DNA REARRANGEMENTS AND VARIATION IN *Bordetella pertussis*.

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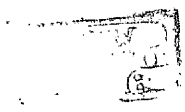

To Honor, Malcolm and Marrianne, for their help, support and guidance throughout my education.

To Mandy for her patience, understanding, humour and love.

That is the essence of science: ask an impertinent question and you are on the way to the pertinent answer.

Jacob Bronowski.

Experience is the name everyone gives to their mistakes.

Oscar Wilde.

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INDEX TO CONTENTS.

LIST OF FIGURES.

LIST OF TABLES.

SUMMARY OF RESEARCH.

Vir$^-$ (avirulent) strains of *B. pertussis* were selected, via increased resistance to erythromycin, which were isogenic with parental Vir$^+$ strains. Chromosomal DNA from these pairs of strains were studied with a number of probes and with methylation-sensitive restriction endonucleases. These experiments suggested that DNA rearrangements were not involved in the Vir$^+$ to Vir$^-$ switch, and that the methylation status of the DNA was not altered during the switch.

A *B. pertussis* gene library was probed with an oligonucleotide specific for the fimbrial subunit genes. This probe revealed that the chromosome of *B. pertussis* strain Taberman contained three fimbrial subunit genes:- i) an active *fim* 3 gene, ii) an inactive *fim* 2 gene, and iii) a gene dissimilar from the previously characterised *fim* X gene. The behaviour of the *fim* 3 gene in various AGG 3$^-$ strains was investigated, and these studies revealed that a *cis*-acting factor was responsible for inactivation of the chromosomal *fim* 3 gene in these strains. It was also shown that the *fim* 3 locus encoded fimbrial structures when conjugated into an afimbrial *B. pertussis* strain. No evidence was found to suggest that DNA rearrangements were involved in serotype variation.

A probe made from the *hin* gene, which controls the invertible region of *Salmonella* typhimurium, was found to hybridise to chromosomal DNA from *B. pertussis*, under reduced stringency conditions.

The gene library was probed with this probe and a cosmid clone isolated. This clone did not appear to produce an active invertase in *E. coli*, when tested in two different inversion assays. An oligonucleotide probe was produced which was homologous with the recombination sites recognised by the *hin*-like family of invertase proteins and this also hybridised to *B. pertussis* chromosomal DNA under reduced stringency conditions.

LIST OF ABBREVIATIONS.

AC = adenylate cyclase enzyme.

ACT = adenylate cyclase toxin.

AGG = agglutinogen.

bp = base pairs.

BG = Bordet Gengou.

BSA = bovine serum albumin.

bvg = Bordetella virulence gene.

CAMR = Centre for Applied Microbiological Research.

cix = *cin* crossover.

DNA = deoxyribonucleic acid.

DTP = diptheria, tetanus, pertussis.

FHA = filamentous haemagglutinin.

gix = *gin* crossover.

hix = *hin* crossover.

HLY = haemolysin.

IS = insertion sequence.

kb = kilobase.

Kd = kilodalton.

LOS = lipooligosaccharide.

LPS = lipopolysaccharide.

McAb = monoclonal antibody.

NA = nutrient agar.

PBS = phosphate-buffered saline.

PcAb = polyclonal antibody.

PHLS = Public Health Laboratory Service.

pix = *pin* crossover.

PT = pertussis toxin.

PTd = toxoided pertussis toxin.

SDS = sodium dodecyl sulphate.

SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis

WHO = World Health Organisation.

1. INTRODUCTION.

1.1. THE GENUS BORDETELLA.

The genus Bordetella is currently made up of four species, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*. All members of the genus cause upper respiratory tract infections in mammalian or avian species.

1.1.1. Bordetella pertussis.

B. pertussis was first isolated by Bordet and Gengou (1906) and subsequently shown to be the causative organism of the disease whooping cough. The bacterium was originally isolated on a medium composed of glycerated potato extract and 50% (v/v) defibrinated blood. This apparent requirement for blood resulted in placement of the pertussis bacilli in the genus Haemophilus. However, the work of Hornibrook (1940) revealed that blood was not essential for growth and this led to placement of the species in the novel genus Bordetella.

B. pertussis is a strictly aerobic Gram-negative, minute coccobacillus. It is chemoorganotrophic, requiring organic nitrogen, in the form of glutamate, organic sulphur, in the form of cysteine, and nicotinamide.

In vivo growth of the pathogen is highly specific and normally only occurs on and among human ciliated respiratory cells. *In vitro* growth is supported by a number of media, some of which are chemically defined; growth conditions and media have been reviewed by Stainer (1988). The organism is fastidious and growth is easily inhibited by numerous factors. Little is known of the metabolism of *B. pertussis* as research

has concentrated on the genetics and molecular mechanisms of virulence.

Several repeated DNA sequences have been isolated from the genome of *B. pertussis*. McPheat and McNally (1987) isolated a sequence present as approximately twenty copies throughout the genome. The sequence of two of these copies revealed that they were related to the insertion sequence (IS) element IS481 isolated by McLafferty *et al.* (1988). The chromosomal location of the two copies was established; IS481v1 is located 3kb upstream of the adenylate cyclase locus, whilst IS481v2 is located immediately adjacent to the C-terminal end of the agglutinogen 2 fimbrial subunit gene (McPheat *et al.*, 1989). Probing of *B. pertussis* chromosomal DNA isolated from strains which varied in expression of AGG 2 (section 1.4.1) and from avirulent phase variants (section 1.5.1) revealed no rearrangements around either of these two insertion sequences. However, DNA rearrangements were observed at other uncharacterized loci using a probe internal to the IS (McPheat *et al.*, 1989). Park *et al.* (1989) have isolated a highly conserved 530bp repeat sequence from the *B. pertussis* genome. The sequence is specific to *B. pertussis* and is thought to be present in approximately 60-80 copies throughout the genome, accounting for almost 1% of the DNA.

Alsheikhly and Löfdahl (1989) have identified a repeat sequence differing from IS481 within the genome of *B. pertussis*. The sequence consists of an inverted repeat sequence surrounding a 200bp central segment. This sequence is also present in the *B. parapertussis* genome. Alsheikhly and Löfdahl

(1989) suggest that this IS-like element exists as an integral entity in just a few locations within the genome, but that the inverted repeat sequence is present in multiple copies.

1.1.2. Other bordetellae.

B. parapertussis was first recognised as a separate species by Eldering and Kendrick (1938) and is believed to cause a similar but less severe disease than *B. pertussis* (Lautrop, 1971).

B. bronchiseptica has been implicated as the primary cause of numerous veterinary diseases such as kennel cough and swine atrophic rhinitis . The biology of *B. bronchiseptica* has been reviewed by Goodnow (1980).

B. avium was proposed as a member of the genus by Kersters *et al.* (1984). *B. avium* is the causative agent of turkey coryza and respiratory disease in other birds. The species had previously been identified as "bronchiseptica-like" bacteria (Hinz *et al.*, 1978).

1.1.3. Evolutionary relationships within the bordetellae.

Several studies have been performed on the genetic relatedness of the species within the genus Bordetella. DNA-DNA reassociation experiments (Kloos *et al.*, 1979; 1981) have revealed a high degree of homology between the chromosomal DNA from *B. pertussis* , *B. parapertussis* and *B. bronchiseptica*. Auxotrophic mutants of *B. pertussis* could be complemented with DNA from both *B. parapertussis* and *B. bronchiseptica* (Kloos *et al.*, 1979). Musser *et al.* (1986) studied the electrophoretic mobilities of 15 metabolic enzymes from strains of all three

species. The results of this study split the genus into three groups: i) all *B. bronchiseptica* strains, ii) all *B. parapertussis* strains and *B. pertussis* strain 18-323, a strain commonly used in vaccine production, and iii) all remaining *B. pertussis* strains.

A major phenotypic difference between *B. pertussis* and other members of the genus is the production of pertussis toxin (PT). PT genes are present in *B. parapertussis* and *B. bronchiseptica* (Locht & Keith, 1986) but are not expressed. Sequencing of these genes revealed DNA homology of over 95% between the PT genes of all 3 species (Aricò and Rappuoli, 1987). The major difference between the sequences consisted of the accumulation of deleterious mutations in the promoter regions of PT genes from *B. parapertussis* and *B. bronchiseptica*. Computer analysis of this sequence information allowed Aricò *et al.* (1987) to propose a phylogenetic tree of the genus Bordetella. This study supported the conclusions of Musser *et al.* (1986) by finding that strain *B. pertussis* 18-323 was more genetically related to *B. parapertussis* and *B. bronchiseptica* than to current clinical isolates of *B. pertussis*. However, Altschul (1989) has re-assessed the sequence data of Aricò and Rappuoli (1987) and proposed an evolutionary tree which places *B. pertussis* 18-323 within the boundaries of other *B. pertussis* strains.

1.2. THE DISEASE.

Pertussis is primarily a paediatric infection, affecting children under the age of twelve. The disease is endemic with epidemics occuring every three to four years (Cherry *et al.*,

1988). The transmission dynamics of the disease have been modelled mathematically with regard to the efficacy of vaccination programmes (Grenfell and Anderson, 1989). This investigation concluded that vaccination of 88% of each birth cohort before the age of 1 year would eliminate bacterial transmission if the protection conferred by the vaccine was lifelong. However, if the vaccine-induced immunity is significantly less than lifelong, repeated cohort vaccination would be required to prevent transmission.

Pertussis is highly contagious, having an attack rate exceeding 90% in unimmunised individuals (Lambert, 1965). The classical disease syndrome has three symptomatic stages: catarrhal, paroxysmal and convalescent.

After initial infection there is an incubation period of 6-20 days (Lapin, 1943) before the disease enters the catarrhal stage. Symptoms during the catarrhal stage are non-specific and resemble a common cold with low fever and slight cough. This stage may last several weeks, during which *B. pertussis* can be recovered from the patient. Severe violent coughing fits begin as the disease enters the paroxysmal stage. The violent coughing serves to clear the airways of accumulated mucus and cell debris. Immediately after a coughing bout air is inspired past the swollen glottis and causes the characteristic whoop. Antibiotic therapy at this stage has little or no effect.

