brennan *et al.*, 1988).

1.iii.c. Filamentous haemagglutinin

B. pertussis produces two distinct haemagglutinins, pertussis toxin and filamentous haemagglutinin (FHA) (Arai and Sato, 1976), both

of which are secreted into the culture medium during growth (Arai and Munoz, 1979). Polyclonal and monoclonal antibodies against both haemagglutinins have been shown to confer passive protection against intranasal challenge in mice (Sato *et al.*, 1981; Munoz *et al.*, 1981), suggesting a role for these factors in pathogenesis. Tuomanen *et al.* (1985) have also found that transposon mutants lacking FHA or PT [BP353 and BP357 respectively - see Section 1.viii.d.] show decreased adherence to rabbit and human ciliated cells in an *in vitro* assay (Tuomanen and Weiss, 1985). However, Weiss *et al.* (1984) have shown that mutant BP353 (FHA^-) causes a lethal pulmonary infection in an infant mouse model, with virulence not significantly reduced from that of the wild-type.

FHA appears to be a non-fimbrial protein (Ashworth *et al.*, 1982), with a molecular mass of 210-220kD (Irons *et al.*, 1983), which forms filamentous aggregates 2nm in diameter and 40-100nm long. Brown and Parker (1987) have cloned and expressed the gene for FHA in *E.coli*, and Relman *et al.* (1989) have sequenced the FHA structural gene (*fha*B) which lies adjacent to the *bvg* locus [see Section 1.vi.d.]. The nucleotide sequence of *fha*B has suggested that co- or post-translational processing may play a role in FHA synthesis.

1.iii.d. Heat-labile toxin

Bordetella species produce a heat-labile, dermonecrotic toxin (HLT). HLT causes necrotic lesions when injected subcutaneously into mice at low doses (Livey and Wardlaw, 1984), but can be inactivated by heating at 56°C for 5 minutes. The toxin has a constrictive effect on vascular smooth muscle and may cause inflammation of the respiratory tract mucosa during the disease process (Nakase and Endoh, 1988).

1.iii.e. Lipopolysaccharide endotoxin

B.pertussis endotoxin is composed of two distinct lipopolysaccharide types, LPS-I and LPS-II (Le Dur *et al.*, 1980). The lipopolysaccharides of *B.pertussis* have alternatively been called lipooligosaccharides (LOS) because they do not contain the long-chain polysaccharide characteristic of the LPS of enteric organisms (Li *et al.*, 1988). *B.pertussis* LPS, in common with LPS from other bacteria, is heat-stable, antigenic, pyrogenic and toxic. However, a definite role for endotoxin in pertussis pathogenesis has not yet been assigned.

1.iii.f. Pertussis toxin

Irons and Gorringe (1988) have stated the widely held view that "pertussis toxin (PT) is probably the major contributor to the pathogenesis of pertussis", although the exact role of PT in the virulence of *B.pertussis* is not known. PT has been the subject of numerous reviews including Wardlaw and Parton, (1983); Sekura *et al.* (1985); Ui, (1988); Furman *et al.* (1988); and Munoz, (1988). The toxin has alternatively been known as lymphocytosis promoting factor (LPF), histamine-sensitising factor (HSF), islets-activating protein (IAP) (Yajima *et al.*, 1978a, b), and pertussigen (Munoz and Bergman, 1977), giving some indication of its wide range of biological activities.

Pertussis toxin is a hexameric protein composed of five different subunits, the genes of which are arranged in an operon (Locht and Keith, 1986; Nicosia *et al.*, 1986). An A-B subunit structure has been proposed for the toxin, the A subunit being the active protomer, the B subunit the binding oligomer (Tamura *et al.*, 1982). The A subunit is active in ADP-ribosylation of a guanine nucleotide-binding protein

(G_i), resulting in loss of inhibition of adenylate cyclase in target cells (Katada and Ui, 1982a. b.).

Ui (1988) has classified the biological activities of PT, with respect to underlying mechanisms of action, by susceptibility to acetamidination of lysine residues. 'Class 1' activities are not impaired by acetamidination and include ADP-ribosylation of G-proteins, potentiation of adenylate cyclase and stimulation of insulin secretion. The acetamidinated toxin being as effective as the native toxin in 'Class 1' activities suggests that no free amino groups are required for the binding of the B-oligomer to the target cell surface and translocation of the toxin into the cell. 'Class 2' activities, including lymphocytosis, histamine-sensitisation, mitogenicity and adjuvanticity, are, however, abolished by acetamidination. During 'Class 2' activities the B-oligomer has been suggested to bind to cells 'divalently', using the amino groups of lysine residues which are modified during acetamidination.

The pertussis toxin genes have been cloned (Locht *et al.*, 1986; Nicosia *et al.*, 1986), sequenced and the amino-acid sequences of the individual subunits deduced (Locht and Keith, 1986). Additionally, PT subunits have been subcloned and expressed in *Escherichia coli*, both as fusion proteins and as native proteins (Locht *et al.*, 1987; Burnette *et al.*, 1988). Recently, Pizza *et al.* (1989) have constructed an inactive toxin by site-directed mutagenesis. The replacement of key amino acids in the enzymatically active S1 subunit allowed the production of a non-toxic and immunogenic molecule which protected mice from intracerebral challenge with virulent *B. pertussis*.

The PT genes present in *B. bronchiseptica* and *B. parapertussis*

display a high (over 96%) degree of nucleotide sequence homology to those in *B. pertussis*, although neither species expresses the genes, probably due to multiple mutations in the promoter region (Marchitto *et al.*, 1987).

1.iii.g. Tracheal cytotoxin

Tracheal cytotoxin (TCT) is a recently discovered toxin of *B. pertussis*, which has been shown to have cytopathic effects on cells of the respiratory epithelium of hamsters (Goldman and Herwaldt, 1985). Since ciliary activity is the normal clearance mechanism in the respiratory tract, destruction of ciliated cells allows accumulation of debris, and leaves coughing as the only means of clearing the airways. All bordetellae produce TCT, and Goldman (1988) has suggested that "the complete conservation of this virulence determinant provides strong evolutionary support for its central and essential role in the pathogenesis of *Bordetella* infections".

Goldman (1988) has proposed that TCT derives from the enzymatic cleavage of peptidoglycan. A similar peptidoglycan-derived toxin is produced by the pathogen *Neisseria gonorrhoeae* (Melly *et al.*, 1984), suggesting that TCT may be a virulence determinant common among mucosal pathogens.

1.iii.h. Virulence factors and pathogenicity

The virulence factors produced by *B. pertussis* have so far been reviewed individually, however, during the disease process these factors presumably contribute synergistically to produce the 'classical stages' of pertussis. A possible sequence of events, both for host and parasite, is shown in Fig. 3.

Fig. 3 Virulence factors and pathogenesis

A diagrammatic representation of *B. pertussis* virulence factors and their contribution to pertussis pathogenicity and immunity. Events affecting both host and parasite are shown. Rearranged from Wardlaw and Parton (1988).

Host	Virulence Factors	Parasite
Exposure		Transmission
↓		↓
Incubation	FHA, PT, AGG? OMP?	Attachment & growth
↓		↓
Catarrhal stage	TCT, PT, ACT, HLT	Secretion of toxins
↓		↓
		Encounter with host immune response
Paroxysmal stage	Protective antigens - FHA, PT, AGG, LPS ?	↓
↓		Expulsion
Convalescence		

1.iv Pertussis Vaccines

The introduction of pertussis vaccines into widespread use in the UK during the 1950s caused a marked reduction in the incidence of this previously common disease. Present day control of whooping cough still relies on vaccine prophylaxis. The vaccines currently used in the UK are biologically complex suspensions of killed, whole cells of *B.pertussis* with representative serotypes. Inoculation with such vaccines may involve some risk, including neurological illness, encephalopathy and death (Kulenkampff *et al.*, 1974; Manclark and Cowell, 1984; Ross, 1988). Such reactions have been the cause of much public concern over the past decade, and have made the production of a defined component or acellular vaccine a high priority (Robinson *et al.*, 1985a).

The high protective efficacy (Griffiths, 1988), low toxicity and relatively low cost of the current whole-cell vaccine does make the introduction of a demonstrably improved vaccine difficult. However, it has been suggested that children inoculated with the whole-cell vaccine may act as asymptomatic carriers of the disease, since the vaccine may protect against disease but not colonisation (Fine and Clarkson, 1982). An acellular vaccine could possibly be designed to protect against both infection and disease.

Robinson *et al.* (1985a) proposed that multicomponent vaccines should include filamentous haemagglutinin, because of its low toxicity and good protective activity against animal infections (Munoz *et al.*, 1981); pertussis toxoid (PTd) (Sato and Sato, 1984); and all three major serotypes of agglutinogens. Further studies are required on the role in the disease process and immunogenicity of both heat-labile

toxin and tracheal cytotoxin before they may be considered as vaccine components. Outer-membrane proteins (OMP) have been recognised as playing an important role in pathogenesis in a number of Gram-negative bacteria (Levine *et al.*, 1983), and the biological activities of these components, including the 69kD protein, require further assessment. Weiss *et al.* (1984) have shown that mutants deficient in adenylate cyclase / haemolysin are avirulent in the infant mouse respiratory infection model and, if proven to be protective, these components should be considered for inclusion in any new vaccine.

Defined acellular vaccines have been in use in Japan since 1981 and have been given mainly to two-year-old children. The antigenic composition of these vaccines is either FHA: PTd: AGG-2 in the ratio 90: 10: 1, or FHA: PTd in the ratio 1: 1. This latter type of vaccine has also recently been used in Swedish field trials in six-month-old infants. Kallings (1986) compared the acellular vaccine with the whole-cell vaccine and found it less reactogenic and able to elicit good antibody responses to PT. The introduction of such vaccines in Japan has resulted in a decline in the incidence of pertussis (Kimura and Hikino, 1985), although results from the Swedish field trials (Kallings *et al.*, 1988) have suggested that the acellular vaccine has lower protective efficacy than a whole-cell vaccine. However, acellular vaccines have proved to be less toxic, less pyrogenic and have been shown to cause fewer local reactions upon injection. Such favourable information has stimulated further studies. Robinson and Ashworth (1988) have documented the production of a vaccine, consisting of equal amounts of separately purified PTd, FHA and agglutinogens 2 and 3, to be used in British field trials.

The preliminary testing of the efficacy of protective antigens (or antibodies) for use in human vaccines still relies on animal models, which are considered in the next section.

1.v Experimental Infections in Animals

The respiratory tract of a variety of animals can be colonised by *B. pertussis*. However, identification of a relevant animal model for pertussis has proved difficult. Only primates, when challenged intranasally (in), have been reported to develop the characteristic cough and mucus production indicative of human pertussis (Weiss and Hewlett, 1986). Marmosets do not develop the paroxysmal cough, but show the presence of catarrh and nasopharyngeal colonisation by *B. pertussis*. Currently, expense and lack of availability prohibits the widespread use of primate models.

Young mice, inoculated in, are susceptible to colonisation with *B. pertussis* and share a similar course of infection to the human disease (Pittman *et al.*, 1980), as well as exhibiting pathophysiological changes including leukocytosis, histamine sensitisation, hypoglycaemia and hyperinsulinaemia. This model has proved valuable for studies of pathophysiology and immunity in human pertussis and will find continued use in the evaluation of protective activity of *B. pertussis* antigens and antibodies (Robinson *et al.*, 1985b).

Studies with mice showed the intracerebral (ic) mouse protection test to correlate well with the efficacy of the whole-cell vaccines in children (Standfast, 1958). This mouse-brain model is increasingly recognised as being inadequate since studies with purified antigens (for use in acellular vaccines) have shown only pertussis toxin to be

protective ic (Munoz *et al.*, 1981). However, Sato and Sato (1988) suggest for the Japanese acellular vaccine that "while it is difficult to justify the ic-challenge method .... there is currently no other more suitable or correct method as a national control test for protective potency".

1.vi Antigenic Variation in *B. pertussis*

B. pertussis regulates the production of toxins and other virulence factors associated with its ability to cause disease in three distinct ways [for recent reviews see Robinson *et al.*, 1986; Coote and Brownlie, 1988].

1.vi.a. Serotype variation

B. pertussis isolates have been shown to display heterogeneity in serotype. Agglutinogen 1 is always present in phase I isolates, whilst the serotype 2 and 3 antigens may be lost or regained independently. This phenomenon has been noted in the laboratory (Stanbridge and Preston, 1974), in experimental animals (Preston *et al.*, 1980), and in the child (Preston and Stanbridge, 1972). There is clearly a substantial advantage for pathogens in being able to alter the antigenic stimulus which they present to the host, thus avoiding the defences of the immune system and enabling repeated or prolonged infections.

The molecular mechanisms of relatively rapid changes in serotype have been studied in other pathogens including *E. coli* (Freitag *et al.*, 1985), *Neisseria gonorrhoeae* (Meyer *et al.*, 1984), and *Salmonella typhimurium*. In the latter there is a reversible switch involving an invertible region of DNA (Zeig and Simon, 1980). In *N. gonorrhoeae*

phase variation of fimbrial antigens involves a cassette-type mechanism of DNA rearrangement whereby silent copies of genes encoding pilus antigens are moved into expression sites (Meyer *et al.*, 1982). Recent research indicates that serotype variation in *B. pertussis* may be due to single nucleotide insertion or deletion mutations in the regulatory regions upstream of agglutinogen subunit genes (Willems *et al.*, 1990).

1. vi. b. Antigenic modulation

Antigenic modulation was first described in *B. pertussis* by Lacey (1960) as a freely reversible antigenic transition caused by changes in the bacterial growth environment. Growth on NaCl-containing media produced the virulent, or X-mode phenotype, whereas growth on $MgSO_4$-containing media, or on Bordet-Gengou agar at 25°C, produced the avirulent, or C-mode phenotype. Pusztai and Joó (1967) also observed antigenic modulation, induced by increasing the concentration of the growth factor, nicotinic acid, in the medium. However, Wardlaw *et al.* (1976) showed that increasing the nicotinamide concentration (an alternative growth factor to nicotinic acid) does not similarly induce modulation.

During antigenic modulation several virulence factors and surface properties of *B. pertussis* are lost, including PT, X-mode specific polypeptides (Parton and Wardlaw, 1975; Wardlaw *et al.*, 1976), agglutinogens (McPheat *et al.*, 1983), AC (Parton and Durham, 1978), HLT (Livey *et al.*, 1978), HLY (Lacey, 1960), cytochrome d629 (Ezzell *et al.*, 1981), adhesion (Gorringe *et al.*, 1985), hydrophobicity (Robinson *et al.*, 1983) and uptake of Congo red dye (Parton, 1988).

The rates of conversion of X-mode to C-mode have been studied in

both batch and continuous culture. In modulation caused by high levels of $MgSO_4$, Idigbe *et al.* (1981) suggested the selective destruction of virulence factors. However, Robinson *et al.* (1983) and Brownlie *et al.* (1985a) found no inactivation of virulence factors , but that further synthesis was repressed. During nicotinic acid-induced modulation in a chemostat, Robinson *et al.* (1983) showed the loss of PT and hydrophobicity to be quicker than the theoretical washout rate, again suggesting destruction of X-mode characteristics.

Parton and Durham (1978) and Hewlett *et al.* (1979) suggested that cAMP, the product of adenylate cyclase, could play a role in the control of expression of virulence factors in *B. pertussis*. However, Brownlie *et al.* (1985b) indicated that expression of adenylate cyclase is influenced by the same control mechanisms that act on other virulence factors during modulation. Moreover, transposon insertion mutants lacking adenylate cyclase produce normal levels of other virulence factors (Weiss *et al.*, 1983). Therefore a regulatory role for AC in antigenic modulation is unlikely.

1.vi.c. Phase variation

Leslie and Gardner (1931) first described phase variation in *B. pertussis* as the loss of virulence associated with laboratory passage of the bacterium. Four antigenically distinct stages were noted, phases I and II being pathogenic, phases III and IV being relatively non-virulent. The phase I / phase IV classification system is still in use to denote the extremes of phase types. Lawson (1939) proposed additional intermediate stages, dependent on strain and environmental conditions.

There is still no definitive description of the process of phase

variation, although Parker (1976) introduced the terms 'fresh isolate', 'intermediate strain', and 'degraded strain' for the process, while Weiss and Falkow (1984) have introduced the more descriptive designations, 'virulent phase' and 'avirulent phase', as concise terms for the potential of the bacterium to cause disease when expressing all of the virulence-associated properties. Phase transition appears not to be merely an *in vitro* artifact since avirulent organisms can be isolated from patients in the later stages of infection (Kasuga *et al.*, 1954). Weiss and Falkow (1984) proposed that avirulent *B.pertussis* variants might act as a carrier state. Organisms in such a state could increase their survival capacity by switching off an array of factors useful only *in vivo*.

Phase variation involves the loss of the same virulence-associated factors as antigenic modulation, but not in the same freely reversible manner. Avirulent variants have an increased resistance to certain antibiotics (Bannatyne and Cheung, 1984), and fatty acids (Peppler and Schrumpf, 1984). The latter may explain their ability to grow on media not supplemented with charcoal, blood, starch or cyclodextrin. The colony morphology of the phase IV organisms is flat, rather than the domed colonies of the virulent organism (Peppler, 1982). Phase I organisms can also be identified as red (Crb^+) colonies on media containing the dye Congo red, whereas variants grow as pale (Crb^-) colonies (Parton, 1988).

The mechanism of change from virulence to avirulence in *B.pertussis* is only now becoming fully understood. Standfast (1951) and Parker (1976; 1979), proposed that phase variation resulted from a non-ordered accumulation of point mutations which selected for

variants deficient in virulence properties, but with an enhanced capacity to grow *in vitro*. A multi-step, non-random process for change of phase was discussed by Leslie and Gardner (1931). Goldman *et al.* (1984) selected organisms able to grow on defined synthetic media known to be unable to sustain growth of phase I organisms, and screened the variants for production of HLY, PT and FHA. Only four out of a possible eight combinations of these traits were found, suggesting phase variation to be an ordered process involving a multi-step disappearance of virulence factors. However, the existence of organisms of intermediate phenotype does not necessarily mean that they are intermediates in the process of phase variation. C.J. Duggleby (personal communication) selected for variants on a batch of agar unable to sustain the growth of phase I organisms, screening for seven virulence-associated factors. These intermediates showed no ordered loss of factors.

Goldman *et al.* (1987) have suggested that DNA modification may be involved in phase variation, having found variant DNA to be less methylated than that in virulent strains and more amenable to restriction enzyme digestion. Alternatively, McPheat and McNally (1987b) have suggested that DNA rearrangements at the sites of repeated DNA sequences may be involved in the regulation of virulence factors, since differences in hybridisation patterns of chromosomal DNA digests, probed with repeat sequences, have been shown between phase I and phase IV strains.

An alternative mechanism for phase variation is the single-step, reversible change proposed by Weiss and Falkow (1984), for producing the fully avirulent phase variants. Increased tolerance to

erythromycin was used to select for avirulent organisms, while regained haemolysis was used to screen for the reverse event. Two complete cycles of phase variation were demonstrated indicating the process to be fully reversible, although reversion appeared to be influenced by environmental factors. The single-step nature of this event was further confirmed by the observation that a single insertion of the transposon Tn*5* at a specific site in the *B. pertussis* chromosome caused the concomitant loss of LPF, FHA, HLT, HLY and AC in BP347 [see Section 1.viii.d.] (Weiss *et al.*, 1983).

1.vi.d. Mechanisms of variation

Weiss and Falkow (1984) discussed the finding that a single insertion of the transposon Tn*5* into the *B. pertussis* genome could co-ordinately turn off the expression of virulence factors associated with phase I organisms. However, the transposon mutant (BP347) was not equivalent to phase IV strains, as it was unable to grow on nutrient agar. These authors proposed a model for phase variation, based on the avirulent (Vir-) transposon mutant BP347, relying upon the existence of a *trans*-acting gene product. The *vir* gene product was proposed to act as a positive inducer for the virulence-associated genes, allowing co-ordinate expression at multiple loci. The model also provided an explanation for the failure to obtain expression of *B. pertussis* virulence factors when fragments of *B. pertussis* chromosomal DNA were cloned in *E. coli* (Weiss and Falkow, 1983; Brownlie *et al.*, 1988; C.J. Duggleby, personal communication). Similar genetic control systems have been identified in *Vibrio cholerae* (Mekalanos, 1985), *Staphylococcus aureus* (Recsei *et al.*, 1986) and *Pseudomonas aeruginosa* (Wozniak *et al.*, 1987).

A potential binding site for the *vir* gene product was suggested to be a set of tandem repeats upstream of the PT operon (Locht and Keith, 1986). Gross and Rappuoli (1988) identified the regulatory elements involved in the transcription of the PT operon as including the 170bp DNA sequence upstream of the start site of transcription. A 21bp repeated sequence in this region was proposed to be the binding site for the *trans*-activating factor(s). However, similar sites have not been shown to be present upstream of the adenylate cyclase operon (Laoide and Ullmann, 1990), nor the FHA structural gene (Roy *et al.*, 1989).

The reversibility of phase change could be accounted for by the *vir* locus or its products being subject to regulation. Foxall *et al.* (1990) have proposed that expression could be controlled by an invertible region of DNA, while Lax (1985) has suggested that phase variation could result from a random mutation in the control region, followed by selection. Stibitz *et al.* (1988) have cloned and mapped the *vir* locus (Fig. 4) from a series of virulent and avirulent phase variants derived from the Tohama III strain, then sequenced this region in both types. The 5kb *vir* locus was identified juxtaposed to the *fha* genes and showed no genetic rearrangements between the *vir+* and the *vir-* forms of the loci. However, sequence data revealed virulent organisms to have a stretch of six cytosine (C) residues in a particular region, whereas avirulent variants had a single insertion at this point, giving seven cytosine residues (Fig. 5) (Stibitz *et al.*, 1989). Hence it appears that one mechanism for phase variation in *B. pertussis* strain Tohama involves a frame-shift mutation at an unstable stretch of nucleotide bases within a central regulatory

Fig. 4 Restriction map of the *bvg-fha* region

The restriction map of the genes encoding the *trans*-activating virulence regulatory proteins (*bvg*A, B and C) and filamentous haemagglutinin (*fha*B, *fha*A and *fha*C) was rearranged from Aricó *et al.* (1989). The transposon insertion site in mutant BP347 is shown in the 2.7kb EcoRI fragment within *bvg*C.

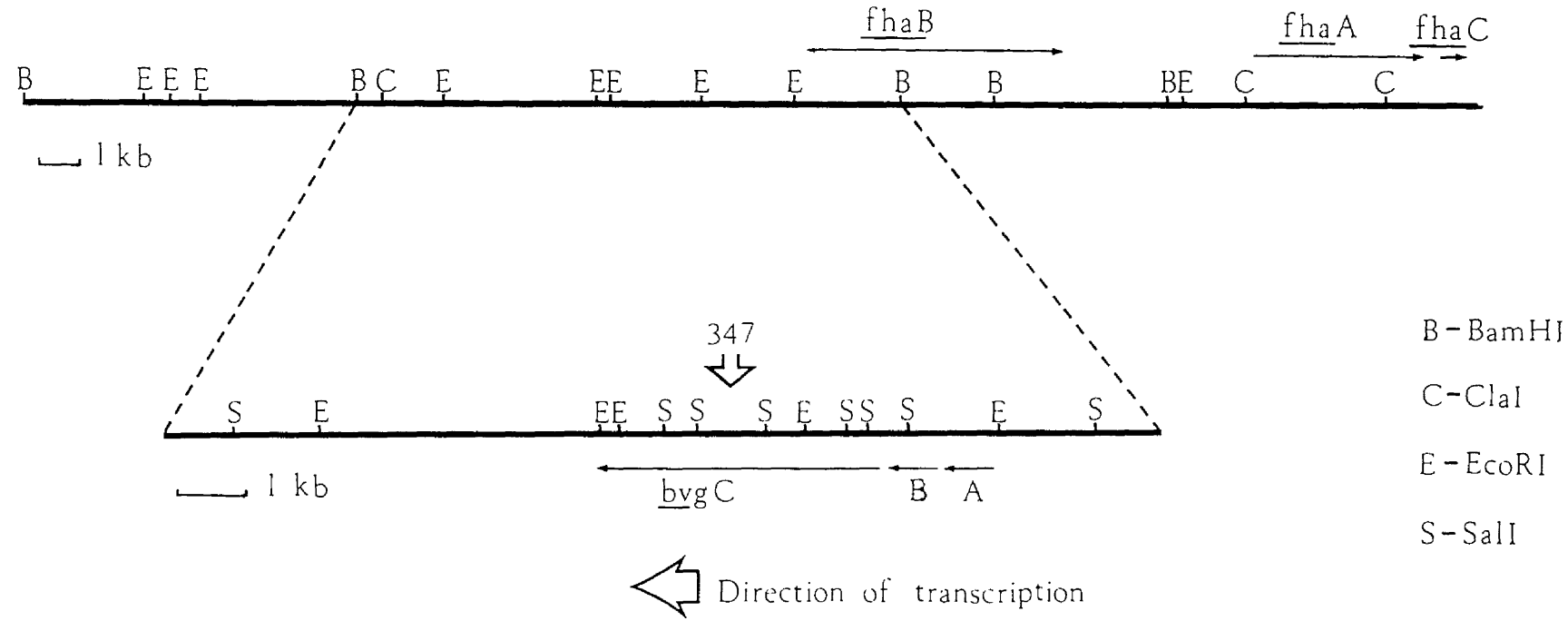
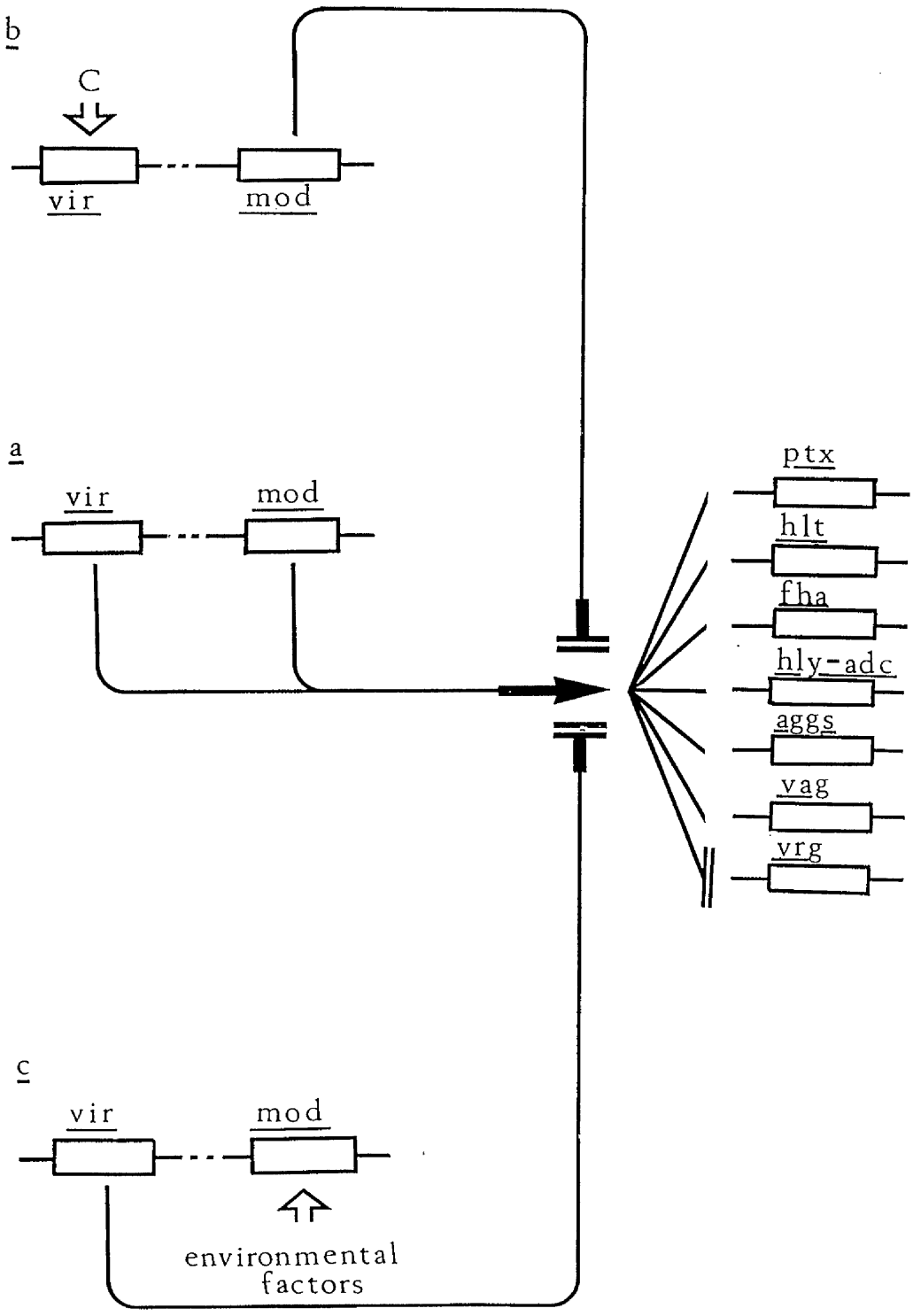

fhaB
fhaA
fhaC
B E E E B C E E E E B B B E C C
1 kb
347
S E E E S S S E S S S E S
1 kb
bvgC
B A
B - BamHI
C - ClaI
E - EcoRI
S - SalI
Direction of transcription

Fig. 5 A model for antigenic variation in *B. pertussis*

Expression of the *vir* activated genes (*vag*) including *ptx*, *hlt*, *fha*, etc., is shown in (a), where the products of both the *vir* and *mod* genes (renamed *bvg*A and *bvg*B: Aricó *et al.*, 1989) and *bvg*C (not shown) are required. This combination of genes does not allow the expression of the *vir* repressed genes (*vrg*). The model for phase variation proposed by Stibitz *et al.* (1989) is shown in (b), where insertion of a single cytosine residue (C) in a string of such residues inactivates the *vir* gene. Knapp and Mekalanos (1988) have shown how environmental factors can repress the *mod* gene as seen in antigenic modulation, not allowing expression of *vag*, but allowing expression of *vrg* (not shown).


b
C
vir
mod
a
vir
mod
ptx
hlt
fha
hly-adc
aggs
vag
vrg
c
vir
mod
environmental
factors

locus.

Knapp and Mekalanos (1988) isolated mutants of *B. pertussis* which constitutively expressed virulence factors in the presence of modulator stimuli. The gene carrying such mutations was designated *mod* and shown to be distinct from the *vir* gene (or *bvg*C), although in close physical proximity. The *mod* gene product has been suggested to have sensory functions for the environmental signals that cause antigenic modulation, co-ordinately regulating the *vir* gene or its product (Fig. 5), and as such may be considered to have a function in the persistence of *B. pertussis* in some, as yet unidentified, alternative environment.

The gene originally designated *vir* has recently been renamed as part of the *bvg* (*Bordetella* virulence gene) locus. Aricò *et al.* (1989) have reported the complete nucleotide sequence of the *B. pertussis bvg* region, identifying three open reading frames, *bvg*A, *bvg*B and *bvg*C. The deduced protein products of *bvg*C and *bvg*A share homology with a family of bacterial signal-transducing proteins and have been suggested to correspond to the *vir* and *mod* genes respectively [see below].

B. bronchiseptica EcoRI-digested DNA has been shown to have a similar hybridisation pattern to *B. pertussis* EcoRI-digested DNA when probed with the cloned *bvg* locus on pRMB2 (McGillivray *et al.*, 1989) [see Section 1.ix.]. Additionally, a derivative of pRMB2, pDM1, has been observed to complement avirulent phase variants of *B. bronchiseptica* to restored expression of virulence determinants. Monack *et al.* (1989) have recently reported that phase variants of *B. bronchiseptica* arise by spontaneous deletions (of 50 - 500bp) in the

bvg locus. The conservation of the *bvg* locus in both *B. bronchiseptica* and *B. pertussis*, suggests that spontaneous deletions may be an alternative mechanism for phase variation in both strains.

1.vi.e Bacterial signal-transducing proteins

Regulatory proteins have been identified in numerous bacteria; such regulatory proteins, which are members of two-component sensory transduction systems, consist of a sensory (transmitter) protein and a regulatory (receiver) protein (Ronson *et al.*, 1987). The sensory component, usually a *trans*-membrane protein with cytoplasmic and extracytoplasmic domains, detects certain environmental stimuli and relays the information to the regulatory component, by phosphorylation of the receiver, which in turn regulates gene expression by the phosphorylated protein binding to specific DNA sites. The transmitter and receiver modules are conserved in all signal-transducing proteins, while the sensor and regulator modules vary according to the stimuli they respond to and the DNA structure with which they interact. Such systems have been found which respond to osmolarity in *E. coli* (*envZ* / *omp*R genes), and control virulence in *Agrobacterium tumefaciens* (*virA* / *virG* genes) and *Vibrio cholerae* (*tox*R). The *tox*R system has been proposed to be a one-component analogue of the two-component systems (Miller *et al.*, 1987). Gross *et al.* (1989b) have classified the proteins belonging to these systems on the basis of their sequence homology.

Aricò *et al.* (1989) have proposed a model for the three-component system of signal-transducing proteins in *B. pertussis*. Both BvgB and the N-terminal portion of BvgC are located in the periplasm where environmental signals are recognised and transduced to the cytoplasmic

transmitter module of BvgC. The receiver portion of BvgA is then activated, which in turn allows the regulatory module of BvgA to act as a typical transcriptional activator. Additionally, BvgC contains a receiver domain fused to the C-terminal part of the transmitter module, the role of which is unclear but may possibly be involved with DNA binding, or the transmission of other signals which affect the activity of BvgA.

1.vii Genetic Transfer in *B.pertussis*

Branefors (1964) first demonstrated transformation in *B.pertussis* by transferring streptomycin resistance to a sensitive host. Since then Weiss and Falkow (1982) have transformed *Bordetella* species with plasmids using cold-shock or freeze-thaw procedures, but only at low frequencies, and Lax (1987) has shown improved transformation of the bordetellae, obtaining 10^4 transformants per microgram of plasmid DNA.

Broad-host-range plasmids, from incompatibility (Inc) groups P and W, have been introduced into *B.pertussis* by conjugation (Weiss and Falkow, 1982). Plasmids with a ColE1 replicon along with IncQ group plasmids were, however, found not to be maintained in *B.pertussis*. Based on this information Weiss and Falkow (1983) used the broad-host-range transfer (*tra*) genes of an IncP plasmid and the narrow-host-range replication genes of ColE1 to construct a 'suicide' vector (pUW964) for use in transposon mutagenesis of *B.pertussis* [see Section 1.viii.b.1. Smith *et al.* (1986) also showed the transfer of various IncP group plasmids into *B.pertussis* and used the transconjugants for chromosome mobilisation studies.

Electroporation, the introduction of DNA into cells using a high

voltage pulse, has recently been demonstrated with *B. pertussis* (Zealey *et al.*, 1988).

1.viii Transposon Mutagenesis

The mutagenic properties of transposons have provided a sophisticated technique to investigate the structure and function of bacterial genes. In contrast to classical chemical mutagenesis, single insertions of transposons can be obtained at a large number of sites throughout the bacterial chromosome, causing total inactivation of target genes and linking these with the phenotype of the transposon. Thus, inserted transposons can provide a genetic marker for the identification of their insertion sites, and as such can be used to define the limits of a gene. Cloning of interrupted genes utilizing the marker genes carried by the transposon permits the screening of gene libraries and identification of wild-type genes. For reviews see Simon (1989) and Duggleby *et al.* (1990).

1.viii.a. Transposable genetic elements

Transposable elements are a family of mobile genetic entities able to promote their own movement to new genomic locations. Transposition is independent of host cell recombination systems and does not require extensive areas of DNA homology (Kleckner, 1981).

The smallest transposable elements are the insertion sequences (or IS elements) which encode only transposition functions. In addition to being found as independent genetic entities, the IS elements are also found in the inverted or direct repeated termini of composite transposable elements (transposons) (Calos and Miller, 1980).

Transposon Tn*5* has been used extensively for mutagenesis and has many advantageous properties (Berg and Berg, 1983). Initially isolated from *Klebsiella pneumoniae* (Berg *et al.*, 1975), Tn*5* has been introduced into many bacterial species and shown to have a high transposition frequency (10^{-2} - 10^{-5} per generation) (Berg and Berg, 1983), little insertional specificity and low excision frequency (Berg *et al.*, 1980). The transposon is composed of inverted repeats of IS*50* (termed IS*50*L and IS*50*R) encompassing resistance genes to the aminoglycoside antibiotics kanamycin, neomycin and streptomycin (which is expressed only in certain bacteria), and to the anti-cancer drug bleomycin (Fig. 6). The IS elements in Tn*5* are independently mobile (Berg *et al.*, 1982), IS*50*R encoding the functional transposase gene (Rothstein *et al.*, 1980). During Tn*5* mutagenesis experiments, independent IS*50* transposition events have been noted in *Pseudomonas solanacearum* (Boucher *et al.*, 1985) and *Erwinia carotovora* (Zink *et al.*, 1984).

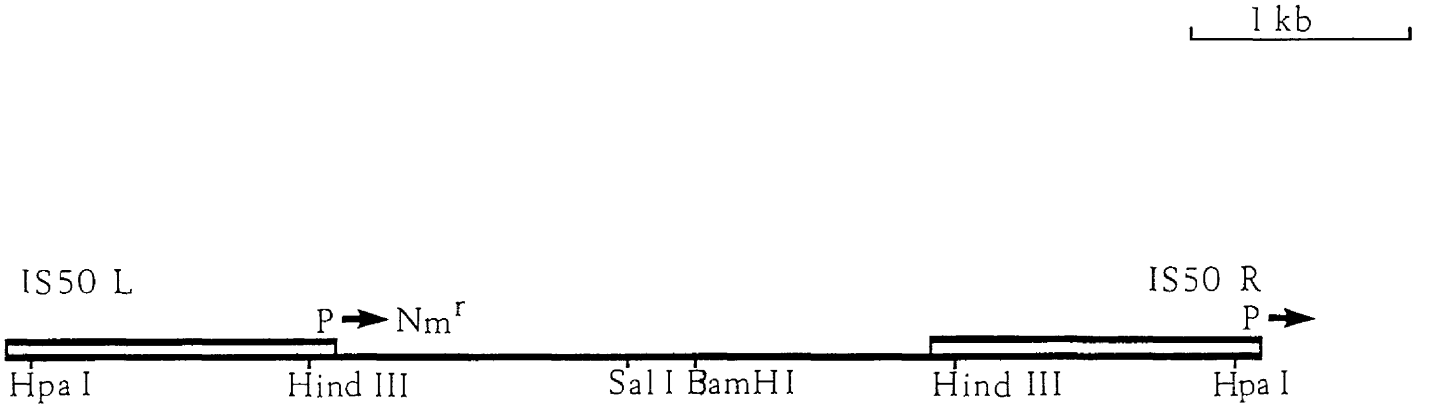
Insertions of Tn*5* usually have polar effects. In operons the transcription of genes distal to the insertion site is interrupted. However, occasionally insertions in operons can give rise to a low level of constitutive expression of distal genes, due to a promoter present near the end of IS*50*R (Berg *et al.*, 1980).

A variety of Tn*5* derivatives have been constructed which offer specialised properties. Numerous derivatives carrying alternative antibiotic resistance genes are available, including Tn-Ap (ampicillin), Tn*5*-Cm (chloramphenicol) and Tn*5*-TC1 (tetracycline) (Sasakawa and Yoshikawa, 1987). An assortment of fusion elements have also been constructed to monitor gene expression, or to produce

Fig. 6 Restriction map of transposon Tn*5*

Tn*5* has been well-characterised physically (Jorgensen *et al.*, 1979; Auerswald *et al.*, 1981; Beck *et al.*, 1982; Mazodier *et al.*, 1985). The central antibiotic resistance genes are flanked by inverted repeats of the insertion sequence IS*50* (IS*50*L and IS*50*R). The promoter (P), for the neomycin phosphotransferase gene (which confers resistance to kanamycin and neomycin) and the genes encoding resistance to bleomycin and streptomycin, is present on the internal end of IS*50*L. A second promoter (P) is present on the external end of IS*50*R and may be the cause of low level constitutive expression of some transposon-interrupted genes.

Tn5



P - promoter

Nm - neomycin phosphotransferase

chimaeric proteins eg. Tn*5-lac* (a promoter probe utilizing *lacZ*) (Kroos and Kaiser, 1984), and Tn*5-phoA* (which creates gene fusions with *phoA* and hence can be used to detect protein export) (Manoil and Beckwith, 1985).

1.viii.b. Transposon delivery vectors

Transposon mutagenesis requires movement of the transposon from the delivery vector, followed by its stable insertion into the host genome. Selective pressure against the vector, and for the transposon, allows for the phenotype of the transposon eg. drug-resistance, to be obtained by the recipient only after a transposition event. Many specialised vectors are available for introducing transposons into recipient bacterial species, but many have only a limited range of use.

One strategy for the production of 'suicide' vectors has been the use of chimaeric plasmids comprising broad-host-range transfer genes and narrow-host-range replication functions eg. pUW964 (ColE1 replication origin and IncP conjugation genes: Weiss *et al.*, 1983), and pGS9 (p15A replicon and IncN conjugation genes: Selvaraj and Iyer, 1983). Boulnois *et al.* (1985) based the vector pLG221 on the narrow-host-range Inc$I\alpha$ plasmid ColIb-P9*drd*-1, a mutant which constitutively expresses (by derepression) conjugation functions. Vectors showing instability outside of *E. coli* due to the insertion of phage Mu DNA have been characterised eg. pJB4JI (Beringer *et al.*, 1978). Meade *et al.* (1982) have, however, shown that in some species Mu can co-transpose with Tn*5*, with the potential to cause genetic rearrangements at the insertion site. Both temperature-sensitive mutants of transmissible plasmids (Jacob *et al.*, 1963; Laird and

Young, 1980) and bacteriophages (Berg *et al.*, 1975; Kleckner *et al.*, 1977) have also been utilized as transposon delivery vehicles.

Simon *et al.* (1983) described a novel transposon delivery system comprising narrow-host-range plasmids containing the specific IncP-type recognition site for mobilisation (*mob* site) within specific donor strains having the IncP RP4 transfer genes integrated into their chromosome. These plasmids are therefore mobilisable into bacteria which are normally receptive to broad-host-range IncP plasmids.

1.viii.c. Transposon mutagenesis in pathogenic bacteria

Kleckner *et al.* (1977) first used transposon mutagenesis, inserting Tn*5* or Tn*10*, to generate auxotrophs of *E.coli* and *S.typhimurium*. Since then a plethora of experiments have been conducted, many on bacterial pathogens. Insertion mutations in bacterial pathogens may allow the identification of genes specifying virulence determinants. Such genes can be cloned and expressed in *E.coli*, and the purified product of the cloned gene can be studied in animal models, or modified to identify important structural properties. Additionally, mutants with phenotypes altered from the wild-type parent strain can be evaluated in animal models and the contribution of particular virulence factors to the disease process examined (Weiss and Falkow, 1983).

Among the pathogens investigated, Leung and Stevenson (1988) have reported Tn*5*-induced protease-deficient strains of *Aeromonas hydrophila*, which have reduced virulence in fish. In mammalian pathogens, transposon mutagenesis has been reported in *Brucella abortus* (Smith and Heffron, 1987), *Legionella pneumophila* (Keen *et al.*, 1985) and the 140-megadalton invasion plasmid of *Shigella*

flexneri (Sasakawa *et al.*, 1986). Tn*5* mutagenesis has been used in *Bordetella avium*, the causative agent of coryza in turkey poults, to identify non-haemagglutinating mutants (Leyh *et al.*, 1988), but of the bordetellae, the human pathogen *B. pertussis* has undoubtedly undergone the most extensive investigation by transposon insertion mutagenesis.

1. viii. d. Transposon mutagenesis of *B. pertussis*

Weiss *et al.* (1983) and Robinson *et al.* (1986) have reported Tn*5* mutagenesis of *B. pertussis* and the identification of mutants unable to express virulence factors (Table 1). Mapping studies around the transposon insertion sites suggested no clustering of the virulence factors around specific areas of the genome, although a mutant deficient in both adenylate cyclase and haemolytic activity suggested that the genes expressing these functions were linked on an operon. Weiss *et al.* (1984) used intranasal infection of infant mice to test the virulence of Tn*5*-induced mutants (Table 2). The avirulent transposon mutant BP347 was utilized by Stibitz *et al.* (1988) to identify the interrupted *bvg* locus and to clone the wild-type genes into cosmid pUW21-26. Tn*5* mutagenesis in *E. coli* was then used to define the limits of the locus.

Knapp and Mekalanos (1988) used Tn*5*-*phoA* gene fusions to define two sets of genes whose expression is either activated (*vag* loci) or repressed (*vrg* loci) by modulation signals. Both *vag* and *vrg* loci were shown to be regulated by the *bvg* locus gene products, since insertion mutations in *bvg* lead to repression of *vag* gene fusions and derepression of *vrg* gene fusions.

Weiss *et al.* (1989) introduced Tn*5*-*lac* insertions into *B. pertussis* *vir*-regulated genes. Such insertions were identified by

Table 1

Transposon mutants of *B. pertussis*

Genes mutated /fused	Transposon	No. of mutants	Strain	Identification No.	Reference
fha	Tn*5*	1	Tohama	BP353	1
	Tn*5*	4	Wellcome 28		2
hly	Tn*5*	1	Tohama	BP349	1
	Tn*5*	1+/−	Wellcome 28		2
cyaA	Tn*5*	1	Tohama	BP348	1
	Tn*5*	1	Wellcome 28		2
ptx	Tn*5*	1+/−	Tohama	BP357	1
	Tn*5*	2	Wellcome 28		2
*fim*3	Tn*5*	6	Wellcome 28		2
vir	Tn*5*	1	Tohama	BP347	1
vag	Tn*5-phoA*	8	18-323		3
vrg	Tn*5-phoA*	5	18-323		3

+/− Reduced levels.

References

1. Weiss *et al.* (1983).

2. Robinson *et al.* (1986).

3. Knapp and Mekalanos, (1988).

Table 2

Virulence of *B. pertussis* Tn5 mutants

Mutant	Phenotypic characteristics	LD_{50} in suckling mice (cfu/mouse)
BP338 (wt)	Adc^+, Hly^+, Fha^+, Ptx^+, Hlt^+	$2x10^3$
BP347	Adc^-, Hly^-, Fha^-, Ptx^-, Hlt^-	$>3x10^7$
BP357	Adc^+, Hly^+, Fha^+, $Ptx^{+/-}$, Hlt^+	$5x10^6$
BP353	Adc^+, Hly^+, Fha^-, Ptx^+, Hlt^+	$8x10^3$
BP349	Adc^+, Hly^-, Fha^+, Ptx^+, Hlt^+	$4x10^5$
BP348	Adc^-, Hly^-, Fha^+, Ptx^+, Hlt^+	$>3x10^7$

Taken from Weiss *et al.* (1984).

expression of β-galactosidase during X-mode conditions, followed by loss of expression in C-mode growth conditions. Of fourteen mutants only six had insertions in known *vir*-regulated genes.

1.ix Cloning of *B. pertussis* Genes

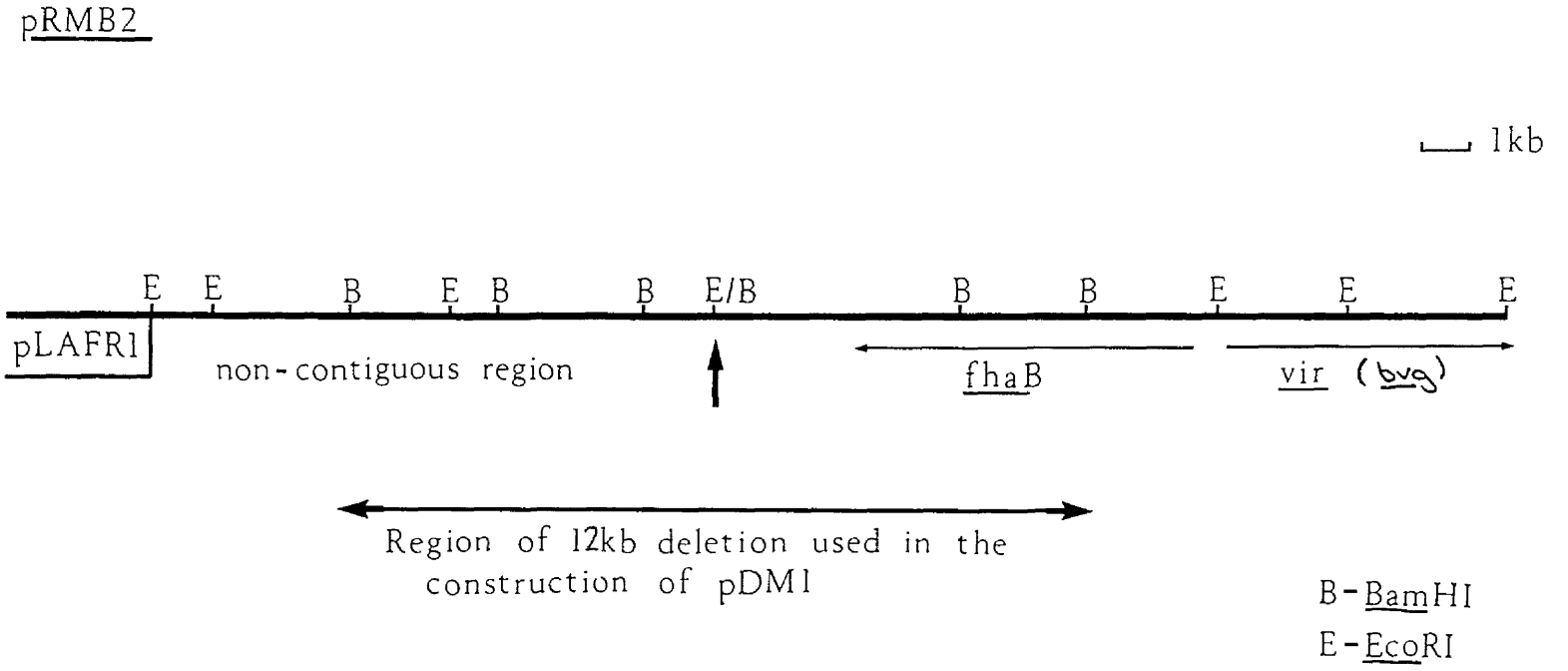
Most experiments involving cloning of *B. pertussis* DNA have been performed on the virulence-related genes eg. *ptx*, *cya*A, *fim*, etc. [see Section 1.iii.]. Sequencing has allowed the deduction of amino-acid sequences and the identification of potential epitopes. For the production of new acellular vaccines, the known epitopes of cloned genes could be manipulated to provide protective but non-toxic components. Cloned genes with increased expression could also prove advantageous for vaccine production. However, expression of the virulence genes in *E. coli* has proved difficult because of the requirement for the *trans*-activating virulence control genes (Weiss and Falkow, 1983). Brownlie *et al.* (1988) identified a plasmid (pRMB2) from their gene library which contained the *bvg* locus. Mapping and subcloning of pRMB2 has identified regions of similarity in this clone to the map of Stibitz *et al.* (1988) (Fig. 7) (McGillivray *et al.*, 1989).

Shareck and Cameron (1984) have cloned DNA fragments which code for the synthesis of two outer-membrane proteins of 33 and 30kD, while Brownlie *et al.* (1986) have shown expression of the *trp*E and *glnA* genes of *B. pertussis* in *E. coli*. More recently, Maskell *et al.* (1988) have cloned, sequenced and expressed the *aro*A gene in *E. coli*, without the requirement of expression vectors.

Stern *et al.* (1984) have noted the presence of repeated DNA

Fig. 7 Restriction map of the *bvg-fha* region on pRMB2

The restriction map of pRMB2 shows a region of similarity to the map of Stibitz *et al.* (1988), along with a non-matching region, considered to be a non-contiguous fragment (McGillivray *et al.*, 1989). The central arrow denotes the joining point of these two fragments. An 11kb deletion used in the construction of the subclone pDM1 is also shown.


pRMB2
1kb
E E B E B B E/B B B E E E
pLAFR1
non-contiguous region
fhaB
vir (bvg)
Region of 12kb deletion used in the construction of pDM1
B-BamHI
E-EcoRI

sequences in the genomes of many bacteria. A repeat sequence with at least 20 copies has been identified in *B. pertussis*, but shown to be absent in *B. parapertussis*, *B. bronchiseptica* (McPheat and McNally, 1987a), and *B. avium* (McPheat and McNally, 1987b). The sequence has been cloned and may find use as a genetic probe for the identification of *B. pertussis* strains. Repeat sequences have also been identified by Park *et al.* (1988) and Alsheikhly and Löfdahl (1989), the latter being present in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* in more than one copy.

Object of Research

The project was directed towards the establishment of the most suitable Tn*5* delivery system for insertion mutagenesis of *B. pertussis*. Transposon mutants with specific virulence-related phenotypes were to be isolated to further our understanding of the different modes of regulation of virulence factor production in this pathogen and to identify any new genes associated with virulence regulation.

An additional directive of the project was the use of a bank of transposon mutants to identify isolates deficient in production of single virulence-associated factors and to assess the virulence of these strains in animal models. Mutants with relevant properties were to be considered for use as vaccines to study protective activity in infant mice.

MATERIALS AND METHODS

2.i Bacterial Strains and Plasmids

2.i.a. *Bordetella pertussis* strains

B. pertussis Wellcome 28 strain phase I (serotype 1,2,3) was supplied by P. Novotny (Wellcome Laboratories, Beckenham). *B. pertussis* Tohama phase I (serotype 1,2) was obtained from the Centre for Applied Microbiology and Research culture collection (PHLS, CAMR, Porton Down, Salisbury), as was *B. pertussis* 134 phase I (serotype 1,3). These strains were obtained from A. Robinson (Biologics Division, CAMR).

B. pertussis Tohama 347 (BP347), a transposon-insertion mutant pleiotropically negative for the expression of virulence-associated factors (Vir^-) due to a Tn*5* insertion in the *trans*-activating virulence regulatory locus *vir*, was supplied by A. Weiss (Virginia Commonwealth University, Richmond, USA). *B. pertussis* Tohama 348 (BP348), a non-haemolytic (Hly^-) Tn*5*-insertion mutant lacking the ability to express adenylate cyclase (Adc^-), was also supplied by A. Weiss. These strains were obtained from the Microbiology Department culture collection (Glasgow University). *B. pertussis* 18-323 vaccine strain, which was used as the challenge strain in mouse protection tests, was also obtained from this source.

B. pertussis L84 phase I Crb^-, a spontaneous mutant of strain L84 phase I unable to bind the dye Congo red (associated with avirulent Vir^- strains), was supplied by R. Parton (Glasgow University).

B. pertussis, a Category 2 pathogen according to the categorisation scheme of the Advisory Committee on Dangerous Pathogens (ACDP), was handled under the appropriate containment conditions (ACDP, 1984).

2.i.b. *Escherichia coli* strains

E.coli strains used in this study, and their relevant characteristics, are shown in Table 3.

2.i.c. Plasmids

Plasmids used in this study are shown in Table 4.

2.ii Media

The chemically-defined media of Stainer and Scholte (1971), with the growth stimulant heptakis (2,6-O-dimethyl) β-cyclodextrin (SS(MeβCD)) (Imaizumi *et al.*, 1983), was used for liquid culture of *B.pertussis* and *E.coli* strains. This cyclodextrin was obtained from Teijin Limited (Tokyo, Japan). Growth supplements were added to the media prior to use, and 1% (w/v) casamino acids were added where minimal conditions were not required (SS(MeβCD+CAA)). Cyclodextrin solid medium (CSM) or Bordet-Gengou agar (BG) were used for solid-phase growth of *B.pertussis*. Nutrient agar (NA) and nutrient broth (NB) were used for solid- and liquid-phase growth of *E.coli* strains respectively. Bacterial cultures were stored at -20°C in a glycerol-based casamino acids solution. [See Appendix 1 - Media preparation].

2.ii.a. Cyclodextrin solid medium for selection of avirulent mutants

CSM for use in the selection of potentially avirulent transposon mutants was prepared using a batch of agar - Difco Bacto No. 726942, previously shown to inhibit the growth of phase I organisms.