The paroxysmal stage usually lasts between 1-4 weeks then symptoms slowly begin to decrease as the patient enters the convalescent phase.

Many of the complications associated with the disease are the result of the force involved in the coughing fits. More serious sequelae such as seizures, encephalopathy, secondary bacterial infections and death, usually from the secondary infections, can occur after the paroxysmal stage.

The severity, duration and likelihood of complications decreases with the age of the patient and although outbreaks in adults are uncommon they can occur (Linneman and Nasenbury, 1977).

1.3. PERTUSSIS VACCINES.

1.3.1. Whole-cell vaccines.

The majority of pertussis vaccines in current use are suspensions of whole killed cells which have been detoxified by heat or chemical methods and adsorbed onto a carrier such as aluminium hydroxide. Detoxification does not remove any lipopolysaccharide endotoxin from the cells, and this component may be linked to reactions against the vaccine (Griffith, 1978; Cameron, 1979). Pertussis vaccine is most commonly administered in combination with diphtheria and tetanus toxoids in the DTP vaccine.

The development and efficacy of whole-cell pertussis vaccines has been reviewed by Griffiths (1988). These vaccines are biologically complex, and their exact composition is rarely known. A number of methods have been used to standardise the potency of pertussis vaccines; these include, a) the use of standard opacity units in the cell suspensions and b) the intracerebral mouse protection test (Kendrick *et al.* 1947).

The methods involved in the control testing of pertussis vaccines and their components are reviewed by Cameron (1988).

Despite the known efficacy of these vaccines, public acceptance of them has dropped since the mid 1970's following the alleged association between pertussis vaccines and rare, but serious, side-effects, the most severe of these being encephalopathy. However, in a recent High Court case the association between whole-cell pertussis vaccines and brain damage could not be proven. The case is reviewed by Dyer (1988) and Griffith (1989). Despite this verdict, acceptance of the vaccine is still low and this has led to research being concentrated on the production of defined component vaccines with high immunogenicity and minimal toxicity.

1.3.2. Acellular vaccines.

Several reasons exist for the development of effective defined component acellular pertussis vaccines. These include the need to increase acceptance of pertussis vaccine and the need for greater protective efficacy (Robinson and Ashworth, 1988).

Acellular pertussis vaccines have been used in Japan since 1981. They consist mainly of toxoided PT (PTd) and filamentous haemagglutinin (FHA), whilst some contain small amounts of other components such as fimbrial agglutinogens (Sato *et al.*, 1984). This vaccine is protective in children (Biritwum *et al.*, 1985; Isomura *et al.*, 1985) and less reactive than whole-cell vaccines (Kimura and Hikino, 1985).

Two acellular vaccines were field-tested in Sweden recently. The vaccines consisted of PTd, either alone or in combination with FHA. The results of the field test (Ad Hoc Group for the Study of Pertussis Vaccines, 1988) indicated that the acellular vaccines had somewhat lower protective efficacy than that reported for established whole-cell vaccines. This finding, and the deaths of three children in the vaccinated group, led to withdrawal of the application for licensing of the vaccine for use in Sweden.

An acellular vaccine composed of equal amounts of PTd, FHA and fimbrial agglutinogens has been developed (Robinson *et al.*, 1986). This vaccine is protective in mice challenged intranasally with *B. pertussis* and may be included in forthcoming field trials of new pertussis vaccines in the UK.

The next generation of acellular vaccines will probably contain either immunogenic peptide fragments or 'genetically toxoided' virulence factors (Robinson and Ashworth, 1988). The catalytic amino acids responsible for the toxic action of several virulence factors such as PT and adenylate cyclase (AC) have been identified (Burnette *et al.* 1988; Au *et al.* 1989; Glaser *et al.*, 1989). Site-directed mutagenesis of the genes encoding these factors may allow the expression of non-functional yet immunogenic toxins. These altered toxins will hopefully retain their protectivity but will have reduced reactogenicity.

1.4. VIRULENCE FACTORS OF *B. PERTUSSIS*

Virulence factors can be defined as 'any bacterial component that promotes pathogenicity by enabling the pathogen to infect the host and cause disease.' (Wardlaw, 1988).

The rational design of a defined acellular pertussis vaccine requires an understanding of the virulence factors responsible for causing disease and the components which elicit a protective immune response.

During typical pertussis infection, *B. pertussis* often cannot be isolated from the patient after the onset of the paroxysmal stage. This, and the failure of antibiotic treatment during the later stages of the disease, led researchers to believe that pertussis was a toxin-mediated disease. It was originally thought that the symptoms of pertussis were mediated by pertussis toxin (PT) alone, with the aid of several adhesins (Pittman, 1979). However, subsequent studies have revealed that a large number of virulence-associated factors appear to play a role in *B. pertussis* infection.

Many *B. pertussis* virulence-associated factors have not yet been assigned definite roles in the disease process. The task of assessing the contribution of individual factors to the disease has been greatly aided by the construction of a number of *B. pertussis* strains containing single Tn5 insertions within genes encoding various virulence-associated factors (Weiss *et al.*, 1983). The properties of a mutant strain lacking a given factor were then compared in an infant mouse model to those of the wild-type strain.

The following is a list of virulence-associated factors implicated in the disease process:-

1.4.1. Agglutinogens.

Agglutinogens (AGGs) are defined as surface antigens able to elicit cell-agglutinating antibodies (agglutinins). The pattern of AGGs produced by a *B. pertussis* strain is referred to as the serotype of that strain.

In the most widely used serotyping system, developed by Eldering *et al.* (1957), *B. pertussis* strains produce eight AGGs: numbered 1 to 7 and 13. AGGs 1 to 6 are specific for *B. pertussis*, AGG 7 is present in both *B. parapertussis* and *B. bronchiseptica*, whilst AGG 13 is shared with *B. bronchiseptica*. *B. avium* shares several heat-labile AGGs with *B. bronchiseptica* (Kersters *et al.*, 1984).

The AGGs of *B. pertussis* have been divided into two classes : AGGs 1, 2 and 3 are known as major AGGs , whilst AGGs 4, 5 and 6 are the minor AGGs (Preston *et al.*, 1982). Purified major AGGs are protective in mice (Zhang *et al.*, 1985; Robinson *et al.*, 1985a). Robinson *et al.* (1989) have found that protection afforded by purified fimbrial AGGs is serospecific. Since the protection afforded by whole-cell pertussis vaccines is correlated with agglutinin production in children and mice (MRC, 1959) the WHO have recommended that whole-cell pertussis vaccines include all three major AGGs (Robinson *et al.*, 1985b).

AGG 1 is typically expressed in virulent-phase *B. pertussis* strains. The expression of the other specific AGGs is subject to serotype variation (Cameron, 1967).

The biochemical nature of several *B. pertussis* AGGs has recently been defined.

1.4.1.1 Agglutinogen 1.

Li *et al.* (1988) have presented evidence that AGG 1 may correspond to the lipooligosaccharide A (LOS A) of *B. pertussis*. However, discrepancies in their data prevent them from definitely associating LOS A with AGG 1.

1.4.1.2. Fimbrial agglutinogens.

Ashworth *et al.* (1982) observed that specific antibodies to AGG 2 bound to the fimbriae of *B. pertussis*. This work was confirmed by the same group (Ashworth *et al.*, 1985) using gold-labelled monoclonal antibodies. However, there has been considerable debate over the nature of AGG-3. Different groups with different antisera have obtained apparently contradictory results. Carter & Preston (1984) used mono-specific antisera to type several strains of *B. pertussis*. Strains positive for AGG 3 in this scheme were found to be afimbriate by electron microscopy. However, Ashworth *et al.* (1985) found that their gold-labelled monoclonal antibody to AGG 3 bound specifically to fimbrial structures on the surface of *B. pertussis* strains. Fredriksen *et al.* (1988) also used gold-labelled antibodies to AGG 3 in electron microscopy studies of *B. pertussis* fimbriae. Anomalous results led them to conclude 'that the term agglutinogen 3 covers more than one structure; or, possibly, that not all agglutinogen 3 is assembled into fimbria-like structures.'

The fimbriae of *B. pertussis* are classed as 'very thin' fimbriae (Paranchych and Frost, 1988), with a diameter of 3-4 nm (Blom *et al.*, 1983). The length of the fimbriae has not been determined accurately. Purified fimbriae aggregate at a characteristic pH; pH 6.0 for AGG 2 fimbriae (Zhang *et al.*, 1985) and pH 7.4 for AGG 3(6) fimbriae (Cowell *et al.*, 1987). Both fimbrial AGGs are composed of subunit proteins arranged in a helical structure. The antigenic heterogeneity between the two fimbrial types is caused by differences in the amino acid composition of the subunits. The molecular weights of the two distinct subunits have been found to be 22 500 for the AGG 2 subunit and 22 000 for the AGG 3 subunit (Irons *et al.*, 1984).

Mooi *et al.* (1987) studied the amino acid sequences of the fimbrial subunit proteins from *B. pertussis* and found that the AGG 2 and 3 subunits are very closely related. In a similar comparative study Cowell *et al.* (1987) reported amino acid sequences for the AGG 2 subunit and the subunit protein of fimbriae they define as AGG 6 which were almost identical to those reported by Mooi *et al.* (1987) for the AGG 2 and 3 subunits respectively. The amino-terminal amino acid sequence of these subunits shares 50% homology with that of *E. coli* K99 fimbrial subunits (Mooi *et al.*, 1987).

The gene encoding the AGG 2 subunit, *fim* 2, has been cloned and sequenced by Livey *et al.* (1987) and a putative AGG 3 subunit gene, *fim* 3, has been isolated (Mooi *et al.*, 1987) and sequenced (Mooi *et al.*, 1990). Pedroni *et al.* (1988) have characterised a gene distinct from those above. The sequence of this gene, *fim* X, has revealed an open reading frame

corresponding to a fimbrial subunit protein of molecular weight 20 000 differing from the AGG 2 and 3 subunits. It is possible that this gene is a silent copy analogous to the silent copies of pilin genes found in the *Neisseria gonorhoeae* chromosome (Meyer *et al.* 1984). The three different fimbrial subunit genes do not appear to be linked on the chromosome (Pedroni *et al.*, 1988).