2.ii.b. Congo red (CR)

Congo red dye (Sigma) for use in differentiation of virulent and avirulent *B.pertussis* strains, was made up as a 1% (w/v) solution in distilled water, filter sterilised and added to CSM to a final

Table 3

E. coli strains

Strain	Relevant Characteristics	Plasmids	Source
E. coli HB101	Leu-, Pro-, Thi-	pRK2013	CAMR culture collection
E. coli HB101	Leu-, Pro-, Thi-	pUW964	A. Weiss*
E. coli HB101	Leu-, Pro-, Thi-	pGS18	V. Iyer*
E. coli 1830	Met-, Pro-	pJB4JI	J. Beringer*
E. coli WA803	Met-, Thi-	pGS9	V. Iyer*
E. coli S17-1	Pro-, Thi- Mobilisation strain RP4-2-Tc::Mu-Km::Tn7 Tp^R, Sm^R.	pSUP2021	R. Simon*
E. coli C600	Leu-, Thi-, Thr-	pLG221	G. Boulnois*
E. coli W3110		pColIb-P9*drd*	CAMR culture collection
E. coli G802	His-, *cyaA*Δ854		S. Garges* (Δ854; Garges and Adhya, 1985)
E. coli DH1	Thi-	pRMB2	R. Brownlie
E. coli JM83	Pro-	pDM1	D. McGillivray
E. coli DH1	Thi-	pRMB11	R. Brownlie

*Obtained by C. J. Dugglebly

Table 4

Plasmids

Plasmid	Size	Relevant Characteristics	Reference
pRK2013	48.0kb	P-group *tra* genes, ColE1 replicon. Mobilisation vector. Km^R	Figurski and Helinski (1979)
pUW964	73.0kb	pRK2013*kan*::Tn*7xyz*::Tn*5* Tp^R, Sp^R, Km^R.	Weiss *et al.* (1983)
pJB4JI	97.2kb	pPH1JI::Mu::Tn*5*. Gm^R, Sp^R, Km^R.	Hirsch and Beringer (1984)
pGS18	29.5kb	N-group *tra* genes, p15A replicon. Tn*1*. Cm^R, Ap^R.	Selvaraj and Iyer (1983)
pGS9	30.5kb	N-group *tra* genes, p15A replicon. Tn*5*. Cm^R, Km^R.	Selvaraj and Iyer (1983)
pSUP2021	13.2kb	pBR325 replicon. Mob+. Tn*5*. Ap^R, Cm^R, Km^R.	Simon *et al.* (1983)
pColIb-P9	93.2kb	Derepressed I_α-group *tra* genes.	Rees *et al.* (1987)
pLG221	99.0kb	ColIb*drd*-1 *cib*::Tn*5*, Km^R	Chatfield *et al.* (1982)

Table 4 (cont.)

Plasmid	Size	Relevant Characteristics	Reference
pRMB2	47.7kb	pLAFR1 derivative containing *B. pertussis vir* locus. Tc^R.	Brownlie *et al.* (1988)
pDM1	33.6kb	Subclone of pRMB2 containing *vir* locus. Tc^R	McGillivray *et al.* (1989)
pRMB11		Vector pIC20H containing the 5.5kb ClaI fragment encoding the transcriptional start site of the adenylate cyclase operon.	Brownlie (personal communication)

concentration of 0.002%.

2.ii.c. ICR 191

ICR 191 (Polysciences, Inc., Worrington, PA, USA) (an acridine-based compound which induces frameshift mutations; Miller, 1972) was made up as a 1mg/ml solution in distilled water and stored in the dark at -20°C. This mutagen was added to liquid media immediately prior to use, or to solid media up to 20 hours before use (to facilitate diffusion into the agar).

2.ii.d. IPTG

Isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) was prepared as a 25mg/ml stock solution in distilled water and stored at -20°C. Stock solution was diluted in media to give a final concentration of 40μg/ml.

2.ii.e. X-gal

5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) (Sigma) was prepared as a 25mg/ml stock solution in dimethyl formamide and stored at -20°C. Stock solution was diluted in media to give a final concentration of 40μg/ml.

2.ii.f. Antibiotics

Antibiotics were made up in distilled water, the exceptions being amphotericin (made up in dimethyl sulphoxide - DMSO), tetracycline (made up in ethanol) and trimethoprim (made up in methanol), filter sterilised and stored at -20°C.

	Final concentration
Amphotericin (Amph)	25μg/ml
Cephalexin (Ceph)	50μg/ml
Chloramphenicol (Cm)	50μg/ml

	Final concentration
Gentamycin sulphate (Gm)	5μg/ml
Kanamycin sulphate (Km)	50μg/ml
Spectinomycin (Sp)	200μg/ml
Streptomycin (Sm)	200μg/ml
Tetracycline (Tc)	20μg/ml
Trimethoprin (Tp)	50μg/ml

2.ii.g. Sterilisation

All equipment and media required sterile were autoclaved at 120°C for 15mins at 15psi. Solutions requiring sterilisation were passed through a Millex GV Millipore 0.22μm disposable filter unit (Molsheim, France).

2.iii Microbiological Techniques

2.iii.a. Filter conjugation

Filter conjugations were performed to introduce Tn*5* into *B.pertussis* from a variety of delivery vectors present in *E.coli* donor strains.

B.pertussis recipient strains were grown on CSM at 37°C for 3-4 days, then transferred to flasks containing 50ml SS(MeβCD+CAA). The flasks were incubated overnight at 37°C with shaking (100rpm). *E.coli* donor strains were streaked onto CSM+Km and incubated over-night at 37°C, then used to inoculate flasks containing 50ml SS(MeβCD+CAA+Km). The flasks were incubated at 37°C, 100rpm. Optical density readings of the donor and recipient bacterial strains were taken every few hours until each had reached an OD_{540nm}=0.4. Purity of the strains was checked by Gram staining.

Cell suspensions for conjugation were prepared from each strain by diluting cultures of standard optical density, then viable cell counts made. The standard suspensions for conjugation were mixed and 2.5ml filtered through a 2cm diameter nitrocellulose filter (pore size 0.45μm) in a presterilised filter unit. Filters were removed from filter units, placed (cells upward) on CSM plates and incubated at 37°C for 18 hours. Approximately ten matings were performed for each different donor strain. A 2ml volume of the *B.pertussis* standard suspension for conjugation was filtered (as above) to identify the frequency of spontaneous resistance to kanamycin.

The growth on the filters was resuspended in 5ml SS(MeβCD). *B.pertussis* transconjugants containing Tn*5* were selected on SSA(MeβCD+Km+Ceph) (resistance to kanamycin selects <u>for</u> the transposon, while minimal media and cephalexin select <u>against</u> the auxotrophic *E.coli* donor strains - Table 3). Final viable counts were made for both *B.pertussis* and *E.coli* strains. Cells from the *B.pertussis* control mating were plated onto CSM+Km.

B.pertussis kanamycin-resistant transconjugants were picked and colony-purified three times, then stored at -20°C in a glycerol based solution.

2.iii.b. <u>Plate conjugation</u> (Smith *et al.*, 1986)

Plate conjugations were performed to increase the yield of transconjugants during matings to obtain Tn*5* mutants unable to bind Congo red (Crb$^-$), and to *trans*-complement the Crb$^-$ Tn*5* mutants with pRMB2 or pDM1.

B.pertussis strains were grown in liquid media for 1-2 days at 37°C, 100rpm, then 100μl of culture was spread on SSA(MeβCD+CAA)

mating plates and incubated for 2 days at 37°C. *E.coli* donor strains (and helper strain HB101 pRK2013 if required) were grown on appropriate antibiotic containing plates overnight at 37°C, then transferred to liquid media and incubated overnight at 37°C, 100rpm.

E.coli strains were diluted 1 in 10 (both donor and helper strains) and 100µl of culture added to the *B.pertussis* mating plates. Cell suspensions were mixed and incubated for 5 hours at 37°C.

Bacteria were resuspended in 1ml SS(MeβCD) and 50µl spread on selection plates. Tn*5* mutants unable to bind Congo red (Crb^-) were selected for on SSA(MeβCD+Km+Ceph+CR). The same selection medium, with the addition of tetracycline, was used for complementation studies (see below). *B.pertussis* and *E.coli* strains were also individually spread on selection plates to act as controls. Selection plates were incubated for 7-10 days to allow for the growth of single colonies of *B.pertussis* transconjugants on minimal media. Transconjugants were colony purified by streaking to single colonies three times, then stored as previously.

2.iii.c. Complementation studies

During conjugations to show complementation in *trans* of Crb^- *B.pertussis* transposon mutants with the cloned virulence-regulatory genes on pRMB2, or pDM1, selection plates comprised SSA(MeβCD+Km+Ceph+Tc+CR). Colonies showing the Crb^+ phenotype were restreaked on BG agar with appropriate antibiotics to ascertain regain of haemolytic activity.

2.iii.d. Antibiotic-resistance screening

B.pertussis kanamycin resistant transconjugants were screened for maintenance of the transposon delivery vector by multi-point

inoculation, from microtitre trays, onto antibiotic containing plates. The multipoint inoculator was alcohol flamed to prevent contamination. Transconjugants were simultaneously inoculated onto CSM to show growth in the absence of antibiotics. Donor and recipient strains were also spotted onto both CSM and antibiotic-containing plates as controls.

2.iii.e. Modulation studies

B.pertussis strains on CSM+CR, or BG overlay plates, were grown at 25°C to show antigenic modulation. Alternatively, plates were prepared containing 20mM $MgSO_4$ (in place of NaCl).

2.iii.f. Frameshift mutagenesis

Frameshift mutagenesis experiments were performed on *B.pertussis* Tn*5* mutants (where the Crb^- phenotype was not associated with the insertion mutation) to identify any spontaneously avirulent mutants. Two methodologies were used:-

i. On solid media.

A central plug of agar was removed from CSM+CR plates and stock solution of ICR191 added to the well. Plates were left over night for the mutagen to diffuse outwards. As controls *B.pertussis* Wellcome 28 (Crb^+) and *B.pertussis* L84 phase I (Crb^-) strains were resuspended from CSM+CR plates, diluted 10^{-4} and 100µl spread evenly onto mutagenesis plates. Plates were incubated for 5-6 days to allow growth to single colonies. Since ICR191 appeared to react with the dye (possibly affecting uptake), colonies were replica plated onto CSM+CR and incubated for a further 5-6 days.

ii. In liquid media (Miller, 1972).

In control experiments *B.pertussis* Wellcome 28 (Crb^+) and *B.pertussis* L84 phase I (Crb^-) were mutagenised in foil-wrapped flasks containing

50ml SS(MeβCD+CAA). ICR191 was added to final concentrations of between 0.5μg/ml and 20μg/ml immediately prior to inoculation with overnight cultures. Control flasks were left free of mutagen. Flasks were incubated with shaking at 37°C and samples removed twice daily for 3 days. Samples were diluted, plated onto CSM+CR and incubated for 7-8 days at 37°C. The ratio of Crb^+: Crb^- colonies from each sample was calculated and plotted against time.

Crb^- transposon mutants were incubated with 0.7μg/ml ICR191 for 2 days. Cultures were diluted and plated onto CSM+CR+Km and any Crb^+ revertants noted.

2.iv DNA Techniques

[See Appendix 2 - Buffers and Solutions for DNA Techniques]

2.iv.a. Chromosomal DNA preparation

Cells from confluent growth on one plate of CSM were suspended in 10ml TE buffer, 2ml lysozyme (20mg/ml) added and cells incubated at 37°C for 15 minutes. EDTA (4ml of 0.5M) was added and cells were left for 5 minutes at room temperature, followed by the addition of 4ml 20% SDS (w/v) and incubation at 60°C for 1 hour with occasional swirling. Lysates were left to cool and then extracted twice with an equal volume of equilibrated phenol/chloroform. After separating the phases by centrifugation for 15 minutes at 5000rpm and discarding the organic phase and interface layer, a further extraction was carried out. Then, one tenth volume of 5M NaCl was added and mixed in by gentle inversions, prior to the addition of two volumes of ethanol. After 30 minutes at -20°C the DNA was spooled out, dissolved in 8ml TE buffer, and left overnight at 4°C.

RNA was removed by adding preboiled (15 minutes) RNase A (0.42ml, 1mg/ml) to the DNA solution and incubated for 2 hours at 37°C. After this any remaining protein was removed by adding 80µl 0.5M EDTA, 0.25ml 20% SDS, and 0.5ml pronase (1mg/ml). After incubation for 1 hour at 37°C the solution was extracted twice with equilibrated phenol/chloroform and once with chloroform (as previously). One tenth of the volume of 5M NaCl was added followed by two volumes of ethanol at -20°C, prior to respooling the DNA on a glass rod. The DNA was dissolved in 4ml TE buffer and dialysed at 4°C, overnight against four changes of TE, each of 1 litre.

2.iv.b. Large scale plasmid preparation (Hansen and Olsen, 1978)

An *E.coli* starter culture was incubated at 37°C, 100rpm, for 6 hours. Of this, 2ml was used to inoculate 1 litre of L-broth, which was grown overnight. Chloramphenicol was added to a final concentration of 170µg/ml and the culture left shaking vigorously for 4-18 hours.

Cells were harvested by centrifugation for 15 minutes 9000g, at 4°C. Supernate was removed and the cells resuspended in 2.6ml of 25% (w/v) sucrose in 0.05M Tris-HCl, pH8.0, and transferred to a polypropylene centrifuge tube maintained on ice. Cells were lysed by the addition of 2ml lysozyme (20mg/ml in 0.25M EDTA, pH8.0) and the tubes kept at 37°C for 5 minutes, with swirling. After this 2.6ml of 0.25M EDTA (pH8.0) was added and the lysate swirled intermittently on ice for 5 minutes. Brij./DOC (4ml) solution was added rapidly from a 10ml pipette, mixed, then left on ice for 20-30 minutes.

Cell debris was pelleted along with the bulk of the

chromosomal DNA by centrifugation for 45 minutes, 15,000g at 4°C. The cleared lysate was carefully decanted into a fresh tube and the volume measured. Solid caesium chloride (CsCl) was added at the rate of 0.95g/ml to the cleared lysate. After this 0.1ml ethidium bromide solution (20mg/ml in dH_2O) was added and the solution transferred to a labelled Ti50 (Beckman) polyallomer tube. Tubes were balanced in pairs to within 50mg, then all the air was displaced and the tops heat-sealed. The tubes were transferred to a Ti50 rotor and centrifuged at 39,000rpm for 36-40 hours, 18°C.

Tubes were carefully removed from the rotor and clamped under ultraviolet (uv) illumination. The lower plasmid band was removed by puncturing the side of the tube with a syringe needle. Plasmid DNA was allowed to drip into a tube, the ethidium bromide removed by extraction with isopropanol (equilibrated with a saturated solution of CsCl), and the DNA dialysed against three changes (1 litre each) of TE buffer at 4°C, overnight.

2.iv.c. DNA concentration and purity

DNA concentration and purity were measured spectrophotometrically by reading the OD_{260nm} and OD_{280nm}. Pure DNA solutions have $OD_{260/280}$ ratios > 1.7. Duplex DNA solutions at 50µg/ml have an OD_{260} of approximately 1.

2.iv.d. Precipitation of DNA

One tenth volume of 3M ammonium acetate and 2.5 volumes of cold absolute ethanol were added to the DNA solution and mixed. DNA was incubated at -70°C for 15 minutes, then centrifuged at maximum speed in a microfuge (10,000rpm) for 15 minutes. The supernate was carefully removed and the pellet dryed *in vacuo*. DNA was

resuspended in TE buffer to the required concentration.

2.iv.e. Restriction enzymes

Restriction enzymes (Table 5) and x10 reaction buffers were obtained from BRL, except HpaI (Pharmacia) which was used in the presence of the medium salt buffer (Maniatis *et al.*, 1982). Digestions normally contained 1µg DNA and 1-5units of enzyme in a 20µl reaction volume, and were incubated overnight. After incubation 5µl of loading buffer was added to 20µl of digested DNA, centrifuged briefly and loaded onto a gel.

2.iv.f. Lambda HindIII markers

For the production of markers 2µg of λ DNA (BRL) was added to 40µl 1x React™ 2 buffer (BRL). The restriction enzyme HindIII (1µl) was added and left to react for 1 hour at 37°C. Restriction fragments were used as molecular weight markers either directly on gels, or were 3'-end-labelled for use as radioactive markers (see below).

2.iv.g. End-labelling of markers (Downing *et al.*, 1979)

Approximately 500ng of a λ-HindIII digest was added to 50µl of 1x Core buffer (BRL). After this, 10µl of A solution (containing 0.1mM each of the deoxyribonucleotide triphosphates dCTP, dGTP and dTTP) was added, followed by 1µl of α-^{32}P-dATP (Amersham: specific activity = 3000 Ci/mmol; radioactive concentration = 10mCi/ml). The reaction was started by the addition of 1 unit of the large (Klenow) fragment of *E.coli* DNA polymerase (Boehringer Mannheim). The mixture was incubated at room temperature for 15 minutes then loaded directly onto a gel.

Table 5

Restriction enzymes

Enzyme	Conditions		Cleavage site
	Reaction buffer	Temperature	
BamHI	REact 3™	37°C	5'-G↓GATCC-3'
EcoRI	REact 3™	37°C	5'-G↓AATTC-3'
HindIII	REact 2™	37°C	5'-A↓AGCTT-3'
HpaI	Medium salt*	37°C	5'-GTT↓AAC-3'
SalI	REact 10™	37°C	5'-G↓TCGAC-3'

*Maniatis *et al.* (1982)

REact™ buffers were obtained from BRL

[See Appendix 2 for composition of reaction buffers]

2.iv.h. Agarose gel electrophoresis

DNA fragments were separated by agarose gel electrophoresis on the basis of size (in 1% agarose the relationship between $\log_{10}$ size and mobility is approximately linear for DNA fragments between 7kb and 0.5kb). Plots of $\log_{10}$ molecular weight versus mobility using standard DNA fragments were used to determine the size of unknown fragments.

Horizontal gel electrophoresis systems (Phamacia) were used throughout. Agarose Type II-A Medium EEO (Sigma) was used except in the production of restriction fragment length probes (see below). Both E buffer and B buffer (1x or 0.5x strength) were used. Agarose was prepared by boiling in buffer solution and poured when the temperature had dropped to around 50°C.

DNA fragments were detected by staining gels with ethidium bromide (1µg/ml) for 20 minutes. The gels were washed in distilled water and examined using long-wave uv light to illuminate the DNA bands. Photographs were taken through a red filter, using Polaroid Type 667 film.

2.iv.i. Southern blotting

Restriction enzyme digests of *B.pertussis* chromosomal DNA were transferred to nylon membrane (Hybond-N™, Amersham) using a vacuum blotting system (LKB 2016 Vacugene™, Vacuum Blotting System).

The nylon membrane was dampened with 2xSSC, placed on the porous support stand within the area cut away from the plastic mask and the gel was cut to a suitable size and laid over the nylon membrane, covering the edges of the plastic mask. The gel was immobilised by application of the vacuum.

Depurination solution was pipetted onto the surface of the gel and the vacuum stabilised at 40cm H_2O. After 4 minutes the solution was aspirated off and replaced by denaturation solution. This was replaced after 3 minutes by the neutralisation solution. After 3 minutes neutralisation, the solution was removed and the gel covered to twice its depth in the transfer solution (20xSSC). Transfer was allowed to proceed for 50 minutes under a constant vacuum.

After transfer of the DNA was complete the filter was washed briefly in 2xSSC, blotted between Whatman 3MM filter paper and wrapped in Saran Wrap™ (Dow Chemical Co.). Damp filters were examined under uv illumination to show transfer and to fix the DNA, then stored at 4°C.

2.iv.j. Hybridisation conditions

Filters were heat-sealed in a plastic bag containing 0.1ml of prehybridisation solution per cm^2 membrane [see Appendix 2] and incubated for 2-3 hours at 42°C in a shaking water bath. For hybridisation the denatured probe (made by boiling for 5 minutes) was added to the prehybridisation solution in the bag, all the air bubbles were removed and the bag resealed. Hybridisation was allowed to proceed overnight at 42°C.

Filters were washed in 50ml 2xSSC for 15 minutes at 65°C, then in 50ml 2xSSC containing 0.1% (w/v) SDS for 30 minutes at 65°C, and finally in 50ml 0.1xSSC for 10 minutes at 65°C, to remove unincorporated probe. Filters were not dried completely, to facilitate reprobing, but were wrapped damp in Saran Wrap for autoradiography.

2.iv.k. Preparation of restriction fragment length probes (Feinberg and Vogelstein, 1984)

Plasmid DNA was cleaved with the appropriate restriction enzyme and the fragments separated by electrophoresis in 1% (w/v) low-gelling-temperature agarose (using E buffer). After electrophoresis, the gel was stained with ethidium bromide and the desired band excised under uv illumination. Both HpaI and HindIII restriction fragment probes of Tn*5* were prepared from a plasmid preparation of pSUP2021 using this technique. Distilled water (3ml H_2O per 1g of gel) was added to the DNA-containing agarose slice in a microfuge tube, which was then placed in boiling water for 7 minutes to melt the agarose and denature the DNA. Such probes were then stored at -20°C. In preparation for subsequent radioactive labelling, the gel was reboiled for 5 minutes and stored at 37°C for 10-60 minutes until starting the labelling reaction.

2.iv.l. Random hexanucleotide labelling of probes (Feinberg and Vogelstein, 1983)

The labelling reaction was carried out overnight at room temperature. The following reagents were added in the stated order: (i) distilled water (to a total volume of 50µl); (ii) 10µl oligo-labelling buffer [see Appendix 2]; (iii) 2µl of bovine serum albumin (10mg/ml); (iv) DNA in agarose (up to 32.5µl); (v) 5µl of α- [^{32}P]dATP (Amersham, 10µCi/µl); (vi) 2units of Klenow enzyme (Boehringer Mannheim). The reaction was stopped by the addition of 200µl STOP buffer.

2.iv.m. Autoradiography

Filters were covered with Saran Wrap and mounted in a light

proof cassette. A sheet of X-ray film (X-OMAT S) (Kodak) was placed next to the filter. Autoradiographs were stored at -70°C with intensification screens or at room temperature.

Film was developed (Kodak DX80) for 4 minutes in a dark room, fixed for 4 minutes (Kodak FX40), then washed extensively under the tap and hung to dry.

2.iv.n. Removing probe from filters

Hybond-N filters were incubated at 45°C for 30 minutes in 0.4M NaOH, then transferred to 0.1xSSC, 0.1% (w/v) SDS, 0.2M Tris-HCl, pH7.5, and incubated at 45°C for a further 30 minutes to allow total removal of probe. Membranes were wrapped in Saran Wrap and exposed to X-ray film to ensure the successful removal of probe. Damp filters were stored, wrapped in Saran Wrap, at 4°C in the dark.

2.iv.o. Colony hybridisation

B.pertussis and *E.coli* strains were grown up in microtitre trays and the OD_{492nm} of the cultures recorded in an ELISA plate reader. Culture samples of 75µl were filtered onto nitrocellulose using a 96 well mini-fold apparatus (Schleicher and Schuell). To lyse the cells and bind the DNA to the filter, the nitrocellulose was placed on Whatman No.3 paper soaked in 0.5M NaOH, cells upward, for 10 minutes. Then the filter was neutralised by placing on Whatman No.3 soaked in 1M Tris-HCl pH7.5, twice, each for 2 minutes. The nitrocellulose was then transferred to paper soaked in 0.5M Tris-HCl pH7.4, 1.5M NaCl and left for 5 minutes. The filter was dried at 37°C, then baked for 2 hours at 80°C in a vacuum oven.

Filters were pre-wet in 2xSSC, then prehybridised for 2-3

hours at 37°C in 20ml prehybridisation solution (6xSSC, 0.2% (w/v) SDS, 5x Denhardt's solution, 50% (v/v) formamide, and 100µg/ml preboiled salmon sperm DNA). Half of the prehybridisation solution was removed from the bag and the nick-translated, boiled probe added. The bag was resealed and hybridisation was left to occur overnight at 37°C.

After hybridisation the filters were washed (5xSSC, 0.1% (w/v) SDS) twice for 30 minutes at 65°C, then in 2xSSC at room temperature for 1 hour, dried at 37°C and mounted for autoradiography.

2.iv.p. Nick translation

Probes were prepared using 1-2µg plasmid DNA (pGS18, pColIb, pRMB2 or pRMB11). The reaction mix contained DNA, 20µl A solution [see previously], 10µl 10x nick translation buffer [see Appendix 2], 1µl DNase I (1mg/ml stock), 1µl DNA polymerase (5 units/µl), distilled water to a final volume of 100µl and 2.5µl α-[^{32}P]dATP (Amersham, 10µCi/µl). After incubation at 15°C for 1 hour, the reaction was stopped by the addition of 4µl 0.5M EDTA. Orange G dye was added to the sample, which was then loaded onto a Sephadex G-50 column and eluted with TES buffer. Radioactivity was monitored and the first peak containing the labelled DNA was collected. This was boiled for 5 minutes, cooled rapidly on ice and added to the prehybridisation solution in a sealed plastic bag.

2.v Assays

2.v.a. Agglutination assay

Samples of overnight cultures of *B.pertussis* were mixed with

dilutions of monoclonal antibodies (AG2A and AG3A - raised against AGG2 and AGG3 respectively, provided by L.A.E. Ashworth; Pathology Division, CAMR). Dilutions were made in phosphate-buffered saline (PBS - 0.15M NaCl, 0.066M Na_2PO_4, pH7.0), starting at a 1 in 4 dilution and doubling dilutions were made to a final 1 in 512. This assay was carried out in U-shaped-bottomed microtitre trays, which were covered and left overnight at 37°C. Agglutination was noted when the cells clumped, while non-agglutination lead to the formation of a button at the bottom of the well.

2.v.b. Haemagglutination assay

Horse red blood cells (InterMed) were washed three times in PBS. Concentrated cells were diluted to 2% (v/v) in PBS and 100µl mixed with 100µl of bacterial cells (grown in liquid culture with cyclodextrin). Assays were carried out in microtitre trays with U-shaped-bottoms, which were covered and left overnight at room temperature. Haemagglutination was noted where the red blood cells clumped, while non-ability to haemagglutinate was shown by the formation of a button at the bottom of the well.

2.v.c. Qualitative adenylate cyclase assay

B.pertussis strains were grown on BG overlay plates (the top layer of agar alone containing red blood cells) to show the presence of haemolytic activity associated with the adenylate cyclase toxin. Cells for use in the assay were transferred to 200µl SS(MeβCD+CAA+/-Km) and incubated for 4 days at 37°C. *E.coli* G802 (indicator strain - see Table 3) was streaked onto NA+IPTG+X-gal. A single white colony was used to inoculate a 10ml overnight culture in NB.

Into the wells of a sterile microtitre tray, 50µl of reaction mix

(60mM Tris-HCl pH8.0, 10mM $MgCl_2$, 5mM ATP, 10mM $CaCl_2$, 500units/ml calmodulin, in distilled water) was dispensed, followed by 8µl of *B.pertussis* test culture. The mixture was incubated for 3 hours at 37°C, then 150µl of test suspension was added. The test suspension was composed of NB+IPTG+X-gal and a 1 in 100 dilution of a fresh culture of the *E.coli cyaA*$^-$ indicator strain. The solutions were mixed well and the tray incubated overnight at 37°C. *B.pertussis* cultures producing adenylate cyclase transform ATP to cAMP which is secreted into the culture medium. Cyclic-AMP stimulates the production of β-galactosidase from the *lac* operon in the *E.coli* indicator strain, which in turn utilises the substrate X-gal to produce a dark blue colouration. Wells containing *B.pertussis* strains producing no adenylate cyclase showed no colour formation.

2.v.d. Quantitative adenylate cyclase assay

The cyclic adenosine monophoshate (cAMP) assay kit (Amersham) was used as in the manufacturer's recommendations, being suitable for the detection of 0.2 to 16 picomoles cAMP per incubation tube. Standards of 1, 2, 4, 8 and 16 pmol cAMP were used, and a blank count also performed. Test samples of 50µl were used.

Test samples were prepared using whole cells (without calmodulin) and X-pressed cells (with and without calmodulin). The X-press (Lifesciences Laboratories) was used directly on liquid cultures as per manufacturer's instructions. Cell extract volumes of 40µl were mixed with 120µl 0.002M ATP in 0.02M $MgCl_2$, 80µl 0.18M tricine pH8.0, and an optional 5µl calmodulin (250 units). Controls were also performed with no ATP present. After incubation for 15 minutes at 30°C, the reaction was terminated by the addition of 0.48ml 0.05M

Tris, 0.0005M EDTA pH7.5 and boiled for 5 minutes. Test samples were stored at -20°C.

The cAMP assay is based on competition between unlabelled cAMP (from the test samples) and a fixed quantity of the tritium-labelled compound for binding to a protein which has a high specificity and affinity for cAMP. Samples, of 200µl volumes, from the test mixtures were transferred to scintillation vials containing 5ml scintillation fluid, mixed thoroughly and the percentage of tritiated cAMP remaining in solution counted in a scintillation counter.