Despite the homology between *B. pertussis* fimbriae and the fimbriae of other bacterial pathogens there is little evidence to support the role of fimbriae in the attachment of *B. pertussis* to human ciliated respiratory cells (Tuomanen and Weiss, 1985). Current evidence only implicates fimbriae in adhesion to non-ciliated tissue culture cells (Gorringe *et al.*, 1985; Redhead, 1985).

1.4.1.3. Other agglutinogens.

Little research has been carried out on the 'minor' AGGs of *B. pertussis*. Preston *et al.* (1982) linked AGGs 4 and 5 with AGG 2, deciding that AGG 4 was probably a minor component of AGG 2 and unlikely to be a separate immunogen. This group could not differentiate between AGGs 3 and 6.

A 69 Kd protein has been detected in *B. pertussis* which reacts with the U.S. Reference AGG 3 antisera (Brennan *et al.*, 1988). This protein has not been characterised, but is known to be non-fimbrial. Novotny *et al.* (1985) have also found antigenically-related proteins in both *B. parapertussis* and *B. bronchiseptica*.

1.4.2. Pertussis toxin.

PT has many diverse patho-physiological effects in man and experimental animals. These include histamine sensitization, lymphocytosis promotion, haemagglutination and the activation of pancreatic islets. These effects are reviewed by Ui (1988), Furman *et al.* (1988) and Munoz (1988).

Purified PT is capable of conferring protection to mice against both intranasal and intracerebral challenge with *B. pertussis* (Munoz *et al.*, 1981). This protection is the major reason for the inclusion of detoxified PT in acellular vaccines.

PT is a hexameric protein composed of five different sub-units. The molecule can be divided into two functional moieties (Tamura *et al.*, 1982). The first, comprised of the S1, or A, subunit, is an enzymatic moiety which causes ADP-ribosylation of several host cell proteins. The second, or B subunit, comprised of subunits S2, S3, S4 and S5, is responsible for binding the toxin to target cells and facilitating the entry of the A subunit. The B subunit is also a T-cell mitogen and enhances glucose oxidation in adipocytes. A review of the structure and activity of PT has been written by Burns (1988).

The S1 subunit has a molecular weight of 26kD (Locht and Keith, 1986) and alone is unable to enter cells (Tamura *et al.*, 1982). It acts by ADP-ribosylating several membrane-bound regulatory G proteins. Following ADP-ribosylation the G proteins no longer exert control over intra-cellular processes such as cAMP metabolism, thus PT disrupts normal cell function.

The G proteins affected by PT are different from the G protein (G_s) attacked by cholera toxin.

Cieplak *et al.* (1988) identified the active site of the S1 subunit and showed that it formed part of the protective antigenic determinant. Subsequently the amino acid residues responsible for ADP-ribosylation activity have been identified (Burnette *et al.*, 1988). This information has allowed the construction of a 'genetically toxoided' PT molecule which is fully protective yet possesses no enzymatic activity (Nencioni, *et al.*, 1990).

The genes encoding the various subunits of PT have been cloned and sequenced (Locht and Keith, 1986; Nicosia *et al.*, 1986). The genes are arranged in an operon under the control of a single positively-regulated promoter. Transcriptionally silent copies of the PT operon are present in the genomes of *B. parapertussis* and *B. bronchiseptica* (Aricò and Rappuoli, 1987). Cloned PT genes from *B. pertussis* are expressed in both of these species (Lee *et al.*, 1989) but PT is not secreted, suggesting that the PT transport mechanisms of the species are either different or non-functional.

1.4.3. Filamentous haemagglutinin.

PT and filamentous haemagglutinin (FHA) are responsible for the ability of *B. pertussis* to agglutinate erythrocytes from several species. FHA is 5 - 7 times more active as a haemagglutinin than PT but is sensitive to inhibition by micromolar concentrations of cholesterol (Sato *et al.*, 1983). FHA consists of fine filaments approximately 2nm in diameter and 40 - 100nm in length (Arai and Sato, 1976). The filaments

are composed of highly hydrophobic 220Kd subunits (Sato *et al.*, 1983). The molecule has been purified by a number of methods and confers immunity against aerosol challenge in mice (Oda *et al.*, 1984). A Tn5 mutant of *B. pertussis* deficient in FHA (Weiss *et al.*, 1983) was no less virulent in intranasal challenge of mice than the wild-type parental strain. However, this strain did adhere less well to rabbit ciliated cells than the wild-type (Tuomanen *et al.*, 1985). The addition of exogenous purified FHA to this strain allowed the recovery of full adherence (Tuomanen and Weiss, 1985). FHA and PT are thought to act in concert as adhesins for human ciliated epithelial cells. Both molecules are secreted and will bind to other bacteria; these bacteria are then able to adhere to human ciliated cells (Tuomanen, 1986). This 'piracy' of both FHA and PT is discussed by Tuomanen (1988).

The FHA gene has been cloned by a number of groups, and immunogenic FHA has been expressed in *E. coli* in both its native form (Brown and Parker, 1987) and as a fusion protein (Reiser *et al.*, 1985). Nucleotide sequencing of the locus encoding FHA (Stibitz *et al.*, 1988; Relman *et al.*, 1989) has revealed that the FHA structural gene, *fha B*, is closely linked to the *bvg* locus (see section 1.4.3.). This analysis also revealed two other genes, *fha* C and *fha* A, thought to be involved in the export of FHA to the cell surface.

1.4.4. Dermonecrotising toxin.

First discovered by Bordet and Gengou (1909), dermonecrotising, or heat-labile, toxin causes necrotic lesions in mice when injected subcutaneously at low doses, and is

lethal if the dose is increased (Livey and Wardlaw, 1984). Despite the unstable nature of this toxin several purification schemes exist (Nakase and Endoh, 1985; Endoh *et al.*, 1986). The purified protein has an apparent molecular weight of 102 000, composed of subunits weighing 20Kd and 30Kd (Endoh *et al.*, 1986). Purified toxin causes vasoconstriction in peripheral murine blood vessels followed by local ischaemia and petechial haemorrhage.

The molecular mechanism of toxin action is not known but both dermonecrotising and lethal effects are lessened if the experimental animal is treated with prednisolone (Parton, 1985).

1.4.5. Adenylate cyclase toxin.

B. pertussis produces an adenylate cyclase toxin with unique properties which is secreted into the culture medium during exponential growth (Hewlett *et al.*, 1976). The activity of this ACT is increased a thousand-fold in the presence of the eukaryotic calcium-binding protein calmodulin (Wolff *et al.*, 1980). The properties, purification schemes and possible functions of ACT have been reviewed (Hanski, 1989; Hewlett and Gordon, 1988; Masure *et al.*, 1987).

B. pertussis adenylate cyclase has been identified in two forms; one, the adenylate cyclase enzyme (AC), has the ability to convert ATP into cAMP in solution, whilst the other, adenylate cyclase toxin (ACT), is able to enter eukaryotic cells and increase cAMP levels within them. The molecular weights determined for AC range from 50 000 (Ladant *et al.*, 1986) to 70 000 (Hewlett and Wolff, 1976). The molecular weight

of ACT was determined to be ≃200 000 (Hanski and Farfel, 1985). The 200Kd protein alone is sufficient for penetration into target cells and subsequent intoxication (Hewlett *et al.*, 1988b; Rogel *et al.* 1988). Rogel *et al.* (1988) concluded that the 50Kd non-toxic form was probably formed by proteolytic cleavage of the 200Kd protein. Bellalou *et al.* (1989) have produced evidence that suggests production of the 50Kd form may not occur under physiological conditions.

B. pertussis ACT acts by penetrating the plasma membrane of a target cell (Farfel *et al.*, 1987) and rapidly increasing the intracellular cAMP concentrations. ACT affects a number of cell types including the phagocytic cells of the immune system, whose functions are severely inhibited.

The ACT locus has been cloned (Glaser *et al.*, 1988; Brownlie *et al.*, 1988) and sequenced (Glaser *et al.*, 1988). The sequence data of Glaser *et al.* (1988) revealed four genes *cya* A, -B, -D and -E. The *cya* A gene encodes the 200Kd ACT protein, whilst *cya* B and -D are homologous to the *E. coli hly* B and D genes necessary for haemolysin secretion. This group also found that the carboxy terminal 1300 amino acids encoded by *cya* A are highly homologous to the haemolysin of *E. coli*, suggesting that ACT may be responsible for the haemolytic activity of *B. pertussis*. Brownlie *et al.* (1988) cloned a similar locus and were able to express AC activity in *E. coli*. This group also detected homologous genes in *B. parapertussis* and *B. bronchiseptica* but not in *B. avium*.

A transposon mutant of *B. pertussis* which does not produce ACT was found to be avirulent in mice (Weiss *et al.*, 1984).

Virulence was restored by cloned ACT (Brownlie *et al.*, 1988). Although essential for disease, ACT may not be necessary for colonisation and persistent infection of the host (Brownlie *et al.*, 1988).

Both polyclonal and monoclonal antibodies raised against purified ACT are protective in mice (Brézin *et al.*, 1987; Guiso *et al.*, 1989). A transposon mutant deficient in all virulence factors except adenylate cyclase activity was found to partially protect mice against in-challenge by a fully virulent strain (M.J. Ward, personal communication). The sites and amino acid residues responsible for catalysis (Au *et al.*, 1989; Glaser *et al.*, 1989) and calmodulin binding (Glaser *et al.*, 1989) have been identified. As with PT this information should allow the construction of an enzymatically nontoxic immunogenic ACT molecule.

1.4.6. Tracheal cytotoxin.

The major cytopathological symptom of *B. pertussis* infection is the damage to and subsequent extrusion of ciliated cells from the respiratory epithelium, leading to congestion of the airways with mucus and bacteria. A toxin which may be responsible for this activity was isolated by Goldman *et al.* (1982) and was found to be a low molecular weight (900) glycopeptide, tracheal cytotoxin (TCT). TCT can be purified from log-phase culture supernates of virulent and avirulent strains of *B. pertussis*. TCT has been chemically analysed and is thought to come from the enzymatic degradation of the cell wall component peptidoglycan (Cookson *et al.*, 1989).

The other three members of the genus Bordetella produce identical toxins. *N. gonorrhoeae* also produces a chemically similar toxin which damages ciliated cells (Melly *et al.*, 1984). Goldman (1988) speculates that these toxins 'may in fact represent (one particular example of) a virulence mechanism common among mucosal pathogens'.

1.4.7. Lipopolysaccharide endotoxin.

Lipopolysaccharide (LPS) endotoxins are components of Gram-negative bacterial cell walls which cause toxicity and pyrogenicity. *B. pertussis* produces two chemically and immunologically distinct LPSs (LeDur *et al.*, 1980; Peppler, 1984). Both molecules lack the long chain polysaccharide O antigen characteristic of the LPS of enteric organisms. The *B. pertussis* endotoxin has thus been designated a lipooligosachharide (LOS). Despite this difference, the LOS endotoxin is in many respects similar to the LPS endotoxins of enteric bacteria (Ayme *et al.*, 1980). The chemical structure, isolation and properties of the *B. pertussis* LPS are reviewed by Chaby and Caroff (1988). Purified LPS is pyrogenic in mice and is able to activate mononuclear phagocytes (Haeffner-Cavaillon *et al.*, 1984). An unusual property of *B. pertussis* LPS is the ability to induce non-specific resistance to viral infections in mice (Winters *et al.*, 1985).

Li *et al.* (1988) have presented evidence suggesting that LOS A may correspond to AGG-1.

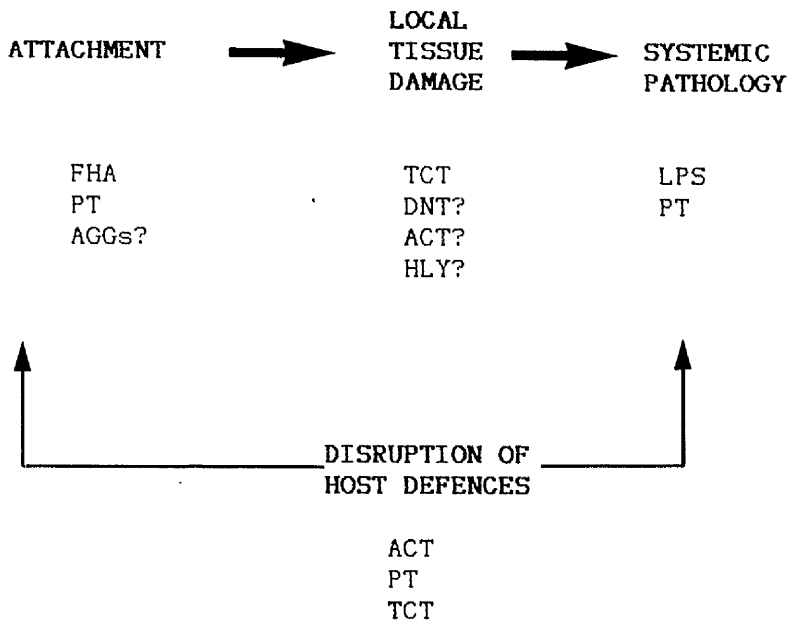
1.4.8. Virulence factors and disease.

Hewlett and Weiss (1986) have proposed a simple three-stage model for *B. pertussis* infection. The model commences with attachment of the bacteria to the ciliated cells of the respiratory tract. This is followed by local tissue damage and, subsequently, systemic pathology. Accompanying these stages are processes carried out by the infecting bacteria to disrupt the host immune defences.

One of the major difficulties encountered in the study of pertussis is the lack of a suitable animal model. The disease is specific for humans and no animal model is capable of mimicking the entire disease process. The animal models used in pertussis research, and their limitations, are reviewed by Sato and Sato, 1988.

Information collected from several lines of research allowed Hewlett and Weiss (1986) to suggest that the virulence factors of *B. pertussis* can be fitted into this three stage sequence, as illustrated in Figure 1. Hewlett and Weiss concede that this model is just a 'rough picture' of the disease process, but, hopefully, one which will 'represent a beginning in the understanding of this disease and how best to design approaches to its control and prevention.'

FIGURE 1. A POSSIBLE SEQUENCE FOR ESTABLISHMENT OF PERTUSSIS AND THE INVOLVEMENT OF VARIOUS VIRULENCE FACTORS IN THE DISEASE PROCESS.


ATTACHMENT
LOCAL TISSUE DAMAGE
SYSTEMIC PATHOLOGY
FHA
PT
AGGs?
TCT
DNT?
ACT?
HLY?
LPS
PT
DISRUPTION OF HOST DEFENCES
ACT
PT
TCT

1.5. ANTIGENIC VARIATION OF *BORDETELLA PERTUSSIS*.

One aspect of *B. pertussis* infection not considered in the sequence illustrated in Figure 1 is the variation in the antigenic nature of the bacteria which may occur during infection. The three types of variation described below have all been observed *in vitro* and are thought to occur *in vivo*.

1.5.1. Phase variation.

Leslie and Gardner (1931) observed that, after repeated subculture, isolates of *B. pertussis* suffered a loss of toxicity for guinea pigs. They called this process phase variation and classified four antigenically-distinct phases ranging from fully toxic, phase I, to fully non-toxic and able to grow on nutrient agar, phase IV. Phase IV strains also possess increased resistance to certain antibiotics (Bannatyne and Cheung, 1984) and to levels of fatty acids in the growth media (Peppler and Schrumpf, 1984). Phase IV strains have been isolated from patients in the later stages of disease (Kasuga *et al.*, 1954). Classification has subsequently become confused by the loss of the original antisera and by the existence of a substantial body of apparently contradictory work concerning the process of phase variation.

Weiss and Falkow (1984) proposed a new classification system for phase variants. Isolates which possessed all virulence factors were labelled virulent (Vir^+) strains whilst those which had lost the involved virulence factors were labelled avirulent (Vir^-) strains. Avirulent strains can be easily identified on a number of solid media by their inability to take up dyes such as Congo red (Parton, 1988). The DNA of

avirulent strains may also differ in the amount of methylation from that of the parent virulent strain (Goldman *et al.*, 1987).

The loss of toxicity during phase variation is caused by the loss of several virulence factors including PT, ACT, FHA, dermonecrotising toxin and the fimbrial AGGs. This loss is generally thought to be irreversible, although Vir^- to Vir^+ revertants have been reported at low frequencies (Weiss and Falkow, 1984; Stibitz *et al.*, 1989).

Weiss and Falkow (1984) observed that a single Tn5 insertion into the *B. pertussis* chromosome was sufficient to create the avirulent phenotype. This led them to propose that a single locus, the *vir* locus, produces a *trans*-acting factor which positively regulates the expression of genes encoding the virulence factors. This *vir* locus has been cloned by several groups (Brown and Parker, 1987; Brownlie *et al.*, 1988; Stibitz *et al.*, 1988; McGillivray *et al.*, 1989), and fully sequenced (Stibitz *et al.*, 1989; Aricò *et al.*, 1989).

1.5.2. Antigenic modulation.

The virulence factors lost during phase variation can also be lost through antigenic modulation. Unlike phase variation the loss of expression is more readily reversible and is dependent upon the presence of modulators in the growth media. Lacey (1960) noticed a loss of antigen expression when growing *B. pertussis* under a wide range of conditions including high concentrations of $MgSO_4$. Subsequently, other workers have confirmed and extended these observations on antigenic modulation, e.g. using nicotinic acid (Pusztai and Joó, 1967), quinaldic acid and other pyridines (Schneider and Parker,

1982), Na_2SO_4, sodium lactate and sodium succinate (Brownlie *et al.*, 1985).

Cells expressing virulence factors are known as X-mode cells; cells which do not express virulence factors due to growth conditions are referred to as C-mode cells (Lacey, 1960). C-mode cells are more hydrophilic than virulent X-mode cells (Robinson *et al.*, 1983) and adhere less well to mouse lung cells (Burns and Freer, 1982) or unciliated human tissue culture cells (Gorringe *et al.*, 1985; Redhead, 1985). The mechanism of antigenic modulation was investigated by Knapp and Mekalanos (1988) using the Tn5 derivative TnphoA (Manoil and Beckwith, 1985). This transposon is designed to allow the detection of genes producing proteins exported to the cell surface. They described a locus, *mod*, which is thought to encode a *trans*-acting factor required for the expression of the *vir* locus or for activity of a *vir* gene product. The *mod* gene is closely linked to the *vir* locus defined by Weiss and Falkow (1984). A constitutive mutant in the *mod* gene was found which produced virulence factors in the presence of known modulators.

1.5.3. A mechanism controlling both phase variation and antigenic modulation.

The sequence of the *vir* locus revealed three open reading frames. These open reading frames were designated *bvg*A, *bvg*B and *bvg*C and were calculated to encode proteins of 23kD, 30kD and 102kD, respectively (Aricò *et al.*, 1989). Homology exists within these genes to previously characterised transmitter and receiver domains within prokaryotic signal transducing proteins (Kofoid and Parkinson, 1988). Aricò *et al.* (1989) proposed a

model for both antigenic modulation and phase variation using this data. The gene product of *bvgC*, BvgC, is a membrane spanning protein and in the absence of modulators this protein activates BvgA (the *bvgA* gene product) which then acts as the transcriptional activator (Roy *et al.*, 1989). In the presence of modulators, detected by either the periplasmic portion of BvgC or the smaller, totally periplasmic BvgB, the activation of BvgA does not occur and transcription of *vir*-dependent genes ceases. Phase variation in this model occurs by the insertion of an extra cytosine residue in a string of cytosine residues (Stibitz *et al.*, 1989) within *bvgC* (Aricò *et al.*, 1989).

Calderwood *et al.* (1988) used TnphoA to detect a number of genes which are positively regulated by the *vir* locus; and several genes which are repressed by the same mechanism.

The environmentally-dependent, coordinate regulation of virulence factors has been observed in a range of bacterial species. The mechanisms behind these phenomena have been reviewed (Miller *et al.*, 1989).