2.vi. Animal Studies

2.vi.a. Assay for heat-labile toxin

B.pertussis strains were grown on BG agar for 2 days at 37°C. Cells were removed and suspended to 10ou (opacity units; WHO 5th International Reference Preparation of Opacity; Perkins *et al.*, 1973) in PBS. Three-week old male mice were injected intraperitoneally (ip) with 0.5ml of 10ou or 2.5ou doses, in groups of five. The number of mice surviving after 5 days was recorded.

2.vi.b. HLT skin reactions

B.pertussis cultures were diluted 1 in 3 in sterile saline and 50µl injected subcutaneously into the dorsal region of 3-week old male mice (after ether anaesthetic). After 24 hours the mice were killed with CO_2 and the skin of the back removed. The size and severity of the zone of haemorrhage was noted.

2.vi.c. Histamine-sensitisation test

B.pertussis strains were grown on BG agar for 2 days at 37°C. Cells were removed and suspended to 10ou in PBS, then heated at 56°C

for 30 minutes. Groups of five male mice, nine weeks old, were injected ip with 0.5ml of 10ou or 2.5ou doses. After 5 days each mouse was challenged by ip injection with 3mg of histamine dihydrochloride (Sigma) in 0.5ml PBS, pH7.4. The number of mice surviving after 3 hours was recorded.

2.vi.d. Virulence as determined by the mouse-weight-gain test (MWGT)

Groups of ten CD-1 strain female mice, of 3-4 weeks of age, were challenged in with 0.05ml volumes of graded doses of test samples in sterile 1% CAA solution. An ether anaesthetic was used. Challenge dilutions were started from a 10ou initial suspension of each strain. The first three 10-fold dilutions were used as challenges, while the 10^{-6} dilution was used to determine viable counts. Control mice were left unchallenged. Mice were weighed prior to challenge and again at 2-3 day intervals for the next three weeks. Fatalities were recorded.

After three weeks, five mice from each challenge group were examined for lung weight (as a percentage of body weight) and pathology. Slices of lungs were streaked onto BG agar to identify remaining infection. Spleen weight as a percentage of total body weight was also examined, and leukocyte counts perfomed.

2.vi.e. Vaccine preparation

Vaccines were prepared from *B.pertussis* Wellcome 28, BP347 and the Crb^{-} Tn*5* mutant 51. Cells were grown with shaking in liquid culture for 24 hours, then centrifuged at 6000g for 20 minutes and resuspended to 100ou in PBS. Suspensions were heated at 56°C for 30mins, thimerosal (Sigma) added to a final concentration of 0.01% as a preservative and the vaccines stored at 4°C.

2.vi.f. Mouse intranasal and intracerebral protection tests

Groups of five, 3 - 4 week old CD-1 strain mice, were injected ip with 0.5ml vaccine (at 10ou, 2ou and 0.4ou). Two groups of mice were left unvaccinated as controls.

Two weeks after vaccination mice were anaesthetised and challenged with virulent *B.pertussis*. Lawn plates of the challenge strain, *B.pertussis* 18-323, were prepared. Cells were suspended in a CAA solution to 10ou (approximately $2x10^9$ cfu/ml) and eight serial 10-fold dilutions made. Highest dilution samples were used for colony counts. The 10^{-2} dilution was used for intranasal (in) challenge, 50µl being passed through the nasal cavities by allowing the anaethetized mice to inhale. For the intracerebral (ic) challenge, 30µl of a 10^{-5} dilution was injected into the brain. One group of controls were challenged, the other left unchallenged.

Mice were weighed every two days and any fatalities noted for a period of nearly 3 weeks. For mice challenged in, lung pathology was recorded. Lungs were weighed, examined for pathological changes and slices spread over BG+Ceph+Amph overlay plates. Plates were incubated at 37°C and signs of bacterial colonisation noted.

RESULTS

3.i. Transposon Delivery Systems in *B.pertussis*

A series of plasmids, proposed as transposon delivery vectors for Tn*5* mutagenesis studies, were introduced into the pathogen *B.pertussis* by conjugation. The resultant populations of *B.pertussis* kanamycin-resistant transconjugants were screened for absence of the delivery vehicle in order to identify the most suitable vector for Tn*5* insertional mutagenesis in this bacterium. The transposon Tn*5* was re-evaluated as a mutagen in *B.pertussis* by screening those kanamycin-resistant transconjugants not maintaining the donor plasmid for the presence of Tn*5* DNA (to identify spontaneously kanamycin-resistant isolates).

3.i.a. Viable count data for donor and recipient strains during filter conjugations

E.coli donor strains carrying Tn*5* delivery vectors [see Table 3] and *B.pertussis* recipient strains were inoculated onto filters with the recipient in excess. The numbers of donor organisms placed on the filters varied between 8.8×10^{6}cfu and 3.0×10^{7}cfu per filter, while numbers of recipients varied between 5.9×10^{7}cfu and 3.5×10^{8}cfu per filter.

After incubation for 18 hours at 37°C, viable counts were determined. Donor strains showed an increase in cell numbers, varying between 2.6×10^{9}cfu and 1.0×10^{10}cfu per filter, while recipient strains showed only slight fluctuations in cell numbers with respect to the initial counts.

Differences in growth of *B.pertussis* strains alone were noted on the control filters used to identify spontaneous kanamycin-resistance

frequencies. Initial viable counts of *B. pertussis* Wellcome 28 inoculated onto filters varied between 7.4×10^7cfu and 4.4×10^8cfu per filter, while final viable counts showed a drop in cell numbers. In a single experiment with *B. pertussis* Tohama, an increase in cell numbers from an initial 1.6×10^8cfu to a final 7.2×10^9cfu per filter was noted.

3.i.b. *B. pertussis* kanamycin-resistant transconjugants

The frequencies of *B. pertussis* kanamycin-resistant transconjugants obtained after filter conjugations with *E. coli* transposon donor strains are shown in Table 6. The values given are a product of the vector transfer frequency and the transposition frequency and have been calculated per recipient.

The Tn*5* delivery vector pSUP2021 did not transfer to *B. pertussis* at a frequency high enough to produce transconjugants and this system was not used further. The self-transmissible plasmids pUW964, pGS9 and pLG221 produced kanamycin-resistant transconjugants at frequencies between 1.1×10^{-6} and 5.5×10^{-6} per recipient, while the larger self-transmissible plasmid pJB4JI transferred at a lower frequency.

3.i.c. Maintenance of 'suicide' vectors

Antibiotic resistance genes carried on the transposon delivery vectors were used to identify the frequency of plasmid maintenance within populations of *B. pertussis* kanamycin-resistant transconjugants. Results of screening transconjugants from matings where vectors pJB4JI (Gm^R, Sp^R), pUW964 (Sp^R, Tp^R) or pGS9 (Cm^R) were transferred are shown in Table 6. The Tn*5* delivery vector pLG221 carries no antibiotic resistance genes and could not be tested. *B. pertussis* controls showed no growth on antibiotic-containing media, whereas *E. coli* controls containing the relevant plasmids grew well. All the transconjugants

Table 6 Transfer of kanamycin-resistance to *B. pertussis* strains from *E. coli* transposon donors

Transposon delivery vectors [see Table 4] were introduced into the *B. pertussis* recipient strains by filter conjugations for 18 hours at 37°C [see Section 2.iii.a]. Ten separate matings were performed with each vector. The percentage of transconjugants maintaining the respective plasmids were deduced by screening for antibiotic resistances encoded by the plasmid [see Section 2.iii.d], or by probing colony dot blots with radiolabelled plasmids closely related to the delivery vector [see Sections 2.iv.o. and 2.iv.p.]. Approximately 80 transconjugants were screened from each different system.

Tn*5* delivery vector.	Recipient strain.	Percentage maintenance of delivery vector from:		Frequency of kanamycin-resistant transconjugants (per recipient).
		i. antibiotic resistance data.	ii. dot blot hybridisation data.	
pUW964	*B. pertussis* Wellcome 28	15.6%	N/D	2.9×10^{-6}
pUW964	*B. pertussis* Tohama	34.9%	N/D	5.5×10^{-6}
pJB4JI	*B. pertussis* Wellcome 28	48.8%	N/D	6.6×10^{-7}
pGS9	*B. pertussis* Wellcome 28	41.8%	30.1%	3.5×10^{-6}
pSUP2021	*B. pertussis* Wellcome 28	N/D	N/D	$<1.9 \times 10^{-8}$
pLG221	*B. pertussis* Wellcome 28	N/D	1.5%	1.1×10^{-6}

N/D - not determined.

tested and the controls grew on media containing no antibiotics.

The vector pJB4JI, which contains phage Mu DNA, was shown to be maintained in nearly half of the kanamycin-resistant transconjugants produced using this system. Similarly, the transposon delivery vector pUW964, which was introduced into two *B. pertussis* strains (Tohama and Wellcome 28), was shown to be maintained in both strains, evident by the presence of plasmid-encoded antibiotic resistance markers. The transposon donor pGS9 was also shown to be maintained in a high proportion of transconjugants.

A second technique for the identification of *B. pertussis* kanamycin-resistant transconjugants maintaining transposon delivery vectors involved filtering liquid cultures onto nitrocellulose filters for use in culture dot-blot hybridisation experiments. Transconjugants from matings where pGS9 was transferred were probed with pGS18 (Figure 8), while transconjugants from matings where pLG221 was transferred were probed with pColIb-P9 (Figure 9). Plasmid pGS18 is closely related to pGS9, and pColIb-P9 is closely related to pLG221, but neither of the probes contain Tn*5* DNA [see Table 4]. Delivery vector maintenance frequencies, obtained by probing the kanamycin-resistant transconjugants with plasmids closely related to the transposon donors, are shown in Table 6. Neither probe hybridised to *B. pertussis* Wellcome 28 DNA (negative control), but both probes hybridised to the appropriate positive controls [*E. coli* WA803 (pGS9) and *E. coli* HB101 (pGS18), for the pGS18 probe, and *E. coli* C600 (pLG221) and *E. coli* W3110 (pColIb-P9), for the pColIb-P9 probe].

The colony dot-blot hybridisation results confirmed that the transposon delivery vector pGS9 was maintained in between 30-40% of

Fig. 8 Culture dot blot hybridisation of *B. pertussis* kanamycin-resistant transconjugants (from matings with *E. coli* WA803 pGS9), probed with pGS18

Seventy-nine kanamycin-resistant transconjugants of *B. pertussis* Wellcome 28 were screened to identify the maintenance of the transposon delivery vector pGS9 by hybridisation with pGS18 [see Section 2.iv.o]. The probe was labelled by nick translation for 1 hour at 15°C [see Section 2.iv.p].

Controls

A. *B. pertussis* Wellcome 28; negative control.

B. *E. coli* WA803 (pGS9); positive control.

C. *E. coli* HB101 (pGS18); positive control.

Interpretation

		Columns									
		1	2	3	4	5	6	7	8	9	10
	1	+	+		+			+			
R	2				+					+	
o	3		+					+			
w	4		+				+				
s	5		+		+		+	+			
	6				+			+		+	
	7	+		+		+	+		+		
	8	/	+						+		

/ - Blank

Delivery vector - pGS9

Probe - pGS18

Transconjugants

Controls

A

B

C

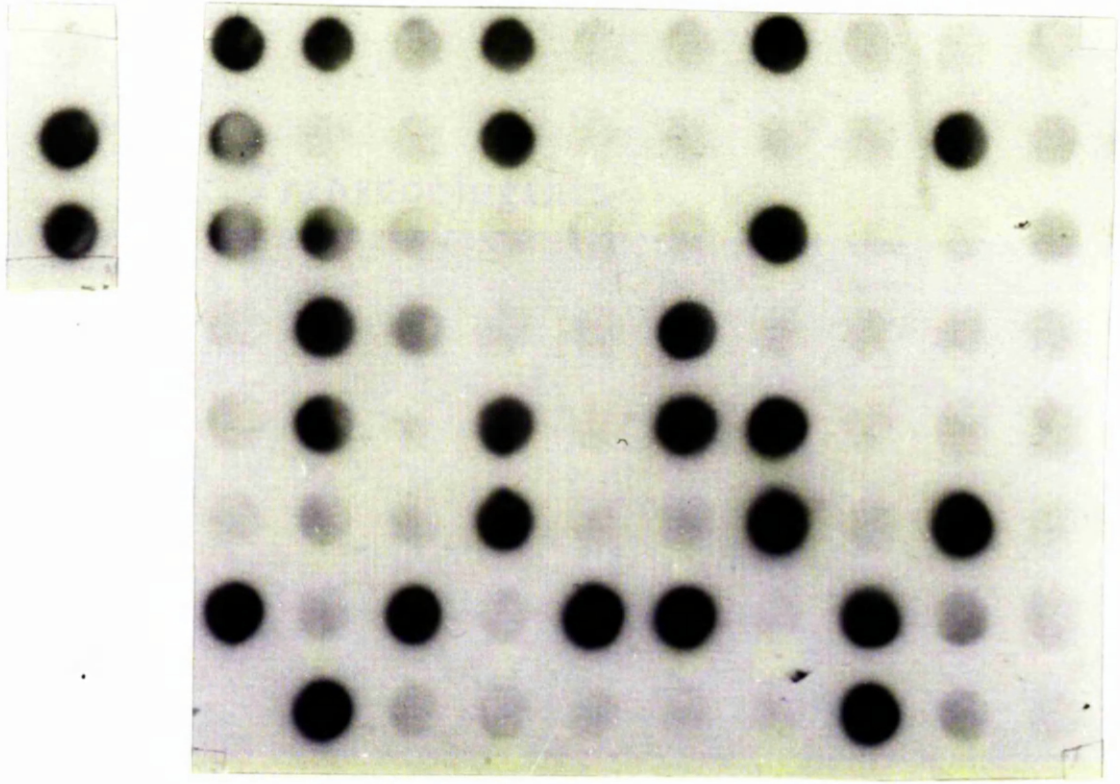


Fig. 9 Culture dot blot hybridisation of *B. pertussis* kanamycin-resistant transconjugants (from matings with *E. coli* C600 pLG221), probed with pColIb-P9

Sixty-five kanamycin-resistant transconjugants of *B. pertussis* Wellcome 28 were screened to identify the percentage maintenance of the transposon delivery vector pLG221 by hybridisation with pColIb-P9 [see Section 2.iv.o]. The probe was labelled by nick translation for 1 hour at 15°C [see Section 2.iv.p].

Controls

A. *B. pertussis* Wellcome 28; negative control.

B. *E. coli* C600 (pLG221); positive control.

C. *E. coli* W3110 (pColIb-P9); positive control.

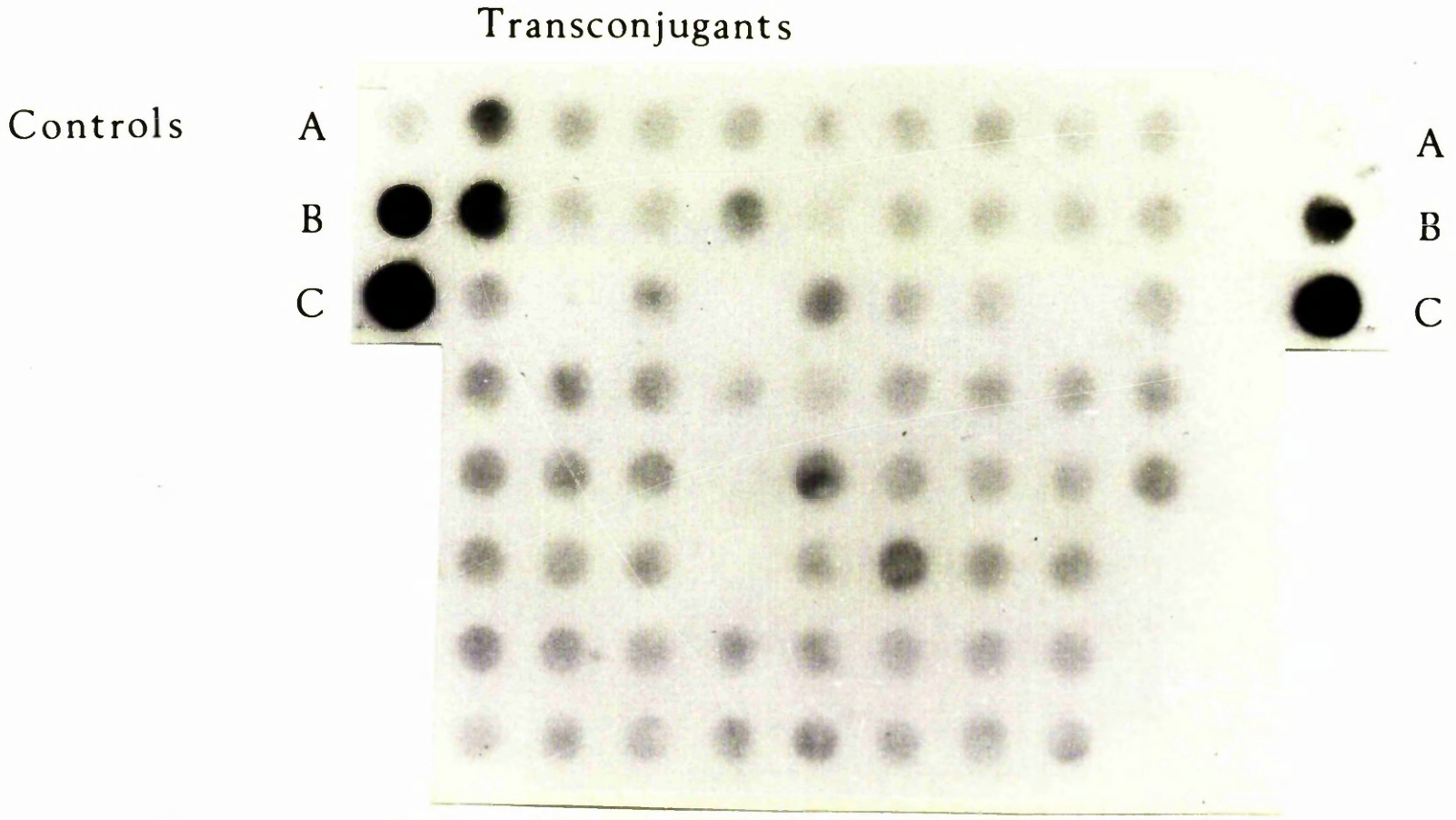
Interpretation

		Columns									
		1	2	3	4	5	6	7	8	9	10
	1										/
R	2	+									/
o	3				/				/		/
w	4										/
s	5				/						/
	6				/					/	/
	7									/	/
	8									/	/

/ - Blanks

Delivery vector - pLG221

Probe - pColIb



the *B. pertussis* transconjugants. However, the ColIb-P9 based vector pLG221 was shown to be a more truly 'suicidal' transposon delivery vehicle for use in this bacterium, since only a single transconjugant from the 65 isolates tested gave a positive hybridisation signal when probed with pColIb-P9 [see Fig. 9; column 1, row 2].

3. i. d. Spontaneous kanamycin-resistance in *B. pertussis*

The frequency of spontaneous kanamycin-resistance in *B. pertussis* Tohama was shown to be less than 1 in 2.9×10^{8}cfu. However, in *B. pertussis* Wellcome 28 the frequency of spontaneous kanamycin resistance was shown to be less than 1 in 8×10^{5}cfu due to poor growth. Since this could be problematic in further Tn*5* mutagenesis experiments, groups of strain Wellcome 28 kanamycin-resistant transconjugants were probed with Tn*5* (to confirm that the antibiotic resistance was associated with introduction of the transposon). The Tn*5* probe did not hybridise to *B. pertussis* Wellcome 28 (the negative control), nor to *E. coli* strains carrying plasmids pGS18 or pColIb-P9. The probe did, however, hybridise to the positive control strains *E. coli* WA803 (pGS9) and *E. coli* C600 (pLG221). All of the putative transposon-insertion mutants tested (over 140 individual isolates) were shown to hybridise to Tn*5* (not shown). This suggests that spontaneous resistance to kanamycin is a rare event in *B. pertussis* Wellcome 28 and unlikely to be a detrimental influence on further mutagenesis experiments.

3. ii. Production and Screening of *B. pertussis* Tn*5* Mutants

The first step in the targeting of specific genes by Tn*5* insertional mutagenesis is the creation of a large library of mutants.

The library may then be either laboriously screened for loss of the desired characteristic, or alternatively, a selective proceedure may be used which eliminates the unwanted mutants, or reduces the number which require screening. Both possibilities were explored during this study.

3.ii.a. Tn*5* mutagenesis of *B.pertussis* Wellcome 28

Tn*5* insertional mutants of *B.pertussis* Wellcome 28 were isolated after 5 hour conjugations on BG agar with *E.coli* C600 (pLG221). Growth to single colonies on SSA(MeβCD+Km+Ceph+CR) allowed the classification of mutants into two groups; i. those able to bind Congo red (Crb^+), and ii. those unable to bind Congo red (Crb^-). A total of 280 mutants, from 31 separate matings, were colony-purified and stored. Mutants 1 - 99 were picked from selective plates prepared with a batch of Difco Bacto agar previously shown to select against phase I organisms [C.J. Duggleby, personal communication; see Section 2.ii.a.]. Mutants 100 - 280 were obtained without this selective pressure.

3.ii.b. Spontaneous mutants unable to bind Congo red

No spontaneous Crb^- mutants were identified from colonies grown on CSM+CR plates, giving a spontaneous frequency of mutation from the Crb^+ phenotype to the Crb^- phenotype of less than 1 in $3x10^4$cfu. However, colony counts on CSM+CR prepared with the batch of agar previously shown to be inhibitory to growth of phase I organisms, were reduced approximately 1000-fold and Crb^- mutants were shown to comprise up to 30% of the residual colonies.

3.ii.c. Transposon mutants unable to bind Congo red

The following identification numbers were given to twelve Crb^- transposon mutants identified during this study:- 15, 51, 73, 97, 98,

99, 100, 101, 104, 113, 115 and 268. Mutants 51 and 73 were obtained from the same filter mating, as were the following pairs, i. 98 and 99, ii. 100 and 101, and iii. 113 and 115. A single mutant (numbered 102) with reduced ability to bind Congo red ($Crb^{+/-}$) was also identified.

3.ii.d. Antigenic modulation in Crb^{+} mutants

Mutants 1 - 120 (excluding Crb^{-} mutants) were grown at 25°C on CSM+CR to show alteration of the Congo red-binding phenotype during antigenic modulation, or, alternatively, to identify mutants unable to modulate. However, all of the Crb^{+} (red) colonies tested showed the ability to modulate, growing as Crb^{-} (pale) colonies under this C-mode growth condition.

3.ii.e. Expression of heat-labile toxin in transposon mutants

The expression of heat-labile toxin (HLT) in over two hundred Crb^{+} mutants was tested. Mice were injected intradermally with live bacterial suspensions and examined for skin reactions after 24 hours. In the majority of cases, the size and severity of the zone of haemorrhage was large, similar to that found with the positive control strain *B.pertussis* Wellcome 28. Injection with the negative control, BP347, caused only very slight haemorrhage. Mutants which caused low levels of skin reactions were retested in the lethal mouse model, in which cell suspensions were injected into infant mice and the number of fatalities after 5 days noted. No Crb^{+} mutants were identified as being unable to express HLT.

3.ii.f. Screening Crb^{-} (and $Crb^{+/-}$) mutants for loss of other virulence-related characteristics

The Crb^{-} mutants, and the $Crb^{+/-}$ mutant 102, were screened for

alterations in other properties related to virulence, including growth on nutrient agar (a characteristic associated with the fully avirulent phase IV strains). None of the mutants grew on nutrient agar. The results of screening for other virulence-associated properties are shown in Table 7.

The wild-type control strain *B. pertussis* Wellcome 28 was positive for all the virulence-associated properties tested, while the negative control strain, BP347, expressed none of these factors. Other strains, used in agglutination assays, expressed only the agglutinogens shown to be present by previous serotyping of the wild-type [see Table 7].

Mutants 15, 97, 98, 99, 100 and 101 were shown to be non-haemolytic and non-haemagglutinating, and did not express adenylate cyclase, heat-labile toxin, nor agglutinogens 2 and 3. Mutants 104, 113, 115 and 268 were shown to be phenotypically equivalent to this previous group of mutants, but were not tested for expression of heat-labile toxin. Both groups were considered to be equivalent to previously classified Vir^- strains. Mutants 51 and 73 were shown to be haemolytic and gave positive results in the qualitative adenylate cyclase assay, but were also non-haemagglutinating and produced neither heat-labile toxin nor agglutinogens 2 and 3. The $Crb^{+/-}$ mutant (102) appeared to produce reduced levels of agglutinogen 3 and showed little haemagglutinating activity, while expressing apparently normal levels of other virulence factors.

Table 7 Virulence-associated properties of a virulent strain, an avirulent strain and the Crb$^-$ Tn*5* mutants

Transposon mutants altered in the Congo red-binding phenotype, along with appropriate controls, were screened for virulence-associated properties. *B. pertussis* Tohama (1,2) and *B. pertussis* 134 (1,3) were used as controls in agglutination assays only. Production of haemolysin (HLY) was assayed for by growth on BG overlay plates, while adenylate cyclase (AC) was screened using the qualitative AC assay [see Section 2.v.c]. Agglutination assays (known serotypes are given in brackets) and haemagglutination (HA) assays were performed in microtitre trays as described in Sections 2.v.a. and 2.v.b. respectively. The assay for heat-labile toxin (HLT) was performed in infant mice, screening for survivors 5 days after injection [see Section 2.vi.b].

Strain (serotype)	Virulence-associated properties: HLY	AC	HA	HLT	AGG2	AGG3
Controls						
B. pertussis Wellcome 28 (1, 2, 3)	+	+	+	+	+	+
B. pertussis 347 (-)	-	-	-	-	-	-
B. pertussis Tohama (1, 2)	N/D	N/D	N/D	N/D	+	-
B. pertussis 134 phase I (1, 3)	N/D	N/D	N/D	N/D	-	+
Crb$^-$ mutants						
15	-	-	-	-	-	-
51	+	+	-	-	-	-
73	+	+	-	-	-	-
97	-	-	-	-	-	-
98	-	-	-	-	-	-
99	-	-	-	-	-	-
100	-	-	-	-	-	-
101	-	-	-	-	-	-
104	-	-	-	N/D	-	-
113	-	-	-	N/D	-	-
115	-	-	-	N/D	-	-
268	-	-	-	N/D	-	-
Crb$^{+/-}$ mutant						
102	+	+	+/-	+	+	+/-

N/D - not determined.

3.iii. Identification of Tn*5* Insertion Sites in Mutants Altered in the Congo Red-Binding Phenotype

Tn*5* insertion sites, in mutants displaying either complete or partial loss of ability to bind Congo red dye, were identified using Southern blots prepared with three different restriction enzyme digests. The blots were probed with ^{32}P-radiolabelled restriction fragments of Tn*5*. Radiolabelled λ-HindIII markers were used, as a reference, to identify the sizes of the restriction fragments which hybridised to the probe [see Section 2.iv.g].

3.iii.a. Tn*5* insertion sites in BamHI restriction fragments

BamHI digests of chromosomal DNA from the Crb$^-$ mutants and the Crb$^{+/-}$ mutant were probed with the internal HindIII fragment of Tn*5* (Figure 10). The probe (which does not cover the major parts of the IS elements of this composite transposon) was shown to hybridise to two distinct bands in each digest, since BamHI cleaves Tn*5* into two slightly asymmetrical fragments [see Fig. 6]. No hybridisation to the DNA of the parent strain *B. pertussis* Wellcome 28 was observed.

The sizes of the transposon-interrupted BamHI fragments in the Crb$^-$ and Crb$^{+/-}$ mutants (shown in Table 8) were calculated after first identifying the sizes of individual bands hybridising to the probe [see Section 2.iv.h.]. To take account of the 5.8kb insertion of Tn*5*, this value was subtracted from the summated values of those bands hybridising to the probe in each mutant. The interrupted BamHI fragment in BP347 was shown to be 15.0kb [(9.4kb + 11.4kb) - 5.8kb = 15.0kb], in agreement with previous studies (Stibitz *et al.*, 1988). Similarly sized fragments were identified in mutants 100 and 101, but

Fig. 10 Tn*5*-hybridisation to Southern blots of BamHI-digested chromosomal DNA from *B. pertussis* Crb$^-$ and Crb$^{+/-}$ mutants

BamHI-digested DNA fragments from *B. pertussis* Crb$^-$ and Crb$^{+/-}$ transposon mutants and radiolabelled λ-HindIII markers [see Section 2.iv.g.] were separated by electrophoresis on a 0.6% agarose gel in 0.5xTBE, at 30mA, for 20 hours. DNA was then transferred by suction to a nylon membrane and probed with the internal HindIII fragment of Tn*5*. X-ray film was exposed to the washed filter for 2 days, at -70°C, then developed.

Controls

A. *B. pertussis* Wellcome 28; negative control.

B. BP347; positive control.

Transposon mutants (this study)

C. 15.
D. 51.
E. 73.
F. 97.
G. 98.
H. 99.
I. 100.
J. 101.
K. 104.
L. 113.
M. 115.
N. 268.
O. 102.

Digest - BamHI

Probe - Tn5 (Hind III)

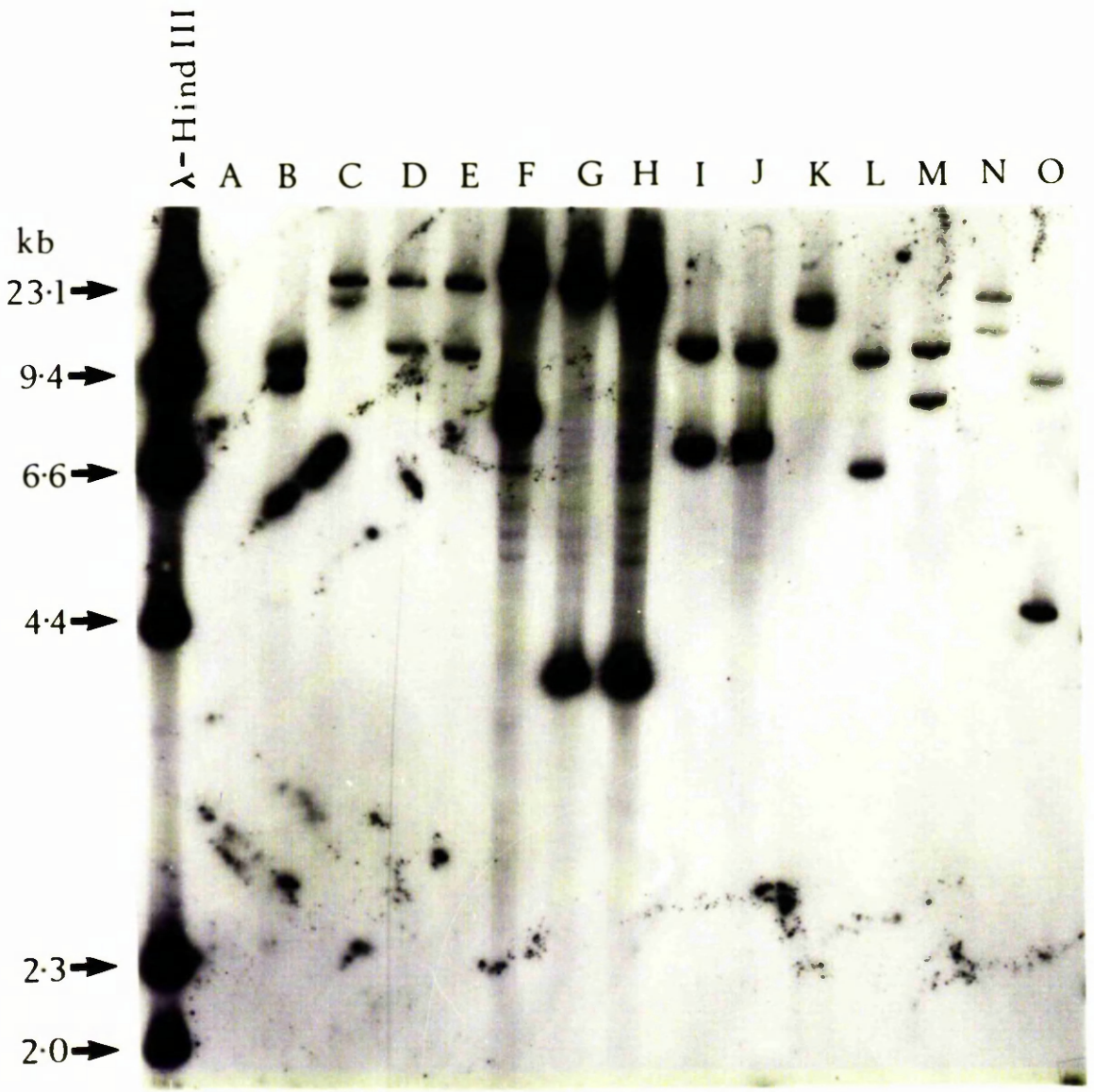


Table 8 Size analysis of restriction fragments, from Crb$^-$ and Crb$^{+/-}$ mutants, interrupted by Tn*5* or IS*50* insertions

Chromosomal DNA preparations from Wellcome 28, BP347 and transposon mutants altered in the Congo red-binding phenotype, were digested with restriction enzymes BamHI, SalI, or EcoRI. DNA fragments were separated on agarose gels along with radiolabelled λ-HindIII size markers [see Sections 2.iv.f. - 2.iv.h.]. DNA fragments stained with ethidium bromide were examined under uv light, photographed and transferred onto Hybond-N™ membrane [see Section 2.iv.i]. Hybridisation conditions were as in Section 2.iv.j. and Appendix 2.

Restriction length fragment probes of Tn*5* were prepared [see Section 2.iv.k.] and labelled using the random hexanucleotide technique of Feinberg and Vogelstein (1983). Hybridisation with the HindIII fragment of Tn*5* identified Tn*5* insertions in BamHI, SalI and EcoRI restriction fragments, while the larger HpaI fragment of Tn*5* was used to identify sizes of restriction fragments interrupted by either Tn*5* or IS*50* in the EcoRI digests alone.

The sizes of restriction fragments interrupted by Tn*5* or IS*50* were deduced from Figures 10, 11 and 12, and are shown after the sizes of the transposable genetic elements (5.8kb and 1.5kb respectively) have been subtracted.

Strains	Restriction enzyme digests (inserted transposable genetic element): BamHI (Tn*5*)	SalI (Tn*5*)	EcoRI (Tn*5*)	EcoRI (IS*50*)
Controls				
B. pertussis				
Wellcome 28	-	-	-	-
B. pertussis				
347	15.0kb	1.0kb	2.7kb	-
Transposon mutants (this study)				
15	40.0kb	7.0kb	5.7kb	1.2kb
51	30.0kb	13.0kb	2.1kb	13.0kb
73	30.0kb	13.0kb	2.1kb	13.0kb
97	30.0kb	11.0kb	10.8kb	2.7kb
98	28.0kb	9.5kb	3.5kb	15.9kb
99	28.0kb	9.5kb	3.5kb	15.9kb
100	15.0kb	0.6kb	2.5kb	-
101	15.0kb	0.3kb	2.5kb	-
104	40.0kb	25.0kb	5.2kb	Multiple
113	14.0kb	4.1kb	9.3kb	-
115	18.0kb	3.8kb	15.1kb	-
268	40.0kb	7.0kb	2.3kb	2.9kb
102	9.0kb	2.8kb	3.5kb	-

with the transposon positioned less symmetrically than in BP347. The sizes of the interrupted fragments in the other Tn*5* mutants were shown to vary from 9.0kb in the $Crb^{+/-}$ mutant 102, to approximately 40kb in mutants 15, 104 and 268. Mutants 51 and 73 appeared to have identical Tn*5* insertion sites, as did mutants 98 and 99.

3.iii.b. Tn*5* insertion sites in SalI restriction fragments

SalI digests of chromosomal DNA from the Crb^{-} mutants and the $Crb^{+/-}$ mutant were probed with the internal HindIII fragment of Tn*5* (Figure 11). The Tn*5* probe was shown to hybridise to two distinct bands in each mutant chromosomal DNA digest, since Tn*5* has a central SalI cleavage site [see Fig. 6]. No hybridisation occured with *B. pertussis* Wellcome 28 DNA.

The sizes of the interrupted SalI restriction fragments (calculated as above) were shown to vary from 0.3kb in mutant 101, to approximately 25kb in mutant 104 (Table 8). Mutants 51 and 73 were confirmed to have identical transposon insertions, as were mutants 98 and 99. The presence of faint bands in addition to the expected doublets were noted in some digests of the mutated DNA. These bands may have been caused by incomplete digestion or, alternatively, by separate IS*50* transposition events (visible since a very small portion of the IS elements were included in the probe).

3.iii.c. Tn*5* insertions in EcoRI restriction fragments

EcoRI digests of chromosomal DNA from the Crb^{-} mutants and the $Crb^{+/-}$ mutant were probed with the internal HindIII fragment of Tn*5* and the internal HpaI fragment of Tn*5* (Figure 12). While the smaller HindIII fragment encompasses only the central antibiotic containing region of the transposon, the larger fragment includes most of the two

Fig. 11 Southern blot of SalI-digested chromosomal DNA from *B. pertussis* Crb$^-$ and Crb$^{+/-}$ mutants, hybridised with Tn*5*

SalI-digested DNA fragments of *B. pertussis* Crb$^-$ and Crb$^{+/-}$ transposon mutants were separated by electrophoresis overnight, at 20mA, on a 0.8% agarose gel, in 0.5xTBE, with radiolabelled λ-HindIII size markers [see Section 2.iv.g]. The DNA pattern was transferred to a nylon membrane and probed with the internal HindIII fragment of Tn*5* [see Sections 2.iv.i - 2.iv.l]. The filter was washed and subjected to autoradiography for 2 days at -70°C.

Controls

A. *B. pertussis* Wellcome 28; negative control.

B. BP347; positive control.

Transposon mutants (this study)

C.	15.	J.	101.
D.	51.	K.	104.
E.	73.	L.	113.
F.	97.	M.	115.
G.	98.	N.	268.
H.	99.	O.	102.
I.	100.		

Digest - SalI

Probe - Tn5 (HindIII)

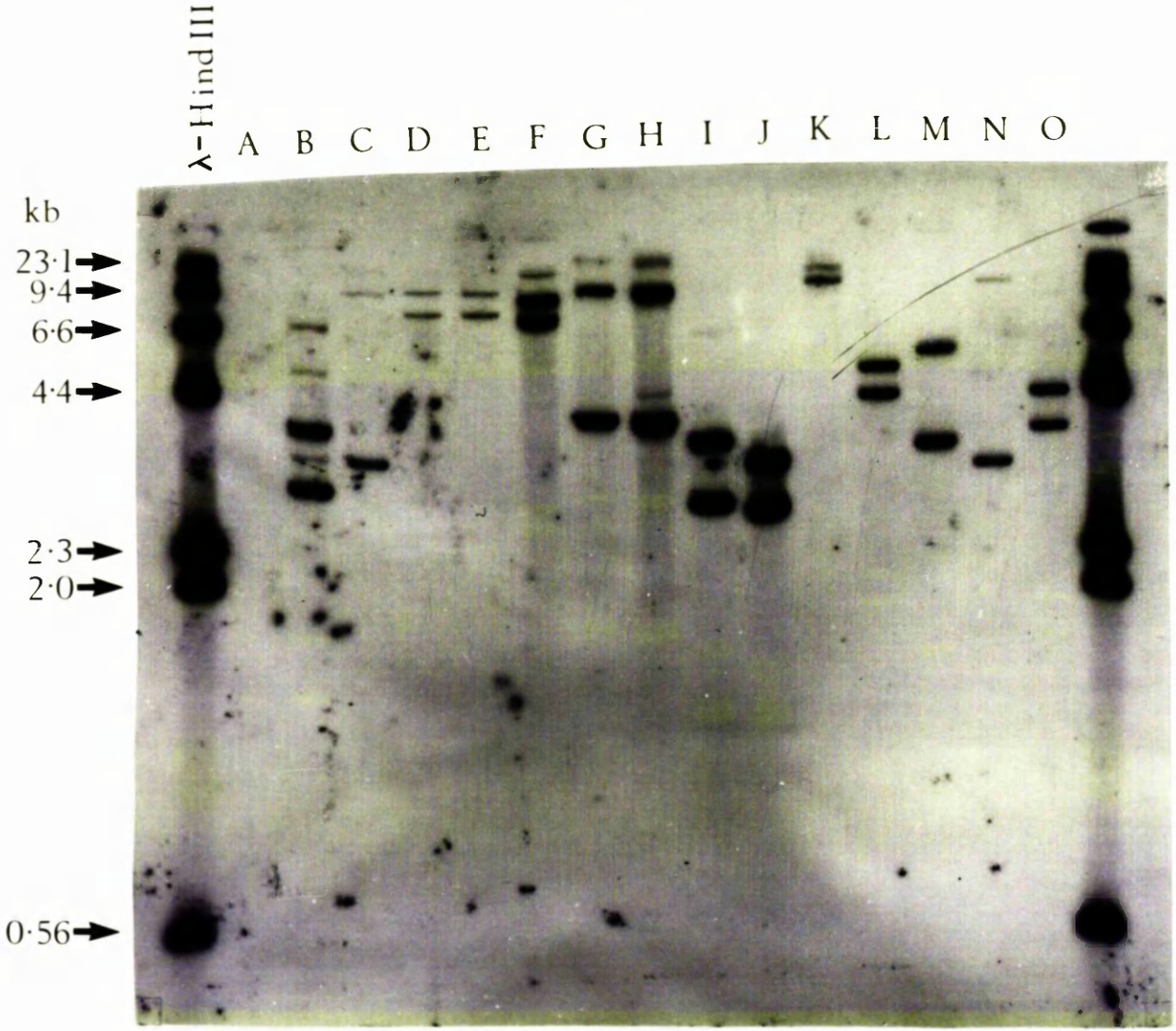


Fig. 12 Hybridisation of a Southern blot of EcoRI-digested chromosomal DNA from *B. pertussis* Crb$^-$ and Crb$^{+/-}$ mutants, with Tn*5*

EcoRI-digested chromosomal DNA fragments of *B. pertussis* Crb$^-$ and Crb$^{+/-}$ transposon mutants were separated by electrophoresis overnight, at 30mA, on a 0.7% agarose gel, in 1xTBE, with radiolabelled λ-HindIII size markers [see Section 2.iv.g]. DNA was transferred to a nylon membrane and probed with the internal HpaI restriction fragment of Tn*5* [see Sections 2.iv.i - 2.iv.l]. Tn*5* insertions were identified by comparison with a Tn*5* HindIII probed blot, while additional bands were proposed to have arisen from IS*50* transposition events.

Controls

A. *B. pertussis* Wellcome 28; negative control.

B. BP347; positive control.

Transposon mutants (this study)

C. 15.
D. 51.
E. 73.
F. 97.
G. 98.
H. 99.
I. 100.
J. 101.
K. 104.
L. 113.
M. 115.
N. 268.
O. 102.

Digest - EcoRI

Probe - Tn5 (HpaI)

terminal, independently mobile, IS*50* elements which flank the central area [see Fig. 6]. Tn*5* contains no internal EcoRI restriction sites, so a single restriction fragment was expected to hybridise to the probe when only a single Tn*5* transposition event had occured. Such expected results were obtained using the Tn*5* HindIII probe and were used to calculate the sizes of the Tn*5*-interrupted EcoRI fragments, which, after subtraction of the 5.8kb transposon DNA, are shown in Table 8. Single Tn*5* insertions were noted in each mutant, while the probe showed no hybridisation to the parent strain.

Faint hybridisation signals, in addition to the single expected bands, were noted in some digests after probing with the HindIII fragment of Tn*5*. These bands gained in strength when the alternative HpaI digested probe, which encompasses almost the entire transposon, was used. The presence of additional bands were presumed to be due to seperate IS*50* transposition events. The size of the IS*50*-interrupted EcoRI fragments are shown in Table 8 and have been calculated by subtracting the size of a single IS*50* element (1.5kb) from the size of hybridising bands not previously ascribed to Tn*5* insertions. A single mutant, 104 (K), appeared to have multiple IS*50* insertion sites. Mutants 51 and 73 were shown to have identical Tn*5* and IS*50* insertion sites, as were mutants 98 and 99. BP347 and mutants 100, 101, 113, 115 and 102 showed no independent IS element transposition events.

3.iv. Complementation of the Crb⁻ Transposon Mutants with the Cloned *bvg* Locus

The cloned *bvg* locus, present on pRMB2 and on the pRMB2 deletion derivative pDM1 [see Fig. 7], was introduced into the *B.pertussis* Crb⁻

transposon mutants. Complementation in *trans* of mutations in the *bvg* locus were screened for by regained expression of certain virulence-associated properties.

3.iv.a. Complementation studies with pRMB2

The cosmid pRMB2 was introduced into *B.pertussis* Wellcome 28 Crb$^-$ Tn*5* mutants by 5 hour tripartite conjugation on BG agar with the *E.coli* donor strain DH1 (pRMB2) and *E.coli* HB101 containing the mobilising plasmid pRK2013 [see Table 4]. Transfer of the cosmid was monitored by the acquisition of tetracycline resistance in the *B.pertussis* recipients.

The kanamycin/tetracycline resistant transconjugants were colony purified, then streaked onto BG overlay plates (containing the appropriate antibiotics) to determine if haemolytic activity had been reacquired. Additionally, transconjugants were grown to single colonies on CSM+Km+Ceph+Tc+CR to screen for Congo red uptake.

Table 9 shows the expression of certain virulence-associated properties by Crb$^-$ transposon mutants after complementation in *trans* with the *bvg* locus on pRMB2. All the mutants regained the ability to take up Congo red dye. Additionally, all the non-haemolytic mutants regained the ability to lyse horse red blood cells.

3.iv.b. Complementation studies with pDM1

The pRMB2 deletion derivative pDM1 was introduced into the *B.pertussis* Crb$^-$ transposon mutants by conjugation with *E.coli* JM83 (pDM1), using the helper plasmid pRK2013. A comparison of the effects of complementation in *trans* with pDM1, against those of pRMB2, could provide information on the role of the 12kb deleted region of pDM1 [see Figure 7] in virulence regulation.

Table 9 Complementation of Crb$^-$ transposon mutants with the cloned *bvg* locus on pRMB2 or pDM1

The cloned *bvg* locus was introduced into the *B. pertussis* Crb$^-$ transposon mutants, on either pRMB2 or its deletion derivative pDM1, during 5 hour plate conjugations utilising the helper plasmid pRK2013 [see Section 2.iii.b.]. Transconjugants containing the *bvg* locus were screened for the regain of Congo red-binding ability and haemolysis (HLY).

Expression of virulence-associated properties by Crb$^-$ mutants containing the *bvg* locus on pRMB2 or pDM1:

Strains	CR-binding		HLY	
	pRMB2	pDM1	pRMB2	pDM1
Control				
BP347	+	+	+	+
Transposon mutants (this study)				
15	+	+	+	+
51/73	+	+	N/D	N/D
97	+	+	+	+
98/99	+	+	+	+
100	+	+	+	+
101	+	+	+	+
104	+	+	+	+
113	+	+	+	+
115	+	+	+	+
268	+	+	+	+

N/D - not determined.

Alterations in the Congo red-binding phenotype and in the haemolytic nature of the transconjugants were noted after complementation in *trans* with pDM1 and are shown in Table 9. All of the previously Crb$^-$ mutants, including BP347, showed regained ability to take up Congo red dye, while those mutants originally non-haemolytic all regained the ability to lyse horse red blood cells.

3.iv.c Instability of *bvg* clones

During complementation studies involving either pRMB2 or pDM1 it was noted that the cloned *bvg* locus on these plasmids appeared to be unstable. Although tetracycline resistant transconjugants of all the Crb$^-$ mutants were obtained which showed regained expression of virulence-associated properties, some transconjugants showed no return to virulent characteristics. The presence of plasmid DNA in these tetracycline-resistant recipients was not screened for, nor was the intrinsic tetracycline-resistance level of *B.pertussis* ascertained.

3.v. Hybridisation of the Crb$^-$ and Crb$^{+/-}$ Transposon Mutants with the *bvg* Locus on pRMB2

The regained expression of virulence-associated factors, in the Crb$^-$ mutants isolated during this study, after complementation in *trans* with the *bvg* locus, indicates that the mutations causing the Crb$^-$ phenotype could all have been within this locus. Previous results, however, have suggested the mutations (if in the *bvg* region) were not all caused by transposon insertions. The Southern blots used to identify Tn*5* insertion sites in the Crb$^-$ mutants were washed to remove the unwanted probe, then reused to identify transposon insertions, or alternative genetic rearrangements, in the *bvg* locus by

probing with labelled pRMB2 DNA.

3.v.a. EcoRI-digested chromosomal DNA blots probed with pRMB2

A Southern blot of EcoRI-digested chromosomal DNA from the transposon mutants was probed with ^{32}P-radiolabelled pRMB2 DNA (Figure 13). Hybridisation patterns from the mutant genomes were compared with the wild-type pattern, to identify the sites of Tn*5* insertion or alternative genetic rearrangements which could have affected expression of those virulence factors under *bvg* control.

Figure 13 shows the labelled pRMB2 DNA to have hybridised to five bands of the following sizes on a Southern blot of *B.pertussis* Wellcome 28 EcoRI digested DNA (A): 2.5kb, 2.7kb, 4.7kb, 5.1kb and approximately 10kb. A further band of 1.5kb was seen only faintly and is not shown on Fig. 13. The hybridising bands define the *bvg* locus (2.5kb and 2.7kb bands), the *fha*B gene (10kb band) and a non-contiguous fragment (4.7kb, 5.1kb and the 1.5kb band not shown) present on pRMB2 [see Fig. 7].

When probed with pRMB2, the 2.7kb EcoRI band, shown in the wild-type strain, was not present in the transposon mutant BP347 (B), which was originally used to define the *vir* (*bvg*) locus. However, this mutant showed hybridisation to an additional band of 8.5kb, which corresponds to an insertion of the 5.8kb transposable genetic element Tn*5* in the 2.7kb EcoRI fragment. Similar transposon insertions in the 2.5kb EcoRI fragment of the *bvg* locus are visible in mutants 100 (I) and 101 (J), where the smaller band is replaced by a signal at approximately 8.3kb. Deletions of genetic material were identified in mutants 97 (F) and 104 (K). In mutant 97 the deletion was shown to consist of approximately 100bp from the 2.7kb EcoRI fragment. A larger

Fig. 13 Autoradiograph of EcoRI-digested chromosomal DNA from transposon mutants probed with the *bvg* locus on pRMB2

A Southern blot of EcoRI-digested chromosomal DNAs from the transposon mutants altered in the Congo red-binding phenotype and *B. pertussis* Wellcome 28 was probed with radiolabelled pRMB2 DNA (plasmid was supplied by D. MacGregor and labelled by nick translation; see Section 2.iv.p). After autoradiography for 2 days at -70°C, with intensification screens, the film was developed.

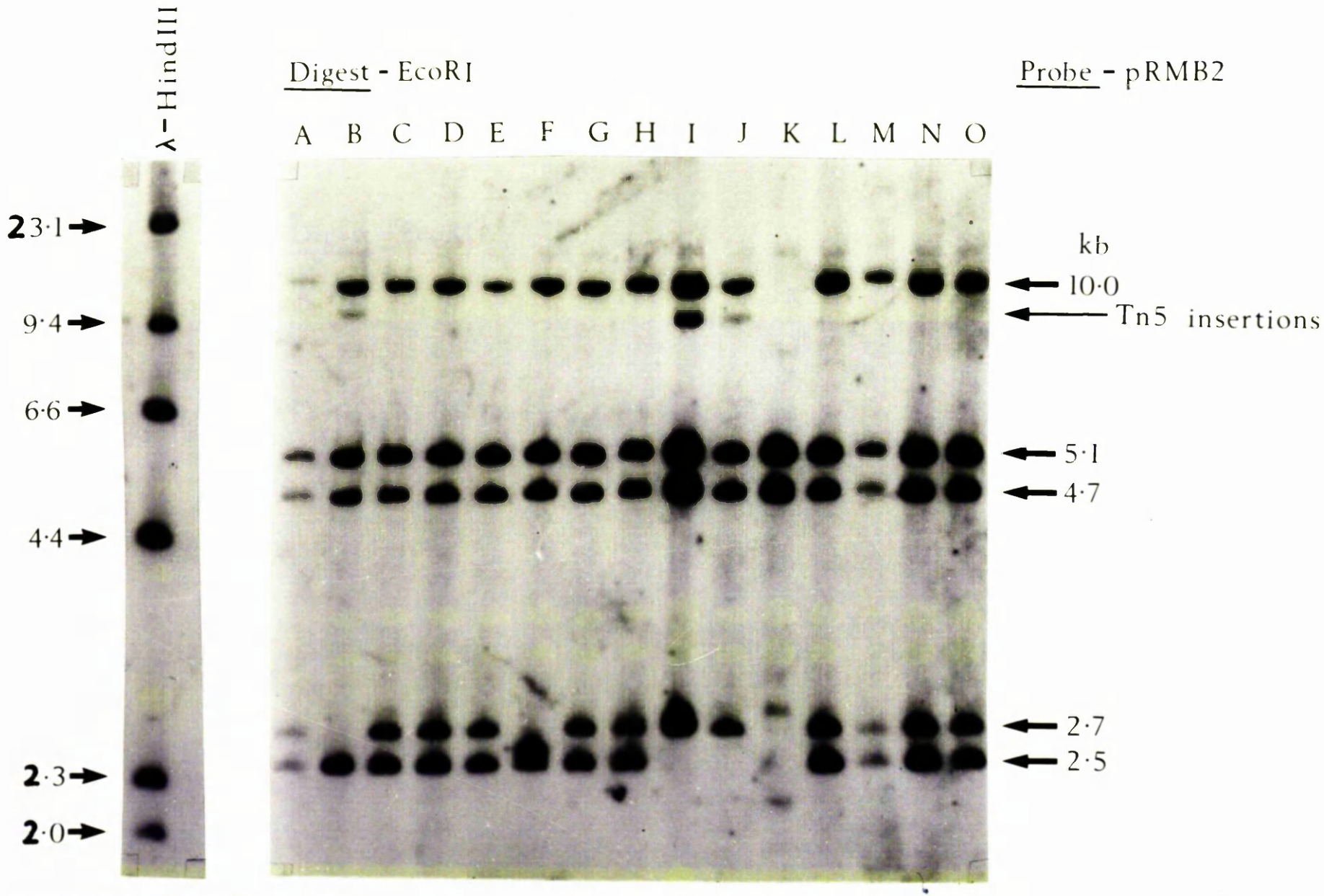
Controls

A. *B. pertussis* Wellcome 28; wild-type.

B. BP347; Tn*5* insertion in *bvg*C.

Transposon mutants (this study)

C. 15.
D. 51.
E. 73.
F. 97.
G. 98.
H. 99.
I. 100.
J. 101.
K. 104.
L. 113.
M. 115.
N. 268.
O. 102 ($Crb^{+/-}$).


λ – HindIII
23·1
9·4
6·6
4·4
2·3
2·0
Digest - EcoRI
Probe - pRMB2
A B C D E F G H I J K L M N O
kb
10·0
Tn5 insertions
5·1
4·7
2·7
2·5

region was missing in mutant 104, the deletion apparently covering the entire *bvg* locus and the *fha*B gene [see Fig. 7]. However, the probe hybridised to two small, additional, fragments (2.3kb and 2.9kb) in the EcoRI digest of mutant 104 DNA, suggesting the ends of the deletion were within the area covered by the probe. The remaining Crb^- mutants showed no transposon insertions or genetic rearrangements, having hybridisation patterns in common with the wild-type strain.

3.v.b. BamHI-digested chromosomal DNA blots probed with pRMB2

Radiolabelled pRMB2 was used to probe a Southern blot of BamHI-digested chromosomal DNA from the Crb^- and $Crb^{+/-}$ transposon mutants and controls (*B.pertussis* Wellcome 28 and BP347). The resultant hybridisation patterns were examined as previously.

When probed with pRMB2, *B.pertussis* Wellcome 28 BamHI-digested DNA showed hybridisation to seven bands of the following sizes: 1.5kb, 2.4kb, 2.8kb, 3.0kb, 5.0kb, 11.5kb, and 15.0kb. A faint additional band, of approximately 4.5kb, was also noted and may have been caused by incomplete digestion at the BamHI site between the 1.5kb and 3.0kb restriction fragments. In the transposon mutant BP347 the probe showed no hybridisation to the 15.0kb BamHI fragment, but alternatively hybridised to two smaller bands of approximately 9.4kb and 11.4kb (identifying and positioning the Tn*5* insertion site within the 15.0kb BamHI restriction fragment, since Tn*5* has an internal BamHI site). Transposon insertions were similarly identified in mutants 100 (I) and 101 (J), both within the 15.0kb BamHI fragment and positioned close to the insertion in BP347. A large deletion was evident in mutant 104 (K), covering both the *bvg* locus and the *fha*B gene [see Fig. 7], whilst the small 100bp deletion, previously noted in mutant 97, was

not apparent in the large 15kb fragment. The remaining mutants displayed hybridisation patterns similar to the wild-type, unmutagenised strain.