1.5.4. Possible roles for phase variation and antigenic modulation.

Evidence exists to suggest that phase variation and antigenic modulation occur *in vivo* (Kasuga *et al.*, 1954; Lacey, 1960). Suggestions for the role of these phenomena during the disease and transmission of the bacteria are purely speculative. Wardlaw and Parton (1988) suggest that children or adults colonised by avirulent *B. pertussis* may serve as carriers in a 'pertussis cycle'. Robinson *et al.* (1986b) observed that C-mode cells adhere less well to mammalian cells

suggesting that antigenic modulation may aid transmission during later stages of the disease.

1.5.5. Serotype variation.

Pure cultures of *B. pertussis* can display heterogeneity with regards to serotype (Cameron, 1967). Variants of a single clone of *B. pertussis* can independently gain or lose fimbrial AGGs 2 and 3 (Stanbridge and Preston, 1974a). This variation can occur in experimental animal infections (Stanbridge and Preston, 1974b; Preston *et al.*, 1980) and in the infected child (Preston and Stanbridge, 1972). The frequency of serotype variation, the loss or gain of AGG 2 or 3, has been investigated only *in vitro*. Stanbridge and Preston (1974a) estimated the frequency to be between 10^{-3} to 10^{-4}, a range of frequencies similar to those quoted for flagellar variation in *Salmonella typhimurium* (Stocker, 1949).

The role of serotype variation in the disease process is not known, but it is believed that the change in fimbrial antigens may allow the bacteria to evade the host immune response. Robinson *et al.* (1989) have demonstrated that immunisation of mice with a purified fimbrial AGG exerts a selective pressure against organisms expressing the homologous AGG. *B. pertussis* recovered from the lungs of mice immunised with AGG 3 were shown to have altered from the serotype 1.2.3 and 1.0.3. of the infecting strains to predominantly 1.2.0. or 1.0.0., respectively. A similar result was observed in mice immunized with purified AGG 2 and challenged intranasally with strains bearing AGG 2. Robinson *et al.* (1989) were unable to determine whether this specific protection represents genuine

serotype conversion or the selection of a small number of organisms with the appropriate serotype present in the infecting inoculum.

The genes encoding the two fimbrial subunits have been isolated (Livey *et al.*, 1987, Mooi *et al.*, 1987) as has a third, homologous gene (Pedroni *et al.*, 1988). These genes are unlinked on the chromosome. There are no published reports on the possible mechanism(s) of serotype variation.

1.6. MECHANISMS OF ANTIGENIC VARIATION.

Antigenic variation can be defined as 'the ability of a single strain to express several antigenic variants of a cellular component with the rate of change of the component being significantly higher than the mutation rate' (Seifert and So, 1988). The primary purpose of antigenic variation appears to be an avoidance of host immune response and it is one of a number of common themes in microbial pathogenicity (Finlay and Falkow, 1989).

In many of these genetic mechanisms, rearrangement of genomic DNA is responsible for the variation in the expressed characters (see Starlinger, 1977; Silverman and Simon, 1983; Plasterk and Van de Putte, 1984; Saunders, 1986 for reviews).

Several distinct types of DNA rearrangements have been observed which include invertible DNA sequences, the shuffling of coding 'minicassettes' and the deletion of specific sequences.

1.6.1. Invertible DNA.

In mechanisms of antigenic variation involving invertible DNA, the orientation of a specific segment of DNA governs the expression of the genes encoding the factors which are varied.

Several of these invertible DNA mechanisms are very closely related. The *hin* system of *Salmonella typhimurium*, involved in flagellar phase variation, the *gin* system of bacteriophage Mu and the *cin* system of bacteriophage P1, both involved in determining the host range of the phages, and the *pin* system of *E. coli*, which has no known function, form a single family of

invertible DNA systems known as the *din* (for ***D**NA **in**version*) systems. All of these mechanisms consist of a recombinase, two inverted recombination sites and a recombination enhancer sequence. The recombinases, and the sequences they interact with, are highly conserved and mutations in one system can be complemented by components from the others. The DNA enzymology of these systems would also appear to be conserved. The invertible DNA systems involved with fimbrial phase variation in *E. coli* and pilin variation in *Moraxella bovis* are not related to the *hin*-like systems.

1.6.1.1. The *hin* system of *Salmonella typhimurium*.

Variation between two serospecific flagellar filaments of *S. typhimurium* was first observed by Andrewes (1923), and shown to occur at the rate of 10^{-3} - 10^{-4} per division (Stocker, 1949). The structure of each flagellar antigen is specified by separate, unlinked genes, H1 and H2.

Initial studies by Lederberg and Iino (1956) revealed that the element responsible for the variation was a DNA structure closely linked to the H2 gene. Further study of this area demonstrated the basic mechanism of flagellar phase variation in *S. typhimurium* (Zeig *et al.*, 1977; Silverman *et al.*, 1979a; Simon *et al.*, 1980). The element responsible for phase determination was found to be a discrete 970bp DNA segment bounded by two inverted 26bp imperfect palindromic repeats, *hix* L and *hix* R. The DNA segment also contains two further loci, the *hin* gene, which encodes a DNA invertase, and a promoter region.

When the DNA segment is in the "ON" orientation the promoter is proximal to the H2 gene and allows transcription. Under the influence of the DNA invertase produced by the *hin* gene, a site-specific recombination event occurs between the two inverted repeats causing inversion of the DNA segment. In this "OFF" orientation the promoter is removed from the H2 gene and transcription cannot occur. A second gene has been characterised which is co-transcribed with the H2 gene. This gene, *rh*1, encodes a repressor protein of the H1 gene (Silverman *et al.*, 1979b). Thus a single recombination event can halt the synthesis of H2 flagella and lift repression of H1 flagellar synthesis. The inversion event, and the genes involved, are diagrammed in Figure 2. The nucleotide sequence of this region has been determined (Simon *et al.*, 1980) and is given in Figure 3.

Each of the two recombination sites consist of two imperfect 12-bp inverted repeats separated by a 2-bp core. The two sites are not identical and they may have different origins (Szekely and Simon, 1983). The sites also appear to have different properties when used in artificial substrates. Inversion of a segment bounded by inverted repeats of *hix* R is less efficient than inversion of segments bounded by both sites or by inverted copies of *hix* L (Scott and Simon, 1982). The Hin protein, 21Kd, binds to these 26bp *hix* (*hin* crossover) sequences as a dimer, one monomer of Hin is thought to bind to each of the inverted repeat half-sites (Glasgow *et al.*, 1989). The 2-bp central core is essential for site function, but the sequence of the 2 nucleotides is unimportant as long as it is

FIGURE 2. A DIAGRAMATIC REPRESENTATION OF THE *hin* SYSTEM OF *S. typhimurium*.

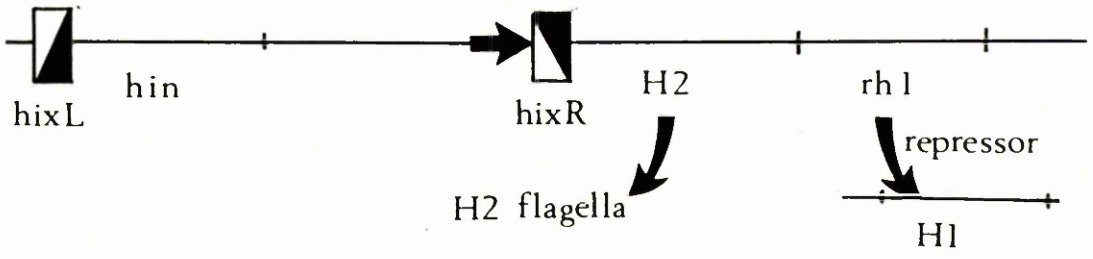
A = H2 'ON' STATE, H1 EXPRESSION REPRESSED.

B = H2 'OFF' STATE, H1 EXPRESSED.

hixL and hixR = RECOMBINATION SITES.

 = PROMOTER.

A.



B.