3.vi. Frameshift Mutagenesis and the Identification of Spontaneous Crb$^-$ Mutants

The insertion of a single nucleotide base at a specific site within the *bvg* locus has been proposed as one mechanism for phase variation in *B.pertussis* (Stibitz *et al.*, 1989). Growth of *B.pertussis* strains in the presence of a frameshift mutagen could, therefore, be expected to cause phase variation (from Vir$^+$ → Vir$^-$ and from Vir$^-$ → Vir$^+$ forms) at frequencies considerably higher than the spontaneous event. The Crb$^-$ mutants created in this study, which had hitherto unidentified mechanisms causing loss of virulence-associated characteristics, could be identified as frameshift mutants if a high frequency of revertants could be induced using the mutagen ICR 191 (since ICR 191 can cause the insertion or deletion of single or multiple bases).

3.vi.a. Spontaneous phase variation in *B.pertussis*

Liquid cultures of *B.pertussis* Wellcome 28 (Crb$^+$) and *B.pertussis* L84 phase I Crb$^-$ (a spontaneous Crb$^-$ mutant [see Section 2.i.]) were incubated at 37°C with shaking for 48 hours, diluted in PBS and plated onto agar containing Congo red. No phase variants were identified for either strain, although over 5000 colonies were screened.

3.vi.b. Resistance of *B.pertussis* to ICR 191 in liquid culture

The resistance of *B.pertussis* Wellcome 28 to ICR 191 was identified by innoculating liquid cultures with different

concentrations of mutagen and incubating, with shaking, in the dark for 24 hours (ICR 191 can induce deletion mutations or alternative genetic rearrangements in bacterial genomes in the presence of light). Flasks containing no mutagen showed good growth, whilst those with an ICR 191 concentration of less than 2.0μg/ml showed some growth. Concentrations of mutagen over 4.0μg/ml totally inhibited growth.

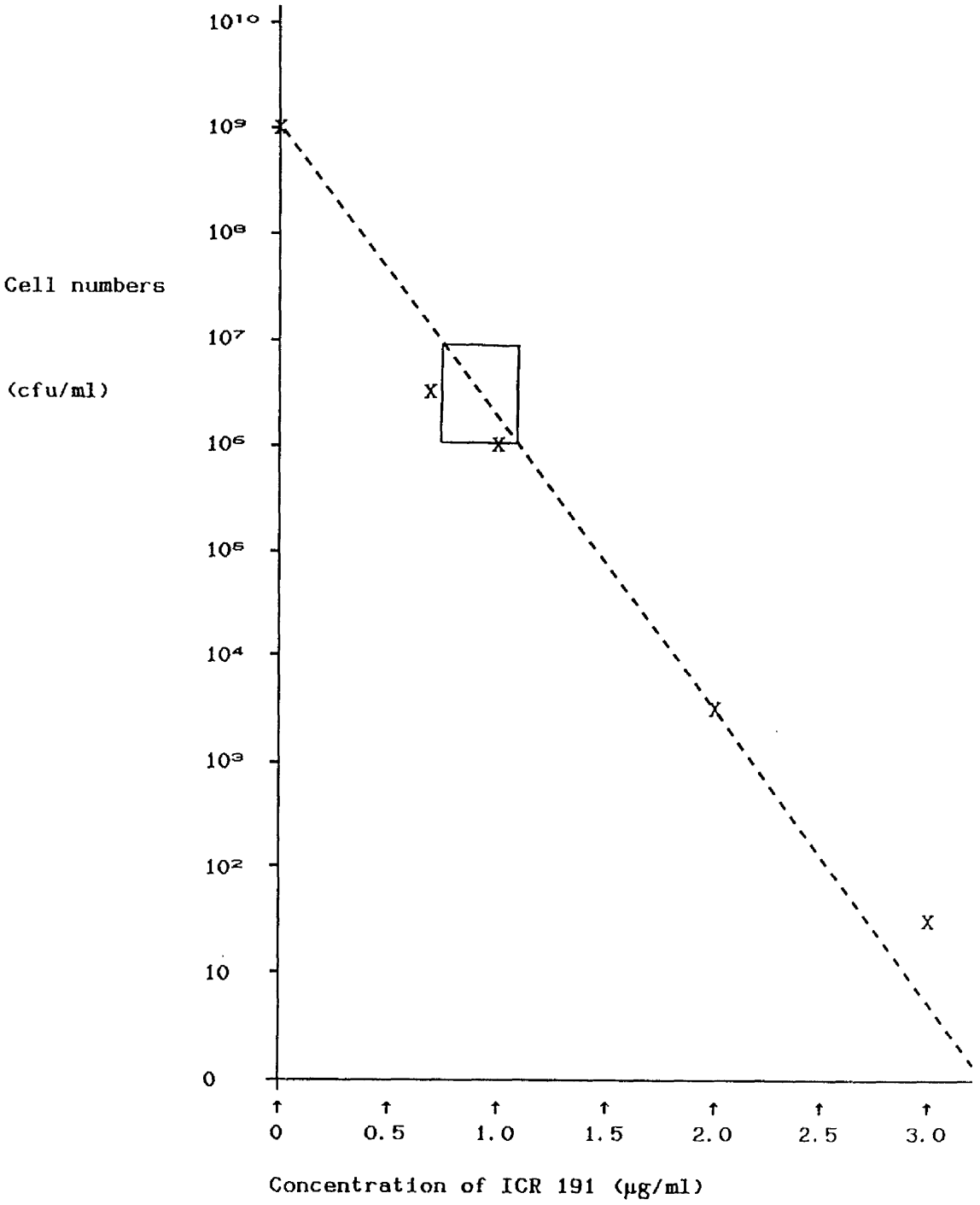
These experiments were repeated using a longer incubation period, of 48 hours, and with only low levels of mutagen, to identify the range of concentrations of ICR 191 which were not so inhibitory as to cause total cell death, but where mutation in the cell was high. Figure 14 shows the effect of increasing ICR 191 concentration on cell numbers. An appropriate range of mutagen concentration for frameshift mutagenesis in *B.pertussis* was identified as being between 0.7μg/ml and 1.0μg/ml, since at these concentrations cell numbers were reduced between 100- and 1000-fold.

3.vi.c. Phase variation in *B.pertussis* by frameshift mutagenesis on agar plates

Liquid cultures of *B.pertussis* Wellcome 28 and *B.pertussis* L84 phase I Crb$^-$ were grown up for 24 hours, diluted in PBS and plated onto CSM+CR, with the following additions to a central well, i. no mutagen, ii. 50μl stock solution ICR 191 (1mg/ml), and iii. 100μl stock solution ICR 191. After incubation for 5-6 days at 37°C the plates were examined. Approximately 2000 colonies grew on each plate, but zones of inhibition in plates containing mutagen were noted around the central well where the mutagen was added (and hence at the highest concentration). However, no phase variants were identified by a change in ability to bind Congo red. The presence of high concentrations of

Fig. 14 The effect of ICR 191 concentration on *B. pertussis* growth

B. pertussis Wellcome 28 was grown with shaking in liquid cultures for 48 hours at 37°C, in the dark, with various concentrations of ICR 191. Cultures were diluted in PBS and used to inoculate BG plates. After incubation at 37°C for 5 days, colony counts were made and the results plotted on the graph.


Cell numbers
(cfu/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10
0
0
0.5
1.0
1.5
2.0
2.5
3.0
Concentration of ICR 191 (μg/ml)

mutagen appeared to affect the CR dye (causing a slight colour change and potentially affecting uptake), so colonies were replica plated onto CSM+CR containing no mutagen. Good growth, in the same patterns as on the original plates was observed, but no phase variants were identified.

3.vi.d. Induced phase variation in *B.pertussis* by frameshift mutagenesis in liquid culture

Liquid cultures of *B.pertussis* Wellcome 28 and *B.pertussis* L84 phase I Crb$^-$ were incubated at 37°C, with shaking, in flasks containing either no mutagen, or 0.7µg/ml ICR 191. After incubation for 22 hours, 28 hours, 45 hours, 52 hours and 70 hours, samples of the cultures were diluted in PBS and plated onto CSM+CR.

In the absence of mutagen no phase variants were identified from *B.pertussis* Wellcome 28 cultures at any of the sampling times. When mutagen was present no phase variants were noted from samples taken at 22 and 28 hours from the *B.pertussis* Wellcome 28 culture. However, at sampling times of 45 hours and 52 hours the same cultures showed a ratio of 1 Crb$^-$ (pale) mutant to every 3000 Crb$^+$ (red) mutants.

In *B.pertussis* L84 phase I Crb$^-$ no phase variants were identified after incubation in the presence, or in the absence, of mutagen.

3.vi.e. Induced phase variation in *B.pertussis* Crb$^-$ transposon mutants by frameshift mutagenesis in liquid culture

Those mutants which might have gained the Crb$^-$ phenotype by spontaneous point mutations within the *bvg* locus were studied under conditions used to induce frameshift mutations in *B.pertussis* Wellcome 28 [see Section 3.vi.d.]. The Crb$^-$ Tn*5* mutants (test isolates and negative controls) and *B.pertussis* Wellcome 28 (positive control) were

grown in liquid culture for 48 hours in the presence 0.7µg/ml ICR 191. *B. pertussis* Wellcome 28 and mutants 51 and 113 were also grown in the absence of mutagen to act as controls to determine the spontaneous mutation rate. Cultures were incubated with shaking in the dark for 48 hours, prior to dilution and plating onto CSM+CR. After 5 days at 37°C colony counts were made to assess the number of red and pink colonies present in each culture.

B. pertussis Wellcome 28 showed the same spontaneous and induced phase variation frequencies as previously [see Sections 3.vi.a. and 3.vi.d.]. Additionally, no spontaneous phase variants were identified on plates of mutants 51 and 113. Mutants 51, 98, 113 and the negative control 104 (a deletion mutant) also showed no induced phase variants. However, mutant 15 showed good growth ($4x10^{8}$cfu/ml) and an induced phase reversion frequency of 1 in 4000cfu. Induced phase variation, due to frameshift mutagenesis, was not studied in mutants 115 and 268.

3.vii. Studies on Crb^{-} Mutant 51

Mutant 51 was identified as a Crb^{-} isolate after transposon mutagenesis experiments. During screening for virulence factors, this mutant (and the identical isolate 73) was shown to be haemolytic and express adenylate cyclase (in a qualitative assay) in the absence of other virulence factors.

3.vii.a. Virulence-associated characteristics

Mutant 51 was previously shown not to express heat-labile toxin, nor agglutinogens 2 and 3, and to be unable to haemagglutinate red blood cells [see Table 7]. Further studies were performed to provide a more complete picture of the virulence-associated characteristics of

this mutant. The results of these studies (Table 10) showed the mutant with all the characteristics of a Vir$^-$ strain, but for the expression of the *cya*A gene.

The haemolytic activity of mutant 51 and the adenylate cyclase production suggested this mutant to be of potential interest for protection studies in animals. The level of adenylate cyclase activity was therefore determined in the quantitative cAMP assay (Amersham), using *B. pertussis* Wellcome 28 and mutant 102 as positive controls, and BP347 (Vir$^-$) and BP348 (Adc$^-$) as negative controls. The assay was performed on X-pressed cell extracts (prepared as previously), with and without ATP, in the presence of calmodulin. Results are shown in Table 11. The control strains tested in this assay showed the expected cAMP levels (high in Wellcome 28 and mutant 102, and low in mutants BP347 and BP348) and little cAMP production when the substrate ATP was absent. However, mutant 51 showed no production of cAMP, either in the presence or absence of ATP. This result suggests that the qualitative adenylate cyclase assay may not be reliable.

3. vii. b. Virulence studies in infant mice

The virulence of mutant 51 was studied in infant mice, using two positive controls (18-323 and Wellcome 28) and BP348 (Adc$^-$). Groups of 10 mice were challenged with 10^{-1}, 10^{-2} and 10^{-3} dilutions of a 10ou (approximately 10^{11}cfu/ml) suspension of each strain. A 10^{-6} dilution of the challenge was used for colony counts. Control mice were left unchallenged. The weights of the groups of challenged and unchallenged mice, over a three week period, are shown in Figures 15 and 16.

After three weeks, groups of five mice previously challenged with the different strains, were examined for signs of infection. Leukocyte

Table 10 Virulence-related characteristics of mutant 51

Transposon mutant 51 was identified as a Crb$^-$ mutant after growth on CSM+CR. However, during growth on BG agar the mutant showed haemolytic activity. Adenylate cyclase was assayed for by a qualitative proceedure (*Dr. C. J. Duggleby, personal communication; see Section 2.v.c). Heat-labile toxin was tested for in infant mice, using both a lethal test [see Section 2.vi.a] and a skin reaction assay [see Section 2.vi.b]. The mutant was shown to produce neither agglutinogen 2 or 3 using monoclonal antibodies in a microtitre tray agglutination assay [see Section 2.v.a]. Pertussis toxin and filamentous haemagglutinin were assayed by ELISA courtesy of Dr. A. Gorringe*[1]. The mutant was additionally shown to be unable to haemagglutinate red-blood cells [see Section 2.v.b]. The lack of X-mode specific polypeptides in mutant 51 was identified by Dr. A. Robinson after SDS polyacrylamide gel electrophoresis*[2].

Virulence factors	Expression
Haemolysin	+
Adenylate cyclase*	+
Heat-labile toxin	-
Agglutinogen 2	-
Agglutinogen 3	-
Pertussis toxin*[1]	-
Filamentous haemagglutinin*[1]	-

Virulence characteristics	Possession of characteristic
Congo red-binding	-
Haemagglutination	-
X-mode specific polypeptides*[2]	-
Growth on nutrient agar	-

Table 11 Cyclic-AMP production by mutant 51

The production of cAMP by the transposon mutant 51 was tested using the Amersham cAMP detection kit. X-pressed cell extracts were incubated for 15 minutes, at 30°C, in the presence of calmodulin, both with and without the substrate ATP. The kit was then used as per the manufacturer's recommendations. Positive controls *B.pertussis* Wellcome 28 and the $Crb^{+/-}$ mutant 102, and negative controls BP347 and BP348, were used to confirm the accuracy of the assay.

Test sample	cAMP levels (pmol cAMP/mg protein)	
	+ATP	-ATP
Positive controls		
B. pertussis		
Wellcome 28	3200	300
Mutant 102	2200	23
Negative controls		
BP347	167	145
BP348	159	N/D
Test isolate		
Mutant 51	8	0

N/D - not determined.

Fig. 15 Virulence studies on *B. pertussis* strains 18-323, Wellcome 28 and BP348, by the mouse-weight-gain test

The average weights of groups of ten mice, after challenge with *B. pertussis* strains, are shown for a period of 20 days.

Challenge strains

A. *B. pertussis* 18-323 (virulent strain).

B. *B. pertussis* Wellcome 28 (vaccine strain).

C. *B. pertussis* Tn*5* mutant 348 (Adc^-).

Challenge dilutions

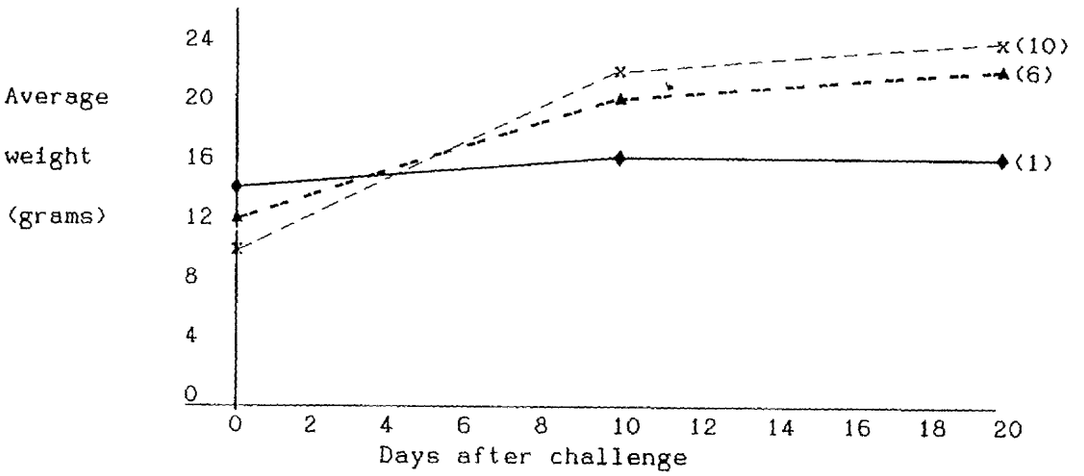
♦ 10^{-1} dilution of a 10ou suspension.

▲ 10^{-2} dilution of a 10ou suspension.

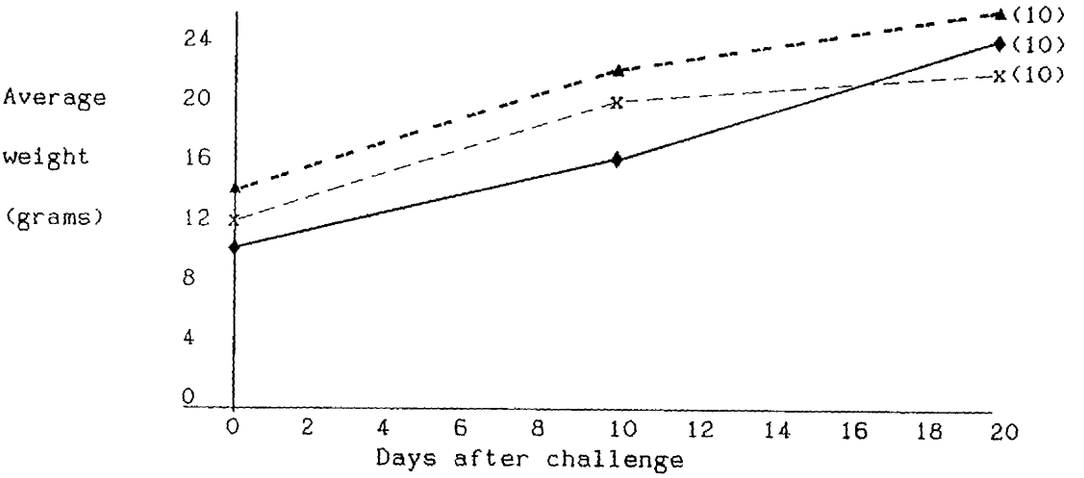
x 10^{-3} dilution of a 10ou suspension.

The challenge was introduced to the mice intranasally, whilst under ether anaesthetic. A single mouse challenged with the 10^{-1} dilution of *B. pertussis* 18-323 died due to the anaesthetic. The numbers of mice remaining alive at the end of the experiment are shown in brackets at the ends of the growth curves. [See Section 2. vi. d.].

A. *B. pertussis* 18-323 challenge



B. *B. pertussis* Wellcome 28 challenge



C. *B. pertussis* 348 challenge

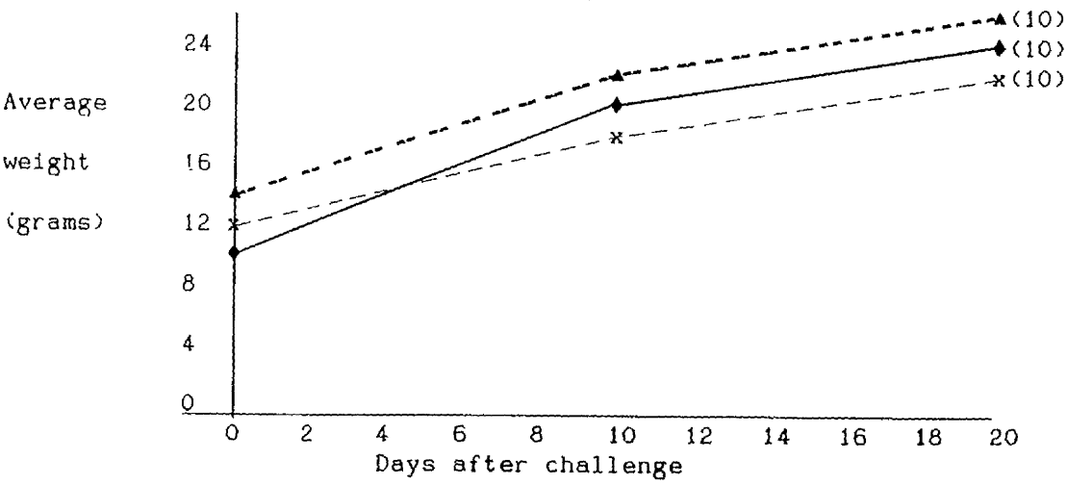


Fig. 16 Virulence studies on *B. pertussis* mutant 51, by the mouse-weight-gain test

The average weights of groups of ten mice, after challenge with *B. pertussis* mutant 51, are shown for a period of 20 days. The average weight gains of a group of unchallenged mice are also shown.

Challenge strains

A. *B. pertussis* 51 (test strain).

B. Unchallenged mice.

Challenge dilutions

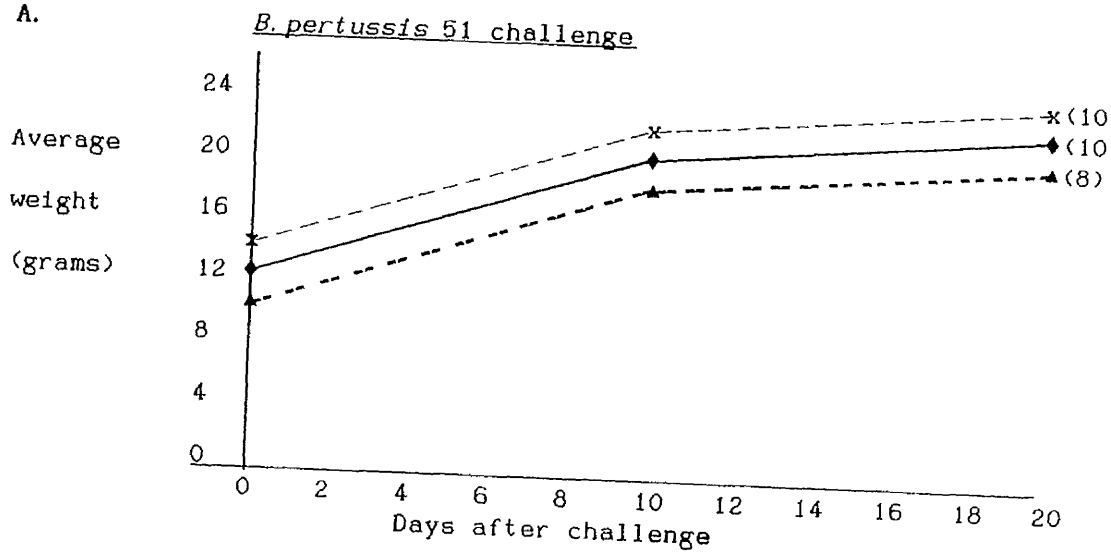
♦ 10^{-1} dilution of a 10ou suspension.

▲ 10^{-2} dilution of a 10ou suspension.

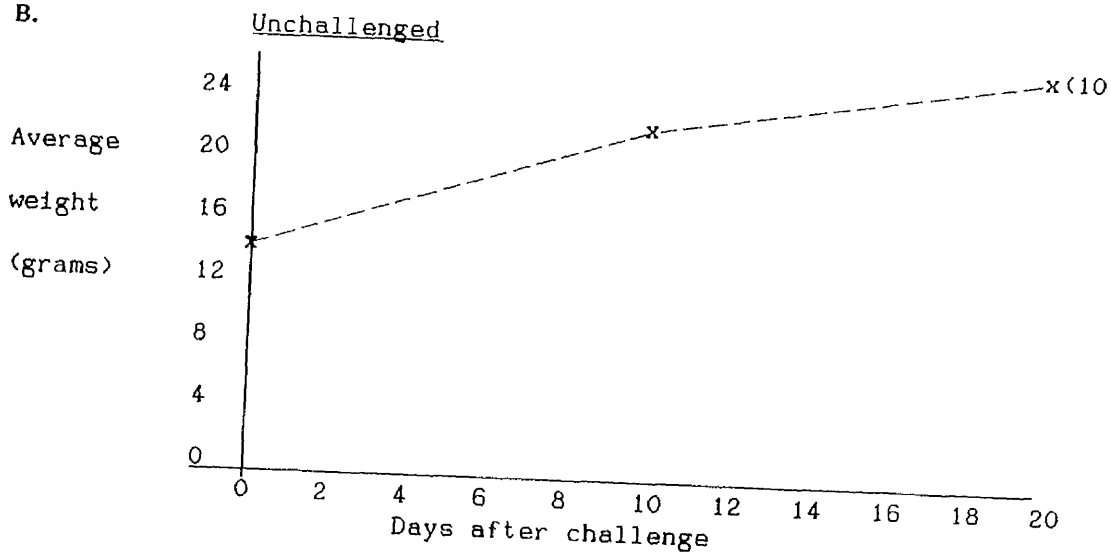
x 10^{-3} dilution of a 10ou suspension.

The challenge was introduced to the mice intranasally, whilst under ether anaesthetic. Two mice challenged with the 10^{-2} dilution of *B. pertussis* mutant 51 died due to the anaesthetic. The numbers of mice remaining alive at the end of the experiment are shown in brackets at the ends of the growth curves. [See Section 2. vi. d.].

A.


B. pertussis 51 challenge
Average weight (grams)
24
20
16
12
8
4
0
0 2 4 6 8 10 12 14 16 18 20
Days after challenge
(10)
(10)
(8)

B.


Unchallenged
Average weight (grams)
24
20
16
12
8
4
0
0 2 4 6 8 10 12 14 16 18 20
Days after challenge
(10)

counts from each group, and spleen weights (as a percentage of body weights) are shown in Table 12, while lung weights as a percentage of total body weight and lung pathologies are shown in Table 13.

The results show *B. pertussis* 18-323 clearly to be a virulent strain, mice surviving the challenge still being infected after 3 weeks. Wellcome 28 was shown to be a weakly virulent strain, with some mice showing signs of infection, including increased lung weights. BP348 was shown to be an avirulent strain, with only very slightly increased lung size, but with leukocyte counts and spleen size closely related to the unchallenged controls. The test isolate 51 was also shown to be avirulent, with spleen and lung weights unaffected by infection, but with some increase in leukocyte numbers.

3.vii.c. Vaccine production and protection studies in infant mice

Heat-killed, whole-cell vaccines were prepared from *B. pertussis* Wellcome 28, BP347 and mutant 51. The vaccines were injected into infant mice in randomised groups. The mice were later challenged with *B. pertussis* 18-323, either intracerebrally (ic) or intranasally (in). Two groups of mice were left unvaccinated in each test. The first unvaccinated group received the challenge, the second group went unchallenged. Mice which died immediately after the challenge were classified as deaths due to anaesthesia.

After ic challenge no mice vaccinated with *B. pertussis* Wellcome 28 died. However, fatalities were observed in mice vaccinated with both BP347 and mutant 51 vaccines. Fatalities were also observed with challenged mice which had not been vaccinated.

After in challenge experiments, infection was monitored by weight gain, lung pathology and lung weights. The lung weights were expressed

Table 12 Leukocyte counts and spleen weights from mice challenged with *B. pertussis* strains

Groups of five mice challenged with different strains of *B. pertussis* in a mouse-weight-gain test, and a set of unchallenged mice, were examined to identify leukocyte counts and spleen weights (as a percentage of body weight) three weeks after the initial challenge.

Challenge strain	Dilution	Mean leukocyte count (+/-SD) (per mm^3)
18-323	10^{-2}	15982 +/_ 4999
Mutant 51	10^{-1}	14446 +/_ 2097
Wellcome 28	10^{-1}	12727 +/_ 3005
BP348	10^{-1}	8735 +/_ 2427
Unchallenged		6852 +/_ 1582

Challenge strain	Dilution	Mean spleen weight (+/-SD) (as a % of total body weight)
18-323	10^{-2}	0.622 +/_ 0.254
Unchallenged		0.584 +/_ 0.108
Wellcome 28	10^{-1}	0.519 +/_ 0.138
BP348	10^{-1}	0.409 +/_ 0.058
Mutant 51	10^{-1}	0.387 +/_ 0.078

Table 13 Lung weights and pathologies from mice challenged with *B. pertussis* strains

Groups of five mice challenged with different strains of *B. pertussis* in a mouse-weight-gain test, and a set of unchallenged mice, were examined for lung pathology (0 - normal, 4 - heavy infection) and maintenance of the challenge strain (A). Increases in lung weights (B; as a percentage of body weight) three weeks after the initial challenge were noted as a sign of virulence in the challenge strain.