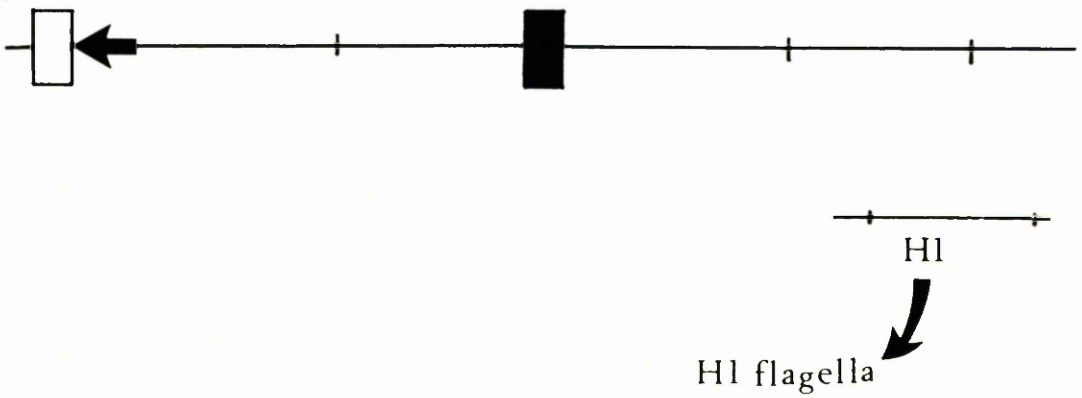
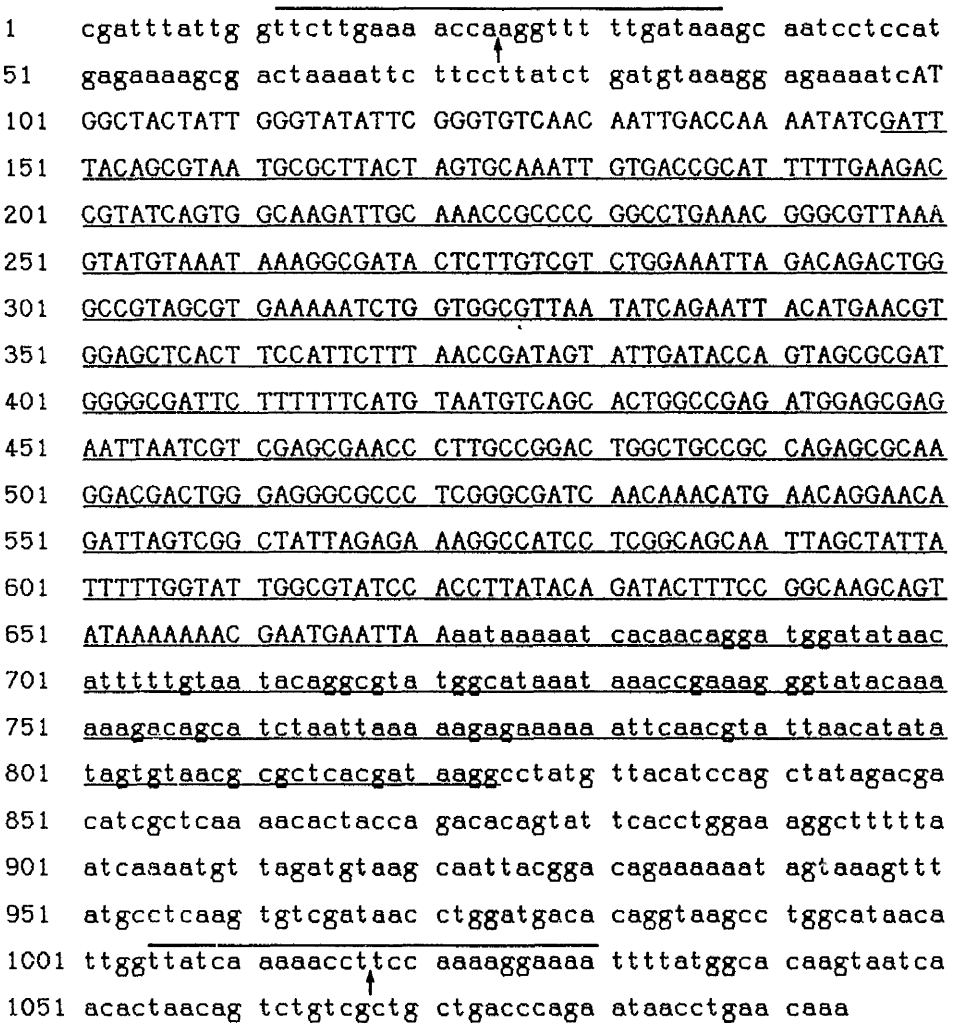


FIGURE 3. NUCLEOTIDE SEQUENCE OF THE INVERTIBLE *hin* SYSTEM OF *S. typhimurium*

This nucleotide sequence is taken from Zeig and Simon (1980). The codons encoding the *hin* gene itself are given in capitals. The residues that make up the 671bp *hin*-specific probe (section 3.4.2) are underlined. The inverted repeat sequences at the boundaries of the invertible region are overlined, and the recombination breakpoint is shown by the small arrow.


1 cgatttattg gttcttgaaa accaaggttt ttgataaagc aatcctccat
51 gagaaaagcg actaaaattc ttccttatct gatgtaaagg agaaaatcAT
101 GGCTACTATT GGGTATATTC GGGTGTCAAC AATTGACCAA AATATCGATT
151 TACAGCGTAA TGCGCTTACT AGTGCAAATT GTGACCGCAT TTTTGAAGAC
201 CGTATCAGTG GCAAGATTGC AAACCGCCCC GGCCTGAAAC GGGCGTTAAA
251 GTATGTAAAT AAAGGCGATA CTCTTGTCGT CTGGAAATTA GACAGACTGG
301 GCCGTAGCGT GAAAAATCTG GTGGCGTTAA TATCAGAATT ACATGAACGT
351 GGAGCTCACT TCCATTCTTT AACCGATAGT ATTGATACCA GTAGCGCGAT
401 GGGGCGATTC TTTTTTCATG TAATGTCAGC ACTGGCCGAG ATGGAGCGAG
451 AATTAATCGT CGAGCGAACC CTTGCCGGAC TGGCTGCCGC CAGAGCGCAA
501 GGACGACTGG GAGGGCGCCC TCGGGCGATC AACAAACATG AACAGGAACA
551 GATTAGTCGG CTATTAGAGA AAGGCCATCC TCGGCAGCAA TTAGCTATTA
601 TTTTTGGTAT TGGCGTATCC ACCTTATACA GATACTTTCC GGCAAGCAGT
651 ATAAAAAAAC GAATGAATTA Aaataaaaat cacaacagga tggatataac
701 atttttgtaa tacaggcgta tggcataaat aaaccgaaag ggtatacaaa
751 aaagacagca tctaattaaa aagagaaaaa attcaacgta ttaacatata
801 tagtgtaacg cgctcacgat aaggcctatg ttacatccag ctatagacga
851 catcgctcaa aacactacca gacacagtat tcacctggaa aggcttttta
901 atcaaaatgt tagatgtaag caattacgga cagaaaaaat agtaaagttt
951 atgcctcaag tgtcgataac ctggatgaca caggtaagcc tggcataaca
1001 ttggttatca aaaaccttcc aaaaggaaaa ttttatggca caagtaatca
1051 acactaacag tctgtcgctg ctgacccaga ataacctgaa caaa

identical in both recombination sites (Johnson and Simon, 1985). The essential bases for Hin binding are those ±4 to ±11 from the point of dyad symmetry. Hin binds only one face of the DNA helix on adjacent major and minor grooves of the *hix* half-sites. The Hin carboxy-terminal region contains a helix-turn-helix motif implicated in binding to the major groove of DNA. The Hin molecule may also contain another structure which recognizes features within the adjacent minor groove. The recognition and binding of Hin to the *hix* half-sites is mediated by the glycine 139 and arginine 140 amino acids.

The -35 region of the *hin* gene overlaps into the *hix* sequences and, consequently, the gene has a different -35 region depending on the orientation of the invertible segment. This obviously influences the rate of *hin* expression since the inversion rates are not equal in both directions.

In vitro studies of this system revealed the need for an acessory DNA sequence to give optimal rates of inversion. In a cell-free system derived from an *E. coli* strain moderately overproducing the *Hin* protein, Johnson *et al.* (1984) could specify the substrate requirements for efficient inversion. Using a relatively crude preparation Johnson *et al.* were able to demonstrate that inversion required a circular supercoiled DNA substrate containing two *hix* sites. The enhancer sequence required for optimal inversion rates is a 60bp sequence normally located within the amino-terminal coding region of the *hin* gene. The positioning of the enhancer sequence is not critical and it is able to function at various sites within a plasmid substrate (Johnson *et al.*, 1987). The Hin protein was

demonstrated to have topoisomerase activity on suitable substrates (Johnson *et al.*, 1984).

Further purification of the above cell extract to near homogeneity revealed a requirement for two host cell proteins (Johnson *et al.*, 1986). One of the required proteins is the major histone-like protein of *E. coli*, HU. This protein promotes unwinding of DNA in the presence of topoisomerase I, and in dimerised form may promote bending of DNA. The other protein involved is Fis (Factor for inversion stimulation). Fis is a 12Kd protein which is not essential for the growth of *E. coli*. The carboxy-terminal 74-93 amino acids of Fis bear similarity to the helix-turn-helix motif known to bind to DNA. The gene encoding Fis in *E. coli* has been cloned and sequenced, and is located at 72min on the chromosome (Johnson *et al.*, 1988).

Fis binds to 2 sites within the enhancer sequence, the sites differ in sequence and are designated the proximal and distal domains with respect to the *hix* L site in the 'ON' position. Fis binds independently to the 2 domains, with a greater affinity for the distal domain. Bruist *et al.* (1987) have presented evidence which suggests that Fis may be able to recruit secondary enhancer sites with partial homology to act in the absence of a functional enhancer, explaining the low rate of inversion observed in substrates with non-functional enhancer sequences. Fis binds primarily to one side of the DNA helix and spans 2 major grooves, making contacts in the major and minor grooves. The pentanucleotide sequence TGACC present in both domains may serve as the recognition sequence. These

two sequences are 48bp apart and separated by ≃4.5 turns of the helix. Thus, it would appear that the Fis molecules must be bound to opposite sides of the helix to stimulate inversion. A binding site for Fis has been described near the *Xis* binding site in bacteriophage λ (Thompson *et al.*, 1987). This binding site does not contain the pentanucleotide sequence and it is possible that Fis may recognise a particular structural feature of the DNA rather than, or in addition to, a specific recognition sequence.

Bruist *et al.* (1987) have proposed a model for the inversion reaction in which the Hin protein and Fis interact to give a synaptenemal complex which holds the DNA in the correct orientation for recombination. This model was investigated by Johnson and Bruist (1989) who isolated various intermediates in the recombination reaction and were able to expand the proposed model. They proposed that the Hin dimers bind to the *hix* sites and make a staggered 2-bp double-stranded cut within the central core. The bound Hin protein forms a phosphoester covalent linkage with the 5' end of the DNA and leaves a free hydroxyl at the 3' end. This transient linkage between the cleaved DNA and the recombinase allows the energy of the broken phosphodiester bond to be conserved and alleviates the need for high energy co-factors in the reaction. The Fis protein bound to the enhancer sequence is thought to mediate the rotation of the DNA molecules to allow religation into the inverted configuration. A possible route for the formation of the recombination complex is shown in Figure 4.