A.

Challenge strain		Mouse 1	2	3	4	5
18-323	Lung pathology	2	1	0	4	4
	Lung culture	+	++	-	+	+++
Wellcome 28	Lung pathology	0	3	0	4	1
	Lung culture	-	-	++	-	-
BP348	Lung pathology	0	0	0	0	0
	Lung culture	-	-	-	-	-
Mutant 51	Lung pathology	0	0	0	0	0
	Lung culture	-	-	-	-	-
Unchallenged	Lung pathology	0	0	0	0	0
	Lung culture	-	-	-	-	-

B.

Challenge strain	Dilution	Mean lung weight (+/-SD) (as a % of total body weight)
18-323	10^{-2}	1.914 +/_ 0.621
Wellcome 28	10^{-1}	1.432 +/_ 0.305
BP348	10^{-1}	1.162 +/_ 0.398
Mutant 51	10^{-1}	0.820 +/_ 0.058
Unchallenged		0.798 +/_ 0.222

as a percentage of the whole body weight and the average value (for each group) was calculated with standard deviations (Figure 17). At a vaccine dose of 2ou.ml vaccines made from both Wellcome 28 and mutant 51 showed good protection (low lung weights as a percentage of total body weight), whilst the BP347 vaccine showed little protective activity. Unvaccinated mice in this test showed lung weights of less than 1% of body weight when unchallenged, and up to 4.5% of body weight when challenged with virulent *B.pertussis*. Lung pathologies of mice vaccinated with *B.pertussis* Wellcome 28 were clear where high vaccine doses were given, but signs of infection were present where lower doses were used. Similar lung pathologies were seen in mice vaccinated with mutant 51. However, in mice vaccinated with the avirulent mutant BP347 the lungs showed signs of heavy infection.

3.vii.d. Antigenic modulation

Haemolytic colonies of *B.pertussis* Wellcome 28 and mutant 51 were picked from BG overlay plates and restreaked on the same media. Plates were incubated in a moist environment at 25°C to induce antigenic modulation. Under these conditions *B.pertussis* Wellcome 28 grew as non-haemolytic colonies, while mutant 51 retained haemolytic activity.

Additionally, mutant 51 showed haemolytic activity on BG sandwich plates containing 20mM $MgSO_4$ when grown at 37°C, whereas under these conditions *B.pertussis* Wellcome 28 was non-haemolytic.

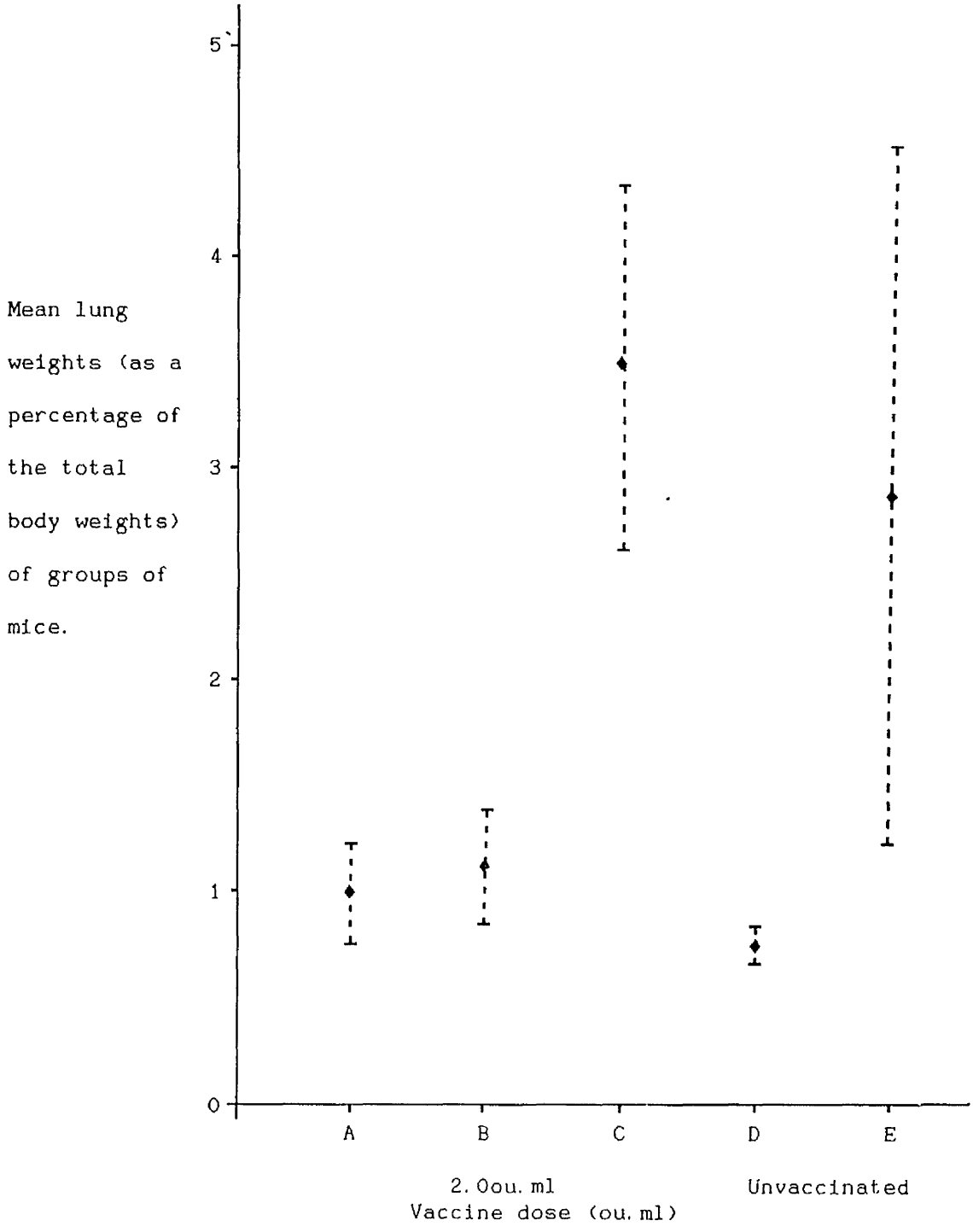
3.vii.e. EcoRI digests of Crb⁻ mutants probed with the adenylate cyclase operon on pRMB11

A Southern blot of EcoRI digests of the chromosomal DNA from the Crb⁻ mutants was probed with pRMB11 [see Fig. 2] to identify genetic rearrangements or transposable genetic element insertions in the Crb⁻

Fig. 17 Mouse intranasal protection test

Vaccines from *B. pertussis* Wellcome 28 (A; positive control), BP347 (C; negative control), and the transposon mutant 51 (B; test isolate) were injected into infant mice in randomised groups. The mice were subsequently challenged in with *B. pertussis* 18-323. Groups of unvaccinated mice were either challenged (E), or left unchallenged (D).

The graph shows the mean lung weights of the mice two weeks after the challenge date, as a percentage of the average body weight per group. Standard deviations were calculated as a measure of variation and are shown on the graph as broken lines.

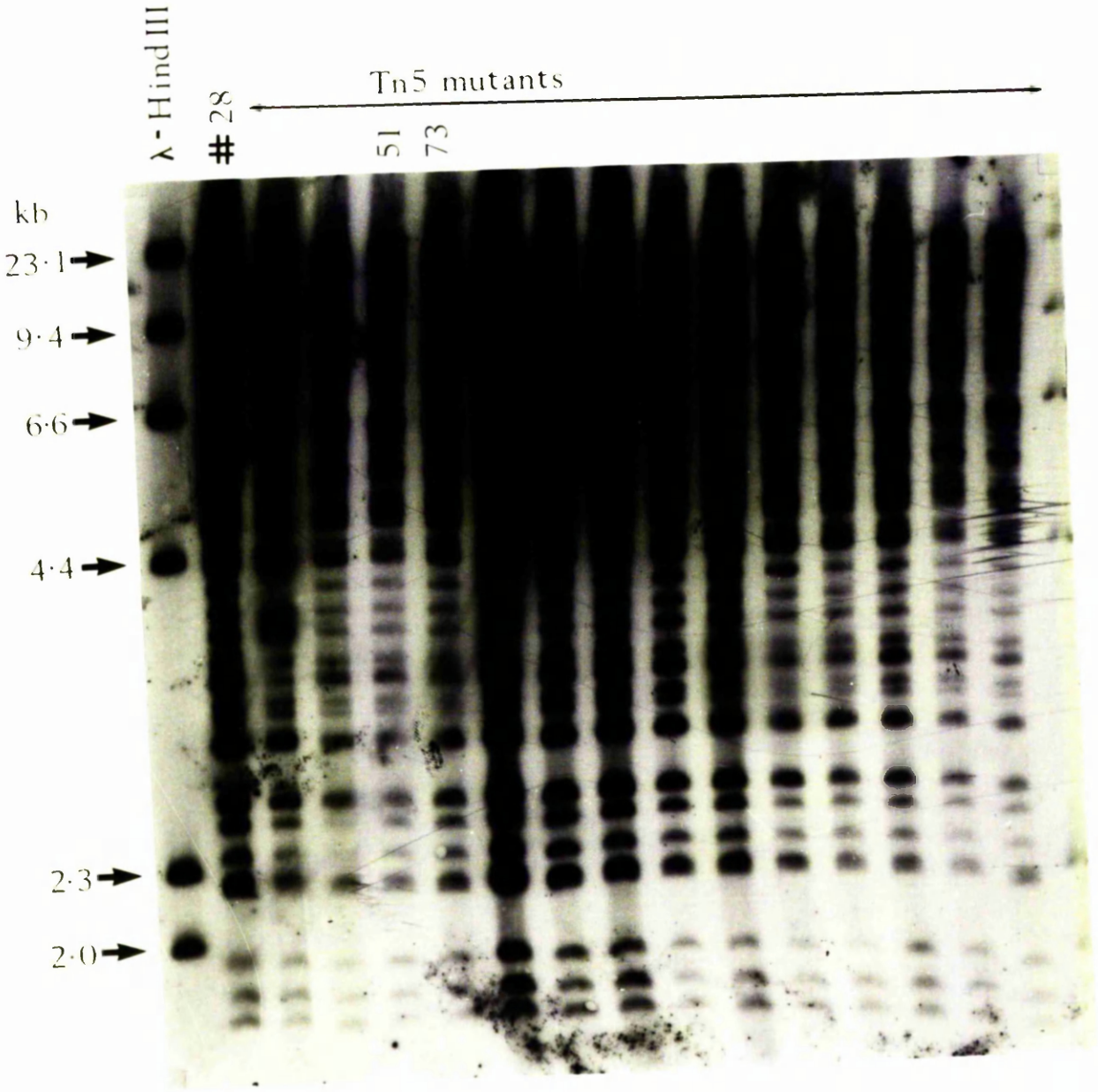

Mean lung weights (as a percentage of the total body weights) of groups of mice.
5
4
3
2
1
0
A
B
C
D
E
2.0ou. ml
Unvaccinated
Vaccine dose (ou. ml)

mutant 51, which could have caused constitutive expression of the haemolysin gene. A film was exposed to the radiolabelled probe on the filter for 2 days at -70°C and then developed (Figure 18). However, the presence of a repeated DNA sequence upstream of the cyclase operon renders the analysis of genetic insertions with this clone impractical.

Fig. 18 Southern blot of chromosomal DNA from Crb⁻ mutants probed with part of the adenylate cyclase operon on pRMB11

The clone pRMB11 [see Figure 2], which contains the 5.5kb ClaI fragment subcloned from pRMB1, was radiolabelled by nick-translation and used to probe a Southern blot of EcoRI digested *B. pertussis* strains including the transposon mutant 51. This subclone contains the start of the cyclase open reading frame, and the repeat sequence IS481v1 (McPheat *et al.*, 1989).

The blot shows the wild-type pattern found when probing *B. pertussis* Wellcome 28 (#28), and the apparently identical banding patterns found in the transposon mutants created in this study. The approximately 12kb EcoRI fragment covering pRMB11, where an IS element insertion may be present in mutant 51, cannot be distinguished from the repeat sequence pattern.



Digest - EcoRI

Probe - pRMB11

DISCUSSION

4.i. Tn*5* Delivery Vectors for Insertional Mutagenesis of *Bordetella pertussis*

The considerable success of Weiss *et al.* (1983) in using Tn*5* mutagenesis to study pathogenicity in *B. pertussis* indicated that this technique could yield information about virulence in other bacterial pathogens. Since this report, generalised transposon mutagenesis has, amongst other work, been used to identify protease-deficient strains of *Aeromonas hydrophila* (Leung and Stevenson, 1988), exoprotease genes in *Pseudomonas aeruginosa* (Stapleton *et al.*, 1984) and mutants of *Salmonella* lacking a virulence-associated characteristic (Finlay *et al.*, 1988). This present study with Tn*5* mutagenesis was aimed at the further elucidation of the mechanisms of virulence regulation in *B. pertussis*.

The proficient use of Tn*5* in mutagenesis depends on the recipient bacterial strain having no intrinsic resistance to the antibiotic resistance gene(s) carried on the incoming transposon. Since several studies have successfully introduced the kanamycin resistance-encoding transposon Tn*5* (or one of its many derivatives) into *B. pertussis* [see Section 1.viii.d.], the level of spontaneous kanamycin resistance in this pathogen was considered to be suitably low. This study re-confirmed that both *B. pertussis* Wellcome 28 and Tohama strains were suitable for Tn*5* mutagenesis by virtue of negligible intrinsic resistance to kanamycin.

The original studies of Weiss *et al.* (1983) on *B. pertussis* virulence genes involved the use of plasmid pUW964, which incorporates the ColE1 replicon, for transposon carriage into strain Tohama.

Preliminary work for this study indicated that this 'suicide' vector might be unsuitable for Tn*5* delivery into *B. pertussis* Wellcome 28 after the identification of transconjugants maintaining pUW964. Simon (1989) reported that the efficacy of transposon donors varies even between closely related strains, which could indicate why pUW964 was maintained in strain Wellcome 28. However, further studies revealed that some transconjugants of strain Tohama also maintain this particular vector. It appears that the inability to transform *B. pertussis* with ColE1 as reported by Weiss and Falkow (1982) does not mean that the replicon is entirely inactive in this bacterium when introduced by conjugation as part of a larger chimaeric plasmid.

The maintenance of transposon delivery vectors could, on analysis, produce both complex and confusing data and their effective use in transposon mutagenesis experiments would require labour intensive screening of transconjugants to exclude all those still containing the delivery vectors. Such methods may have been used in the early experiments of Weiss and co-workers, although this was not reported in either of their papers on Tn*5* mutagenesis (Weiss *et al.*, 1983; Weiss and Falkow, 1983). To facilitate the continuation of this study, alternative Tn*5* delivery vectors, with different genetic backgrounds to pUW964, were introduced into *B. pertussis* Wellcome 28, to identify a truly suicidal plasmid suited for mutagenesis.

The most important characteristics of a transposon delivery vector are a high transfer frequency from the *E. coli* donor strain into the recipient species, combined with an inability to replicate once transferred. The transposon carriers pJB4JI and pSUP2021 used in this study, either incorporate, or can be mobilised by, the broad-host-

range IncP group transfer (*tra*) genes. The *tra* genes for the mobilisable vector pSUP2021 are integrated within the chromosome of the *E.coli* donor strain S17-1 (Simon *et al.*, 1983). However, this strain did not mobilise pSUP2021 into *B.pertussis* at detectable levels and this system was not used further. In contrast, the transposon delivery vector pJB4JI transferred into *B.pertussis* inducing kanamycin resistant transconjugants at reasonably high frequencies. However, this plasmid was shown to be maintained in a high proportion of the resultant transconjugants.

The IncN group transfer genes of pGS9 and the derepressed $IncI_{\alpha}$ group transfer genes of pLG221 conveyed the kanamycin resistance gene of Tn*5* into *B.pertussis* at high frequencies, similar to that of pUW964 transfer. The transposon donor pGS9 was, however, shown to be maintained in the resultant kanamycin resistant transconjugants (both by their acquired resistance to plasmid encoded antibiotics and by their hybridisation to the closely related plasmid pGS18). The transposon delivery vector pLG221 was, however, shown not to be maintained at significant levels in *B.pertussis* Wellcome 28 kanamycin resistant transconjugants.

In the absence of mutagenesis studies using bacteriophage-based transposon delivery vectors, or studies using electroporation to introduce simple narrow-host-range plasmids carrying transposons into *B.pertussis*, this preliminary work indicated that of the transposon delivery vehicles tested, the ColIb based vector pLG221 appeared to be the most useful for further Tn*5* mutagenesis experiments.

4.ii. Transposon Mutagenesis of *B.pertussis*

The Tn*5* insertion mutants of *B.pertussis* Wellcome 28, produced during this study using the transposon delivery vector pLG221, were classified with respect to the Congo red-binding (Crb) phenotype (as red or pale colony types) and to the use of selective pressure, against phase I organisms, used in their isolation. The majority of the transposon mutants created retained the ability to bind the dye Congo red (a characteristic associated with virulent strains of *B.pertussis*; Parton, 1988).

The Crb$^+$ Tn*5* mutants were screened for the loss of expression of heat-labile toxin (HLT) and the loss of ability to modulate when grown at 25°C (since at this time no isolates with insertion mutations in either of these genetic loci had been produced). Mutants not producing HLT were desired for both genetic studies (cloning and sequencing of *hlt*) and for assessment of virulence in the infant mouse model. Although over 200 mutants were screened this study did not identify a mutant solely deficient in HLT. Lodge *et al.* (1985) have discussed the identification of Tn*5* insertion hotspots within target DNA molecules. This insertional specificity of Tn*5* may have been involved in the inability to produce mutants lacking HLT, both in this and in previously published work (Weiss *et al.*, 1983), although recently Weiss *et al.* (1989) have used a Tn*5*-derived transposon to produce a mutant unable to express HLT.

Approximately 110 Crb$^+$ Tn*5* mutants were screened to identify individual isolates which were unable to modulate during growth at 25°C on media containing the dye Congo red. Mutants maintaining the

Crb$^+$ phenotype under such growth conditions would be of use in further studies on the mechanisms of virulence regulation in *B. pertussis*. However, no transposon insertion mutants were identified in this study which were unable to modulate from the Crb$^+$ (red) phenotype to the Crb$^-$ (pale) phenotype after a change from X-mode to C-mode growth conditions.

Spontaneous mutants constitutively expressing X-mode characteristics have, however, now been isolated (Knapp and Mekalanos, 1988) and the mutation causing the loss of regulatory activity was suggested to be within a gene designated *mod*. Knapp and Mekalanos proposed the *mod* gene to be distinct from the *vir* gene, but in close proximity. The recent renaming of the *vir* gene as part of the *bvg* locus (which is composed of three genes) suggests that *mod* may also be part of this locus. Since expression of *bvg*A, *bvg*B and *bvg*C has been shown to be required for the Vir$^+$ phenotype (Aricó *et al.*, 1989), an insertion mutation in *mod* (or *bvg*B, which has a periplasmic sensory protein product and is therefore most likely to correspond to *mod*) would presumably only be identifiable by the change to the Vir$^-$ phenotype. Such a mutant would have been identified in this study as possessing the Crb$^-$ phenotype. Those spontaneous mutants found by Knapp and Mekalanos (1988) to be constitutively expressing *mod*, may have been caused by a mutation in the region of the gene which acts as an environmental sensor. This would allow gene expression, but might modify the proteins ability to act in a regulatory manner.

A number of mutants possessing the pale (Crb$^-$) phenotype were identified from the bank of transposon mutants created in this study. None of the Crb$^-$ mutants proved equivalent to the classical phase IV

strains (being unable to grow on nutrient agar), but, with the exception of mutants 51 and 73, the Crb$^-$ mutants did not express any of the virulence-associated factors tested. Parton (1988) has suggested that the Congo red-binding phenotype is a characteristic whose expression is controlled by the *bvg* locus. The variants created in this study (excluding 51 and 73) will, therefore, be considered as phenotypically equivalent to the avirulent (Vir$^-$) strains described by Weiss *et al.* (1984). Mutants 51 and 73, which express only certain virulence factors, are considered separately in Section 4.vii.

The Crb$^-$ mutants created during this study were divided into two classes, i. those isolated from a fully supportive (non-selective) growth medium, and ii. those isolated after growth on a batch of Difco Bacto agar previously shown (C.J. Duggleby, personal communication) to select against phase I *B.pertussis*. Control plates identified a high frequency of spontaneous mutation to the Crb$^-$ phenotype when *B.pertussis* Wellcome 28 was grown on this selective agar. This high mutation rate suggests that spontaneous mutation to the Crb$^-$ phenotype may be an important factor to consider in those transposon mutants (1-99) which underwent selective pressure against phase I characteristics. Such mutants could have a mutation in the virulence regulatory region of the chromosome, while the Tn*5* insertion could be positioned in a region unassociated with regulatory functions [see later].

Mutant 102, which displayed an intermediate Congo red-binding phenotype (Crb$^{+/-}$), was shown to have a greatly reduced ability to haemagglutinate red blood cells (associated with loss of expression of FHA). Parton (1988) has, however, noted that a transposon mutant with

an insertion in the *fha* structural gene (Fha$^-$ mutant BP353) exhibits the Crb$^+$ phenotype. This suggests that the mutation causing the Fha$^-$ phenotype in mutant 102 may not have been due to a transposon insertion in the *fha* structural gene. This mutant also had reduced levels of agglutinogen 3, suggesting that the transposon insertion may be within genes controlling expression of surface components. Mekalanos (Protein Toxins Meeting, Urbino, Italy, 1989) has identified a transposon mutant with an Fha$^-$ phenotype which produces agglutinogen 2 and 3 subunits, but has no fimbrial agglutinogens 2 or 3. An assembly or export protein which controls the surface expression of both FHA and agglutinogens 2 and 3 was postulated. A possible site for the transposon insertion in mutant 102 would be in the gene which normally expresses this protein.

Alternatively, the mutation in 102 could be within genes associated with iron uptake mechanisms, or surface hydrophobicity (FHA being the main hydrophobic constituent of the cell; Fish *et al.*, 1987), since Congo red-binding may be associated with either of these properties. Crb$^-$ mutants of *Neisseria meningitidis* have been suggested to be defective in their ability to acquire iron *in vivo* (Payne and Finkelstein, 1977), while in *Shigella* the same authors have suggested the Crb$^-$ phenotype to be involved with cell-surface iron-transport components. However, more recently Stugard *et al.* (1989) have suggested a cell surface attachment function for the *crb* gene product in *S. flexneri*.

4.iii. Tn*5* Insertion Sites in Mutants Altered in the Congo red-Binding Phenotype

The region around the Tn*5* insertion sites in the Crb$^-$ mutants and in mutant 102 were mapped for restriction sites to identify insertions within the *bvg* locus, or homologous regions of insertions between mutants. Similarities in the sites of insertions were used to subdivide the mutants into potentially related groups, and to pin-point loci at which Tn*5* insertions could have produced the Vir$^-$ phenotype. The site of insertion in the Crb$^{+/-}$ mutant 102 was analysed after restriction mapping to position the transposon either within the known *fha* genes or at an unknown locus [see Section 4.ii.].

The Tn*5* insertions in the Crb$^-$ mutants 100 and 101 were shown to be within a BamHI fragment of the same size (15kb) as the interrupted fragment in Weiss's mutant BP347 [see Fig. 4]. Due to the internal BamHI restriction site in Tn*5*, the insertions in mutants 100 and 101 could be accurately positioned to an area just upstream of the insertion site in BP347, but still within the *bvg*C gene (Figure 19). This orientation of the insertion sites was confirmed by a comparison of the interrupted SalI and EcoRI restriction fragment sizes (0.6kb and 2.5kb respectively in mutant 100 and 0.3kb and 2.5kb respectively in mutant 101) with the published *bvg* restriction map.

The site of the transposon insertion in mutant 113 was localised to a BamHI fragment of approximately 14kb. However, the similarity in size to the 15kb BamHI fragment interrupted in mutants 100 and 101 appears coincidental since the interrupted EcoRI fragment in mutant 113 is 9.3kb in size and such a restriction fragment does not appear

Fig. 19 Restriction map of the *bvg* locus showing Tn*5* insertion sites identified in the Crb$^-$ mutants 100 and 101

The *bvg* locus was defined by Aricò *et al.* (1989) and the figure shows EcoRI (E) and SalI (S) sites covering the three open reading frames *bvg*A, *bvg*B and *bvg*C. The Tn*5* insertion site in BP347 is shown within the 2.7kb EcoRI fragment and the 1.0kb SalI fragment. The transposon insertion sites in mutants 100 and 101 (isolated in this study) are shown in the 2.5kb EcoRI fragment and in the 0.6kb and 0.3kb SalI restriction fragments respectively. The locations of these insertion sites were determined from Figures 11, 12 and 13. Mutants 100 and 101 were identified from non-selective media by virtue of the Crb$^-$ phenotype.

1 kb
E
S
S
E
S
S
E
E
BP347
100
101
bvgA
bvgB
bvgC

in or around the *bvg* locus. The Vir$^-$ phenotype displayed by this mutant is, therefore, unlikely to be due to an insertion mutation in the known virulence regulatory locus and appears to be in a region unrelated to the insertion sites found in the other Crb$^-$ mutants.

Mutants 51, 73, 97, 98 and 99 all have Tn*5* insertion sites within a BamHI restriction fragment of approximately 30kb. The SalI- and EcoRI-interrupted restriction fragments for these mutants show that two pairs of mutants, 51 and 73, and 98 and 99, have identical insertion sites. Each pair were isolated from the same filter mating and can therefore be considered as clones. Analysis of the simple restriction maps constructed around the transposon insertions in mutants 51, 97 and 98 suggests that, either, the mutations are widely spaced along a single BamHI fragment, or that the transposon insertions are within different BamHI fragments of approximately the same size. In mutant 97 the transposon insertion was positioned not only in a large BamHI fragment, but also within large SalI (11kb) and EcoRI (10.8kb) restriction fragments, indicating that the interrupted locus is unrelated to those found in mutants 51 and 98. In mutant 98 the BamHI restriction site within Tn*5* has allowed the positioning of the transposon to a site very near the end of the 30kb fragment (approximately 1kb from one end), while in mutant 51 the transposon has been identified in a more central position (at least 9kb from one end), indicating that even if the transposons have inserted in the same restriction fragment, the sites of insertions in these two mutants are at least 8kb apart.

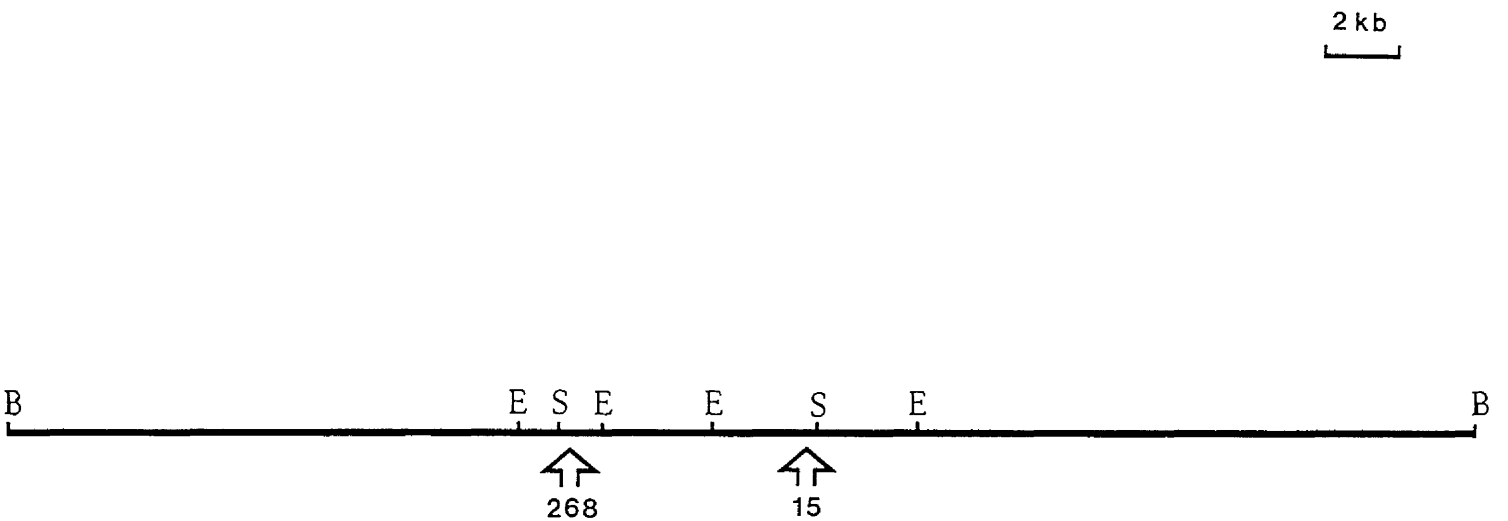
Large sized BamHI fragments (of approximately 40kb) were identified containing transposon insertions in mutants 15, 104 and

268. The internal BamHI site of Tn*5* allowed the positioning of the insertions in these fragments to within a central region (although the large sizes involved makes analysis difficult). Data from the SalI-interrupted fragments show the Tn*5* insertions in mutants 15 and 268 to be within 7.0kb fragments [see Table 8], indicating that the mutations in these isolates could be closely related. The internal SalI site in Tn*5* allowed the positioning of these insertions to, either, near one end of the fragment, or, alternatively, at opposite ends of the 7.0kb fragment. The Tn*5* hybridisation data from the EcoRI digests of both mutants showed the insertions not to be in the same sized EcoRI fragments and, therefore, most probably at alternate ends of the interrupted SalI fragment (Figure 20). The Tn*5* insertions in mutants 15 and 268 appear to be within approximately 7.0kb of each other. This distance is not, however, truly indicative of an alternative locus possibly involved in the regulation of virulence factor expression. The Tn*5* insertion site in mutant 104 was shown to be in a different 40kb BamHI fragment from those in mutants 15 and 268 since the transposon was also shown to be centrally positioned in a large (25kb) SalI fragment.