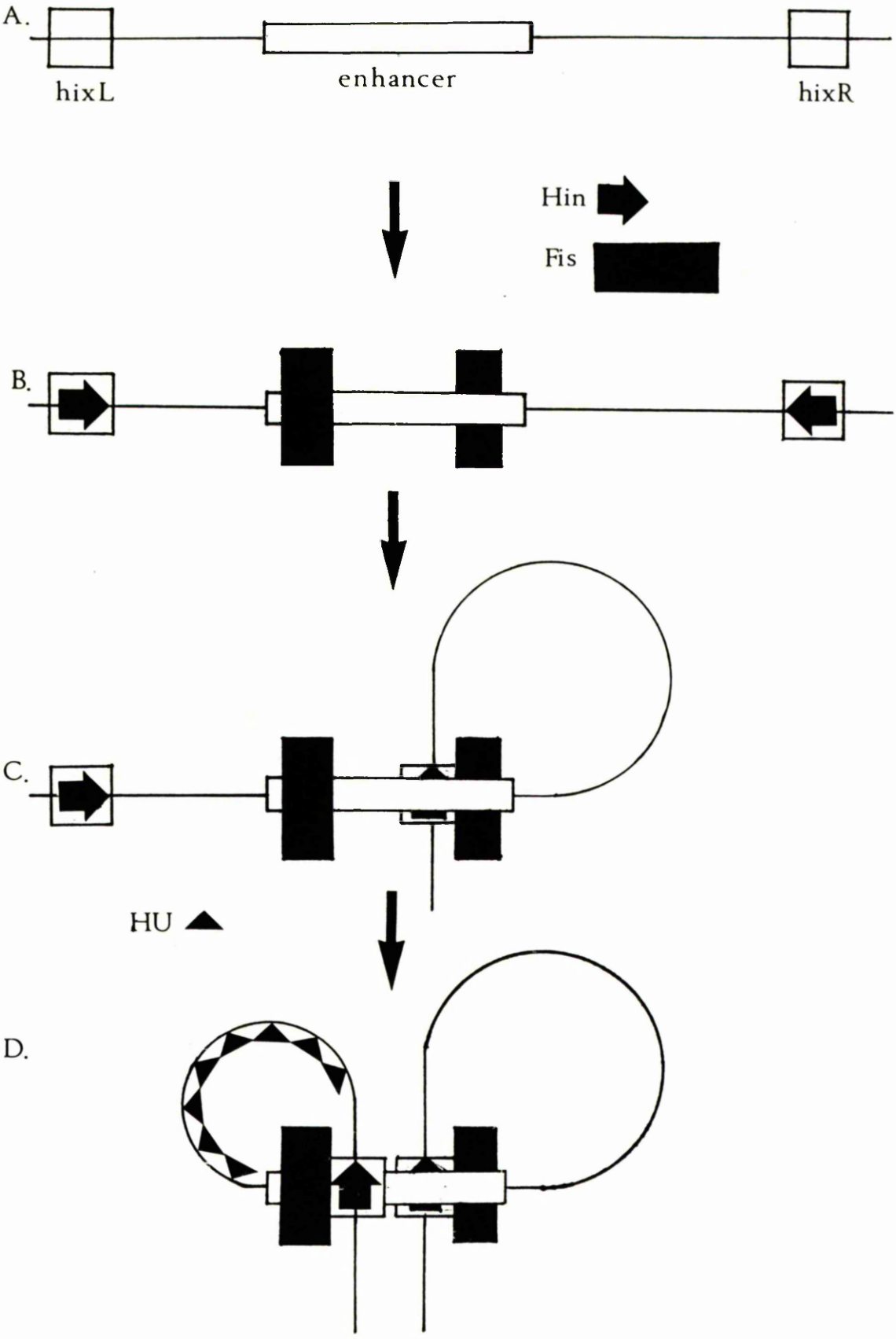
FIGURE 4. DIAGRAM ILLUSTRATING HOW Fis BINDING TO THE ENHANCER SEQUENCE MAY AID FORMATION OF THE RECOMBINATIONAL COMPLEX.

A). The invertible *hin* region.

B). Molecules of Hin bind to the *hix* sites and molecules of Fis bind to the enhancer.

C). The Hin bound to *hixR* is positioned into the nascent recombinational complex.

D). Binding of HU loops the shorter stretch of DNA to bring the Hin bound to *hixL* within the recombinational complex.


A.
hixL
enhancer
hixR
Hin
Fis
B.
C.
HU
D.

Analysis of the sequence of the invertible region reveals that the H2 gene and its 5' proximal sequence are homologous with the flagellin genes from other Salmonella species and *E. coli*. The *hin* sequence on the other hand is closely related to sequences found in bacteriophage Mu. The two *hix* sites may have different origins, with sequence data showing that *hix* L and *hin* are more closely related to *gix* R, the right hand inverted repeat sequence of the invertible DNA segment in Mu, and *gin*, the gene encoding the Mu invertase (Szekely and Simon , 1983). This group have suggested that the *hin* system evolved in three stages. Firstly, a transposon, or a bacteriophage, carrying a site-specific recombinase entered the chromosome approximately 100bp upstream from a single flagellin gene. In the second step the transposon and the flagellin gene are translocated to a different region of the Salmonella chromosome, as compared to the *E. coli* chromosome. The third step involved an aberrant recombination event which could have 'captured' the flagellin promoter. A sequence present in the regulatory region of the flagellin gene which had partial homology with the sequence of the recombinase recognition site may have been used by the recombinase. Since the site was, presumably, in the opposite orientation from that normally used by the recombinase, excision was replaced by inversion. Szekely and Simon (1983) further suggest that this recruitment of novel recombination sites may be a general phenomenon, giving rise to new genetic configurations by exchanging specific functions between transposable elements and chromosomal genes.

1.6.1.2. The *gin* system of bacteriophage Mu.

Bacteriophage Mu contains a 3kb invertible segment known as the G region (Kamp *et al.*, 1978; van de Putte *et al.*, 1980). When this G region is in the (+) orientation the bacteriophage is able to infect a certain set of host species including *E. coli* K12; in the (-) orientation the bacteriophage can infect a different set of host species, including Citrobacter and Shigella strains. This change in host specificity is brought about by differences in the tail fibres expressed by the phage (Giphart-Gassler *et al.*, 1982). In the (+) orientation the tail fibre genes S and U are expressed, whereas in the (-) orientation the genes S' and U' are expressed, altering the tail fibres produced. Unlike the *hin* system the gene encoding the recombinase is not within the invertible segment but is located in the DNA adjacent to the G region.

The S gene is composed of two regions, a constant sequence, S_c and a variable region, S_v. The variable region is contained at one end of the invertible segment, and the constant region in the stable adjacent DNA. The invertible segment also includes two further genes, U and U', completely within its boundaries, and at the other end the gene fragment S_v' located in the opposite orientation to the S_v gene fragment (van de Putte *et al.*, 1980). This arrangement of genes is illustrated in Figure 5.

In the (+) orientation, transcription is initiated at a promoter outside the invertible segment and continues through S_c and S_v and into the U gene. After inversion into the (-) orientation transcription reads through S_c as before but now

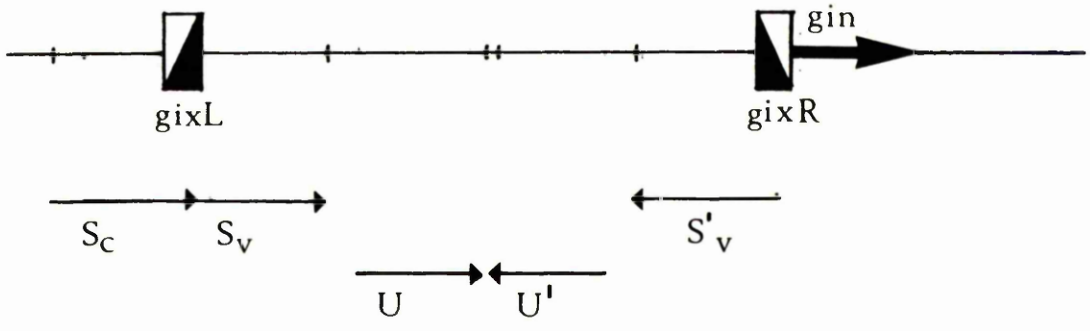
FIGURE 5. DIAGRAM COMPARING THE INVERTIBLE REGIONS OF BACTERIOPHAGES MU AND P1.

1). The G region of the Mu chromosome showing the arrangement of genes.

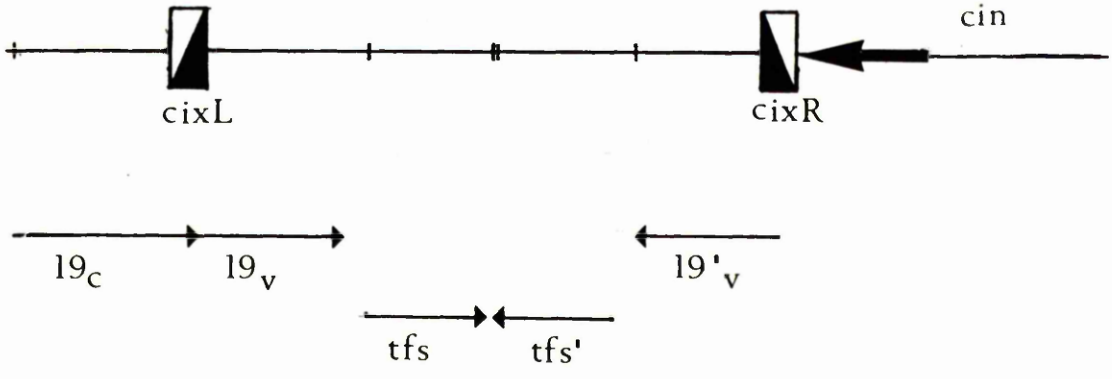
2). The invertible C region of bacteriophage P1, not to scale with the diagram of the G region above.

The functions of the genes are detailed in the text.

1.Mu



2.Pl



continues into S_v' and U'. The inversion is thus responsible for altering the tail fibres produced by the phage.

The G region is flanked by two identical 34bp imperfect inverted repeat sites. Each IR site is composed of two binding sites for the Mu invertase Gin and the two sites do not appear to be equivalent; site II is occupied only if site I is present and occupied (Mertens *et al.*, 1988). G inversion is catalysed by Gin, the product of the *gin* gene. The gene has been cloned, sequenced and compared with the genes encoding the other invertase molecules (Plasterk *et al.*, 1983). As with the *hin* system a recombination enhancer sequence is located within the coding region of the *gin* gene (Kahmann *et al.*, 1985). This enhancer appears to function in the same manner as the enhancer of the *hin* system, requiring the presence of Fis for optimum inversion.