The Crb$^-$ mutant 115, showed no similarities in the site of Tn*5* insertion with the other Crb$^-$ mutants. As such, this mutant (and others with similarly individual insertion sites) is unlikely to have an insertion mutation within a novel virulence-regulatory locus. However, only on the identification of the uninterrupted gene in a gene library, followed by the reintroduction of the functional gene into the mutant, could the true effect of the transposon mutation be resolved. The possibility of having isolated spontaneously Vir$^-$

Fig. 20 Proposed restriction map of the area surrounding the Tn*5* insertion sites in Crb$^-$ mutants 15 and 268

The map shows a possible arrangement of BamHI (B), EcoRI (E) and SalI (S) sites surrounding the Tn*5* insertions in mutants 15 and 268. The map has been constructed from information in Figures 11, 12 and 13 [see Table 8].


2 kb
B
E
S
E
E
S
E
B
268
15

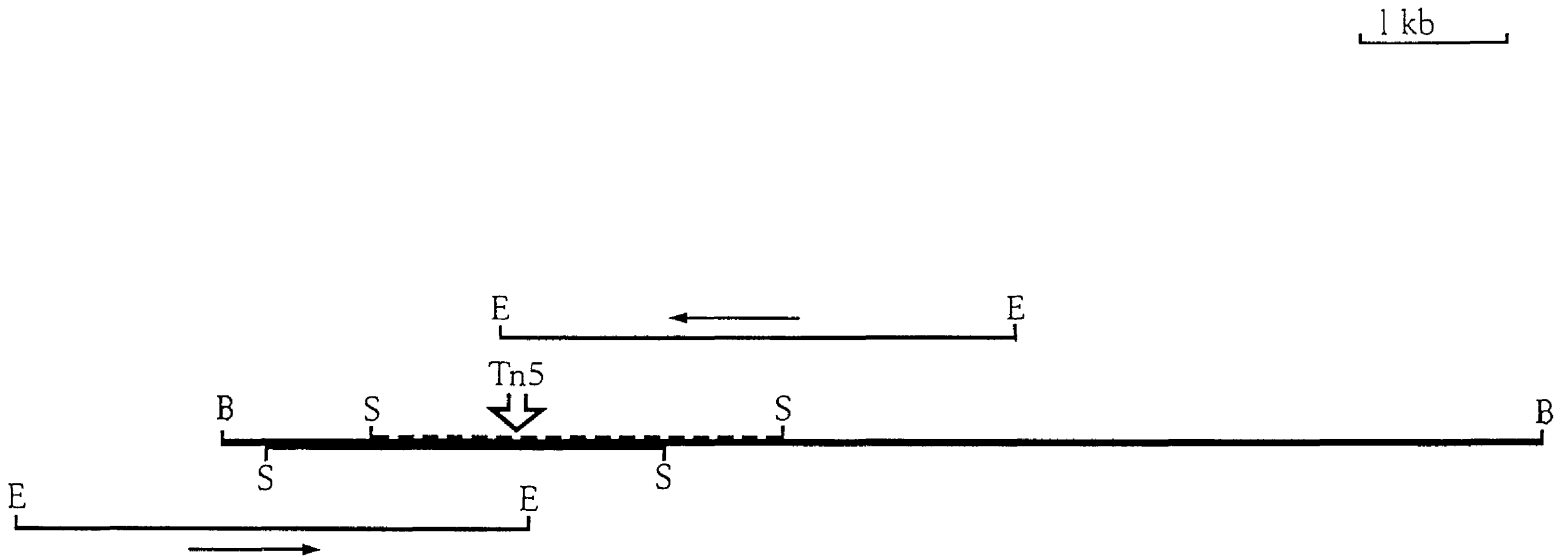
mutants with Tn*5* insertions at sites unassociated with virulence regulation must be considered.

The $Crb^{+/-}$ mutant 102 shows a restriction pattern around the Tn*5* insertion site (Figure 21) which is different from the map of the known *fha* locus (Brown and Parker, 1987). This suggests that the mutation is not within the *fha* structural gene, nor in those genes involved with export functions, but possibly at a locus involved in the control of expression of surface characteristics [see Section 4.ii.].

The Southern blots used to identify the sites of Tn*5* insertions in the Crb^{-} mutants show the presence of more than the expected number of hybridising bands in some of the mutant DNA digests, after a HpaI restriction length fragment probe of Tn*5* was employed (the HpaI fragment of Tn*5* covers both IS*50* elements, whereas the HindIII fragment does not). The additional bands suggest that IS*50*R has been transposing independently of Tn*5*, as has been previously reported in other bacterial species (Berg *et al.*, 1982). One mutant, 104, shows the presence of a large number of additional transposition events within it's genome. The presence of multiple copies of the same transposable genetic element in a single genome is known to cause inversions or deletions of DNA and the possibility of genetic rearrangements must be considered in any analysis of mutant 104. The IS*50* insertions within EcoRI sites have been identified by a comparison of blots probed with the HindIII fragment of Tn*5*, then reprobed with the HpaI fragment. The IS*50* insertions in mutants 97 and 268 are within fragments possibly associated (by size; 2.7kb and 2.9kb respectively) with similarly sized fragments in the *bvg* locus (2.5kb

Fig. 21 Restriction map of the region surrounding the Tn*5* insertion site in mutant 102

The restriction enzyme BamHI (B), EcoRI (E) and SalI (S) sites around the Tn*5* insertion in the $Crb^{+/-}$ mutant 102 are shown. Both possible orientations of the interrupted SalI fragment, with respect to the BamHI fragment are indicated, one above (fragmented line) and the other below (solid line). The possible extreme positions for the EcoRI interrupted fragment are also shown, separate from the restriction map and with arrows to denote their imprecise positioning. This map may represent a novel locus potentially involved with control of expression of cell surface components.


1 kb
E
E
Tn5
B
S
S
B
S
S
E
E

and 2.7kb EcoRI fragments). Reprobing the blots with pRMB2 (which contains the cloned *bvg* locus) would identify IS*50* insertions within this region by the additional 1.5kb fragment size associated with insertions of this element [see later].

The apparent high frequency of IS*50* transposition in *B.pertussis* seen in this study, makes analysis of mutants complex. However, the events described here had not been noted by other workers introducing Tn*5* into this pathogen and have been described as only rare events in other bacteria (Simon, 1989). This suggests that the particular recipient strain used in this study may be the cause of this problem, or that previous studies have mainly ignored IS*50* transposition events. The latter may be the case in the original studies on *B.pertussis* (Weiss *et al.*, 1983), since Tn*5* insertion sites were identified after hybridisation studies using the internal HindIII fragment of Tn*5* as the probe.

4.iv. Complementation of Crb$^-$ Mutants with the Cloned *bvg* Locus

The cloned *bvg* locus, on both the tetracycline resistant cosmid pRMB2 and its deletion derivative pDM1, was introduced into the *B.pertussis* Crb$^-$ transposon mutants isolated during this study and into Weiss's mutant BP347. All the Crb$^-$ mutants could be complemented in *trans* to the Crb$^+$ phenotype on introduction of the cloned *bvg* locus. Additionally, the non-haemolytic Crb$^-$ mutants complemented in *trans* with either pRMB2 or pDM1 regained haemolytic activity. The reversion of the Crb$^-$ transposon mutants to the Crb$^+$ phenotype associated with phase I organisms when complemented in *trans* with *bvg* suggests that all the mutations associated with loss of virulence

characteristics were situated within the *bvg* locus.

Some of the tetracycline resistant transconjugants of the Crb$^-$ mutants did not, however, regain the ability to bind Congo red dye. This instability of the *bvg*-containing clones may have been caused by loss of the insert on repeated subculture, a high spontaneous mutation rate to the *bvg*$^-$ genotype by the previously described mechanism of frameshift mutagenesis (Stibitz *et al.*, 1989), or by alternative mechanisms. However, Lee *et al.* (1990) have recently suggested that the tetracycline resistance gene in pLAFR2 may be poorly expressed in *B. pertussis* strains. These authors suggested that the use of low levels of tetracycline in selective media may allow the growth of plasmid-free segregants. A similar situation could be occuring in this study with the pLAFR1-derived, tetracycline resistant plasmids pRMB2 and pDM1.

Both pRMB2 and its derivative pDM1 complemented in *trans* the Crb$^-$ transposon mutants to Crb$^+$ and Hly$^+$ phenotypes, suggesting that the region deleted during the construction of pDM1 is not involved in the regulation of virulence. It has been proposed that the insert in pRMB2 is composed of two non-contiguous fragments (McGillivray *et al.*, 1989) and these studies support the positioning of the deletion mainly within the non-*bvg* encoding part of the insert.

Mutant 51 regained the Crb$^+$ phenotype after introduction of pRMB2, suggesting that this isolate has one mutation affecting the expression of virulence characteristics (as in the other Crb$^-$ mutants), and another controlling the expression of part of the *cyaA* gene (the haemolysin). The latter could have been caused by an insertion of Tn*5* or IS*50* at the C-terminal end of *cyaA*. An insertion

in this region could have caused the constitutive expression of the genes downstream of the insertion site due to the promoter on the end of IS*50*R (Berg *et al.*, 1980). However, the insertion within the C-terminal part of the gene could have modified the proteins ability to act as an adenylate cyclase.

4. v. Mutations in the *bvg* Locus Causing the Crb⁻ Phenotype

It has been proposed that several of the Crb⁻ mutants isolated during this study have insertions, of either Tn*5* or IS*50*, in the *bvg* locus (based on the sizes of restriction fragments interrupted by these mobile genetic elements - see Table 8). Southern blots of both EcoRI- and BamHI-digested chromosomal DNA from the Crb⁻ mutants, when probed with pRMB2, showed mutants 100 and 101 to have insertions of Tn*5* within the 2.5kb EcoRI fragment of the *bvg* locus. However, the use of pRMB2 as a probe identified no mutants with insertions of IS*50* within the *bvg* locus (as proposed for mutants 97 and 268).

Hybridisation studies with the *bvg* locus revealed that of the remaining eight mutants which possess the Crb⁻ phenotype, two had deletions in or around this region. Mutant 97 has a small (approximately 100bp) deletion within the 2.7kb EcoRI fragment. Similar deletions (of 50 - 500bp) have been identified in phase variants of *B. bronchiseptica* (Monack *et al.*, 1989). The identification of a common event which causes loss of virulence properties in two species of *Bordetella* suggests such a non-reversible event may be of importance to these pathogens. However, the reversible mechanism for phase variation proposed by Stibitz *et al.* (1989), which involves frameshift mutations in the *bvg* locus, would appear of more adaptive

value to a pathogenic bacterium than a non-reversible one. Although both proposed mechanisms for phase variation in *B. pertussis* may occur during the disease process, laboratory studies, on which these mechanisms have been based, have involved certain selective pressures which may have little in common with the *in vivo* situation.

Mutant 104 showed a much larger deletion than that in mutant 97, with the entire *bvg* locus and part of the *fha*B gene excised. A number of additional transposition events have been proposed to have occured in this mutant suggesting that the deletion in mutant 104 may have been less likely to be a spontaneous event, associated with normal mechanisms of phase variation, than that in mutant 97. The mutation may have been caused when DNA positioned between direct repeats of the transposable genetic elements was deleted during a recombination event (Figure 22).

The remaining six mutants (15, 51, 98, 113, 115 and 268) showed no obvious mutations in or around the *bvg* locus which could have caused loss of expression of virulence-associated factors. However, complementation studies have indicated that these isolates are unlikely to have mutations in virulence-regulatory loci distinct from the known *bvg* locus. These mutants may have Tn*5* insertions at loci uninvolved with regulation of virulence and may be Vir$^-$ due to spontaneous frameshift mutations or small deletions within the *bvg* locus which are not apparent on Southern blots.

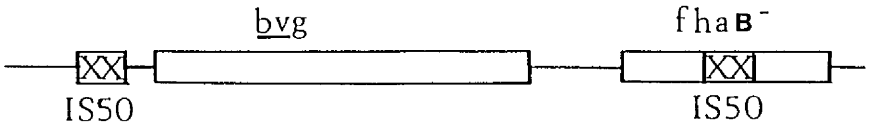
4. vi. Frameshift Mutagenesis of Crb$^-$ Transposon Mutants

The spontaneous mutation of phase I *B. pertussis* to the variant (Vir$^-$) form is a relatively infrequent event. However, the frequency

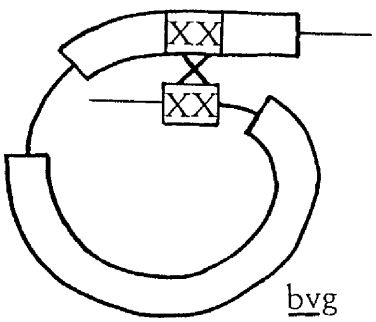
Fig. 22 A possible sequence of events leading to the deletion of the *bvg* locus in Crb$^-$ mutant 104

The multiple transposition events in mutant 104 indicate that the large deleted region in this mutant may have been caused by a recombination event between two closely positioned transposable elements. Two IS*50* elements are shown positioned, one upstream of the *bvg* locus and one within the *fha*B gene (A). Deletion of the central region by recombination (B) would produce a mutant with a *bvg*$^-$ and *fha*$^-$ genotype (C).

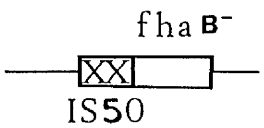
A.



B.



C.



of this variation has been noted to be significantly increased during growth in the presence of a frameshift mutagen, such as ICR 191 (Stibitz *et al.*, 1989). This mutagen has been shown to cause the insertion or deletion of single bases, pairs, or triplets of bases into the genomes of bacteria (Miller, 1972).

After growth for 48 hours in the presence of ICR 191 a culture of *B. pertussis* Wellcome 28 showed a ratio of Crb^-:Crb^+ phenotypic types of 1:3000. Reversion of the Crb^- variants produced in this manner would presumably occur at similar frequencies, as noted in the Tohama strain (Stibitz *et al.*, 1989). However, the spontaneous Crb^- variant used in this study, *B. pertussis* L84 phase I Crb^-, showed no reversion to phase I characteristics after growth in ICR 191. This spontaneous mutant may have arisen by a mechanism unassociated with frameshift mutations (perhaps by a small deletion in the *bvg* locus - see previously).

The Crb^- transposon mutants isolated during this study, which have been identified with transposon insertions (mutants 100 and 101) or deletions (mutants 97 and 104) in the *bvg* locus, were shown (as expected) not to revert to the Crb^+ phenotype after growth in the presence of ICR 191. Mutants 51, 98 and 113, which have no obvious mutations in the known virulence regulatory genes, could also not be induced to revert to the Crb^+ phenotype after growth in liquid cultures containing ICR 191. Hence, these mutants cannot be considered to have arisen by point mutations within the *bvg* locus, and appear to have undergone an, as yet, unidentified mechanism of variation.

Mutant 15, when grown for 48 hours in liquid cultures containing 0.7μg/ml of ICR 191, showed reversion to the Crb^+ phenotype; a ratio

of Crb$^+$:Crb$^-$ phenotypic types of 1:4000 was noted. This suggests that the Wellcome 28 strain, in common with the Tohama strain, undergoes frameshift mutations within the *bvg* locus to produce variants lacking virulence-associated factors. However, mutant 15 was originally isolated on a medium which selects against phase I organisms. Control experiments previously showed that this selective pressure may induce variants, as the spontaneous mutation rate on this medium was high [see Section 3.ii.b.], again indicating that the identification of frameshift mutants of *B.pertussis* under such conditions may have little to do with the *in vivo* situation.

Mutants 115 and 268 were not tested for the ability to revert to the Crb$^+$ phenotype after growth in ICR 191-containing media. Although these mutants were originally isolated without selective pressure, the possibility that they have arisen by frameshift mutation cannot be dismissed.

4.vii. <u>Studies on Mutant 51</u>

Mutant 51 (and the identical isolate 73) was originally identified as a Crb$^-$ transposon insertion mutant from media selective against phase I characteristics. Interestingly, the mutant was unable to haemagglutinate red blood cells and did not produce agglutinogens 2 and 3, heat-labile toxin, pertussis toxin and X-mode specific polypeptides, but retained haemolytic activity. The qualitative adenylate cyclase assay (C.J. Duggleby, personal communication) suggested that the entire cyclolysin was being expressed. However, the more sensitive quantitative cAMP assay (Amersham) showed no cAMP production by mutant 51, indicating that perhaps only a truncated form

of the cyclolysin was being produced in this mutant.

The *cya* operon of *B.pertussis* has been cloned and mapped for sites of restriction enzymes (Glaser *et al.*, 1988a; Brownlie *et al.*, 1988). The known restriction map [see Fig. 2] does not correspond to the simple restriction map constructed around the Tn*5* insertion site in mutant 51. Hence, an insertion of Tn*5*, as the cause of the constitutive haemolytic nature of this mutant, seems unlikely. However, an IS*50* insertion has been identified in an EcoRI fragment of approximately 13kb in the genome of mutant 51 [see Table 8]. A similarly sized fragment (12kb) is present in the *cya* operon, suggesting that the constitutive expression of a truncated form of *cyaA* may have been caused by the insertion of IS*50R* near the start of this gene. The identification of the proposed IS*50* insertion in the *cya* operon by using radiolabelled pRMB11 DNA (a subclone of pRMB1 - see Fig. 2) as a probe proved unsuccessful due to the presence of a repeated DNA sequence in the cloned fragment.

The presence of repeated DNA sequences in the genome of *B.pertussis* provides another alternative explanation for the expression of single virulence-associated factors normally under positive regulatory control. Stern *et al.* (1984) have suggested that repeated DNA sequences may play a role in chromosomal rearrangements, with recombinational events placing intact structural genes under the control of new regulatory regions. However, further studies are required on mutant 51 to identify exactly which genes (complete or truncated) are being transcribed and the event which has lead to the constitutive expression of these genes.

Animal studies on mutant 51 have suggested this strain to be

avirulent in an infant mouse model. Mice were challenged in after ether anaesthesia and weights checked over a three week period. Those challenged with mutant 51 showed growth patterns equivalent to those found in the avirulent control (BP347). Post-infection studies showed that mice challenged with mutant 51 had normal spleen and lung weights, but with slightly increased leukocyte counts.

Additionally, mice immunised with a vaccine made from mutant 51 appeared to be protected after in challenge with *B. pertussis* 18-323, although no protection was derived after ic challenge. These preliminary experiments require confirmation through further studies, but do suggest that the haemolysin could be a protective antigen and as such should be considered as a future component of sub-unit vaccines. *Bacillus anthracis* has an adenylate cyclase with regions of good homology to the *B. pertussis* enzyme (Escuyer *et al.*, 1988). Both enzymes require an attachment factor to facilitate entry into eukaryotic cells, and in *B. anthracis*, the attachment factor is a known protective antigen (PA), which is used in current vaccines.

4. viii. Transposon Mutagenesis of Virulence Regulatory Genes in *B. pertussis*

The mutants which were isolated during this study, after loss of the Congo red-binding phenotype, have been classified with respect to the type of mutation (transposon insertion, point mutation or deletion) shown to have been the cause of this variation (Table 14). Several of the mutants remain with unknown mechanisms having caused the loss of virulence-associated factors. However, no data has indicated that these mutants have arisen by transposon insertions

Table 14 Events causing the Crb$^-$ phenotype in transposon mutants of *B. pertussis* Wellcome 28

Transposon insertions in the known virulence regulatory locus were identified using the *bvg*-encoding plasmid pRMB2 as a probe, as were deletions in this locus. Mutants caused by frameshifts in *bvg* were identified with the frameshift mutagen ICR 191 which caused reversion to the Crb$^+$ phenotype. The mutants with unidentified mechanisms causing the Crb$^-$ phenotype may have been caused by small deletions within the *bvg* locus which are undetectable on Southern blots, or alternative unidentified mechanisms. No common Tn*5* insertion sites were identified in these mutants which makes it appear unlikely that there is an alternative locus controlling virulence in *B. pertussis*.

Crb⁻ Mutants	Mutational Event
15	Frameshift mutation in the *bvg* locus
51 / 73	Unknown
97	100bp deletion in *bvg*C
98 / 99	Unknown
100	Tn*5* insertion in *bvg*C
101	Tn*5* insertion in *bvg*C
104	Deletion of the entire *bvg* locus
113	Unknown
115	Unknown
268	Unknown

within a virulence-regulatory locus independent of *bvg*. Although no other regulatory loci have been identified, a second mechanism for phase variation (associated with the non-reversibility of this event) has been suggested to involve small deletions in the *bvg* locus. The adaptive value of such a mechanism is only suggestive of an alternate, as yet unidentified, environment for *B. pertussis*. Further studies on the *vrg* (*vir* repressed genes) may yet provide information on the site of this alternative environment.

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APPENDICES

Appendix 1 - Media Preparation

a. Stainer and Scholte basal medium with heptakis (2,6-O-dimethyl) β-cyclodextrin (SS(MeβCD))

L-glutamate (monosodium salt)	53.1g
L-proline	1.2g
Sodium chloride (NaCl)	12.5g
Potassium dihydrogen orthophosphate (KH_2PO_4)	2.5g
Potassium chloride (KCl)	1.0g
Magnesium chloride ($MgCl_2.6H_2O$)	0.5g
Calcium chloride ($CaCl_2.2H_2O$)	0.1g
Tris (hydroxymethyl)-aminomethane	30.5g
Heptakis (2,6-O-dimethyl β-cyclodextrin (MeβCD)	5.0g

Media was adjusted to pH7.4 by the addition of concentrated HCl, made up to the final volume with distilled water (5.0 litres or 1.0 litre for 5x concentrate), autoclaved and stored at 4°C.

b. Cyclodextrin solid medium (CSM)

CSM was prepared from SS(MeβCD) by the addition of 15g/l Difco Bacto agar.

c. Casamino acids (CAA)

Casamino acids (Difco) were prepared as a 25% (w/v) solution in distilled water, autoclaved and stored at 4°C (alternatively casein acid hydrolysate (peptone No.5) (Gibco) was used). Casamino acids were added to SS(MeβCD) or CSM to a final concentration of 1%.

d. Supplement

Growth supplements were added to SS(MeβCD) and CSM at 1ml/100ml.

L-cysteine monohydrochloride	400mg
Ferrous sulphate ($FeSO_4.7H_2O$)	100mg
Ascorbic acid	200mg
Nicotinic acid	40mg
Glutathionine (reduced)	1500mg
	100ml in distilled water

Supplement was passed through a disposable Millipore filter (pore size 0.22μm) into sterile universals and stored at 4°C. In media containing the dye Congo red, the concentration of ferrous sulphate was increased 10-fold.

e. Bordet-Gengou agar (BG)

BG agar (Gibco 40g/l) was prepared by the addition of glycerol (10g/l) and distilled water, then autoclaved. Media were allowed to cool to 40-50°C prior to the addition of 15% defibrinated horse blood (InterMed), then poured.

f. Nutrient agar (NA)

NA (Oxoid) was prepared using 28g/l in distilled water.

g. Casamino acid freezing solution

Casamino acids	1000mg
Magnesium chloride ($MgCl_2.6H_2O$)	10mg
Calcium chloride ($CaCl_2.2H_2O$)	1.6mg
Sodium chloride (NaCl)	500mg
Glycerol	20ml
	100ml in distilled water

Solution was adjusted to pH7.1, autoclaved and stored at 4°C.

Appendix 2 - Buffers and Solutions for DNA Techniques

a. Tris - EDTA buffer (TE)

10mM Tris-HCl pH8.0

1mM EDTA

b. Equilibrated phenol/chloroform

Phenol was equilibrated three times with an equal volume of 0.5M Tris-HCl pH8.0, allowing the aqueous layer to separate and be removed. Equilibrated phenol was mixed with an equal volume of chloroform.

c. Brij./DOC solution

1% (w/v) Brij 58

0.4% (w/v) sodium deoxycholate

10mM Tris pH8.0

1mM EDTA

d. Loading buffer

10% (w/v) Ficoll

0.05% (w/v) bromophenol blue

0.005% (w/v) xylene cyanol

0.05% (w/v) orange G

e. 20x SSC

3M sodium chloride

0.3M trisodium citrate pH7.0

f. E buffer (TAE)

40mM Tris-base pH8.2

1mM EDTA

(pH using glacial acetic acid)

g. B buffer (TBE)

90mM Tris-base

90mM boric acid

2.5mM EDTA pH8.2

h. Vacublotting solutions

Depurination solution	0.25M HCl	
Denaturing solution	1.5M NaCl	
	0.5M NaOH	
Neutralising solution	1.0M Tris-HCl	
	2.0M NaCl	pH5.0
Transfer solution	20x SSC	pH7.0

i. Pre-/hybridisation solution

50mM Tris-HCl, pH8.0

1.0M NaCl

50% formamide (Fluka)

10x Denhardt's solution (see below)

1% (w/v) SDS

0.1% tetra sodium pyrophosphate

100µg/ml preboiled salmon sperm DNA

j. 50x Denhardt's solution

0.1% (w/v) bovine serum albumin fraction V

0.1% (w/v) Ficoll

0.1% (w/v) polyvinylpyrrolidone

6x SSC

k. Oligonucleotide labelling buffer (OLB)

Solutions A, B and C were mixed in the ratio 100:250:150 and stored at -20°C.

Solution A: 1ml Solution O (see below)

18µl β-mercaptoethanol

5µl dCTP (100mM in TE)

5µl dTTP (100mM in TE)

5µl dGTP (100mM in TE)

Solution O: 1.25M Tris-HCl

0.125M $MgCl_2$ pH8.0

Solution B: 2M Hepes pH6.6 (using 4M NaOH)

Solution C: Hexadeoxyribonucleotides (Pharmacia) suspended in TE to 90 OD units per ml.

l. STOP buffer

20mM NaCl

20mM Tris pH7.5

2mM EDTA

0.25% SDS

1µM dATP

m. TES buffer

10mM Tris-HCl, pH8.0

1mM EDTA

100mM NaCl

n. Nick-translation buffer (x10)

0.5M Tris-HCl, pH7.2

0.1M $MgSO_4$

1mM dithiothreitol

500µg/ml BSA (fraction V)

Store at -20°C

o. DNase I

A stock solution containing 1mg/ml of DNase I in 0.15M NaCl and 50% (v/v) glycerol was prepared, divided into small aliquots and stored at -20°C.

p. Restriction enzyme buffers (x1)

REact™ 2 - 50mM Tris-HCl, pH8.0
10mM $MgCl_2$
50mM NaCl

REact™ 3 - 50mM Tris-HCl, pH8.0
10mM $MgCl_2$
100mM NaCl

REact™ 10 - 100mM Tris-HCl, pH7.6
10mM $MgCl_2$
150mM NaCl

Medium salt - 10mM Tris-HCl, pH7.5
10mM $MgCl_2$
50mM NaCl
1mM dithiothreitol