G inversion occurs at very low levels, due to the low level of *gin* expression. This low level may be explained by the unusual use of GTG as the initiation triplet rather than the more effective ATG triplet (Plasterk *et al.*, 1983).

1.6.1.3. The *cin* system of bacteriophage P1.

Phage P1 contains an invertible DNA segment 4200bp in length that is highly homologous to the G segment of Mu (Chow and Bukhari, 1976). The segment is flanked by 640bp inverted repeats with the recombination sites, *cix* L and *cix* R, located at the outer ends of the larger inverted repeats (Hiestand-Nauer and Iida, 1983). This C segment performs the same function in P1 as the G segment in Mu, and is organised in the same symmetrical manner, see Figure 5 for a comparative

diagram. The two inner genes, *tfs* and *tfs'*, are highly homologous to the Mu U and U' genes, and likewise encode tail fibres. The larger gene, 19, is split into a constant and two variable regions, again analogous to the S gene in Mu. Significant homology exists between the S gene and 19 at approximately the same known distance from the amino-terminal end of the tail-fibre gene, suggesting that the intervening region, within the large IR sequence, may have been deleted in Mu, leaving only the necessary core sequence.

As with the other *hin*-like systems a 72bp enhancer sequence is required for optimal rates of inversion *in vitro*. The enhancer sequence is also contained within the coding region of the *cin* gene (Kahmann *et al.*, 1985). This enhancer sequence is thought to function in the same manner as the enhancers of the other *hin*-like systems (Huber *et al.*, 1985). Studies by HÜbner *et al.* (1989) have revealed that Fis binds to two sites within the enhancer, and that the sequence between these sites is important for enhancer activity. The enhancer effect appears to involve DNA bending between the two bound Fis molecules. HÜbner *et al.* suggest that this DNA bending may either give rise to DNA perturbations that can propogate along the helix, or, this bent DNA may be required for the three dimensional configuration of the recombination complex suggested by Bruist *et al.* (1987).

The major difference between the P1 *cin* system and the other *hin*-like system lies in the orientation of the invertase gene. The proposed promoters for the other *hin*-like genes lie within the inverted repeat crossover sequences, in the case of

the *cin* gene it is the carboxy terminus which overlaps the *cix* site. Analysis of the area around the *cin* gene promoter reveals a sequence with considerable homology to the *cix* site (Hiestand-Nauer and Iida, 1983). At some point in the evolution of the *cin* system the *cin* gene may have been inverted with the aid of this pseudo-*cix* site. The Cin molecule may also be able to utilise these pseudo-*cix* sites to form co-integrates and this may lead to the fusion of genomes (Kennedy *et al.*, 1983).

1.6.1.4. The *pin* system of *E. coli*.

Enomoto *et al.* (1983) identified a region within the *E. coli* genome that was able to cause inversion in a plasmid carrying a non-functional invertible region from *S. typhimurium*. They named this function *pin* and mapped it to approximately 26min on the *E. coli* chromosome. Enomoto *et al.* found that the product of the *pin* gene was also able to complement mutants in the *cin* system of P1. The *pin* gene was sequenced by Plasterk *et al.* (1983) and found to be homologous to the other invertase genes in the *hin* family. The *pin* gene was found to be adjacent to a 1800bp invertible region.

Van de Putte *et al.* (1984) found that *E. coli* HB101, a derivative of *E. coli* K12, was able to complement mutations in the *gin* system. This group placed the *pin* gene, and attendant invertible region, within the cryptic prophage *e*14. This prophage possess an SOS-sensitive repressor and, consequently the *pin* function is lost in strains which have been exposed to UV radiation or other mutagens. Neither Plasterk *et al.* (1983) nor Kutsukake *et al.* (1985) were able to ascribe a function to this invertible segment. However, it is known that the

inversion determines the differential expression of at least one protein (Plasterk and van de Putte, 1984), although this alteration in protein expression cannot be correlated with any apparent change in the phenotype of *E. coli*.

1.6.1.5. Relationships between the *hin*-like systems.

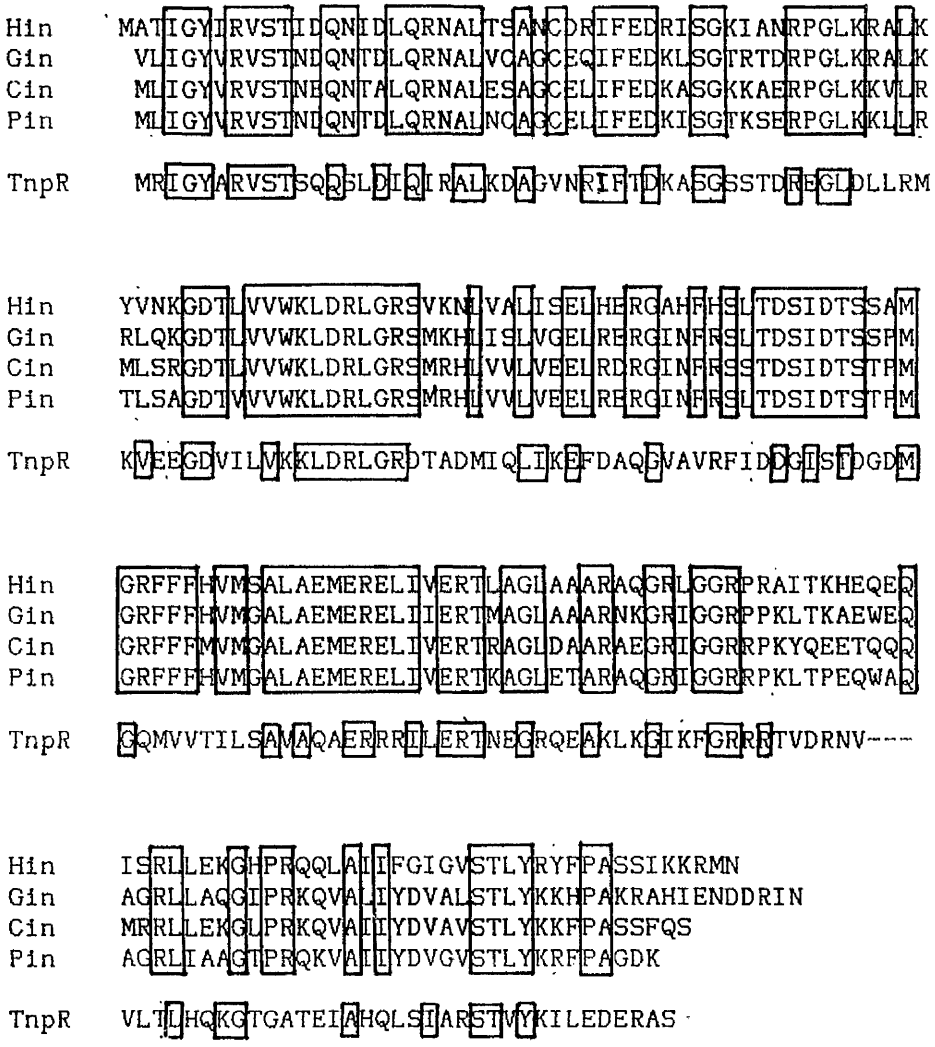
The greatest homology within the family of *hin*-like systems lies in the amnio acid sequence of the recombinases. This homology is demonstrated in Figure 6, in which the amino acid sequences of the invertase proteins are compared. The homology is consistently over 60%, the exact degrees of homology are; 70% sequence homology between Gin and Pin, 62% homology between Hin and Gin and 60% between Hin and Pin. The resolvase protein TnpR shares 33% homology with Hin, which suggests a common ancestry for these proteins (Simon *et al.*, 1980; Kamp *et al.*, 1984). The DNA sequence homology between these genes is not as high, due to the preferred codon usage of the different species. However, the nucleotide sequence homology is high enough to permit DNA probes made from the invertible region of *S. typhimurium* to hybridise under reduced stringency conditions to the invertible regions of Mu and P1 (Szekely and Simon, 1981). This conservation between the proteins implies a selective pressure to maintain protein function, an important observation for the *pin* system which has not yet been assigned a role. Using similar reduced stringency conditions Foxall *et al.* (1990) have observed hybridisation between a probe carrying the invertible region from *S. typhimurium* and chromosomal DNA from *B. pertussis*.

FIGURE 6. ALIGNMENT OF THE AMINO ACID SEQUENCES OF THE HIN, GIN, CIN, PIN AND TnpR PROTEINS.

Amino acids are denoted by the standard one-letter symbols (see Appendix 4).

Amino acids conserved in all four invertase proteins are boxed, as is homology between TnpR and Hin.

Hin sequence from Zieg and Simon, 1980.
Gin and Pin sequences from Plasterk *et al.*, 1983.
Cin sequence from Hiestand-Nauer and Iida, 1983.
TnpR sequence from Heffron *et al.*, 1979.


Hin MATIGYIRVSTIDQNIDLQRNALTSANCDRIFEDRISGKIANRPGLKRALK
Gin VLIGYVRVSTNDQNTDLQRNALVCAGCEQIFEDKLSGTRTDRPGLKRALK
Cin MLIGYVRVSTNEQNTALQRNALESAGCELIFEDKASGKKAERPGLKKVLR
Pin MLIGYVRVSTNDQNTDLQRNALNCAGCELIFEDKISGTKSERPGLKKLLR
TnpR MRIGYARVSTSQQSLDIQIRALKDAGVNRIFTDKASGSSTDREGLDLLRM
Hin YVNKGDTLVVWKLDRLGRSVKNLVALISELHERGAHFHSLTDSIDTSSAM
Gin RLQKGDTLVVWKLDRLGRSMKHLISLVGELRERGINFRSLTDSIDTSSPM
Cin MLSRGDTLVVWKLDRLGRSMRHLVVLVEELRDRGINFRSSTDSIDTSTPM
Pin TLSAGDTVVVWKLDRLGRSMRHIVVLVEELRERGINFRSLTDSIDTSTPM
TnpR KVEEGDVILVKKLDRLGRDTADMIQLIKEFDAQGVAVRFIDDGISTDGDM
Hin GRFFFHVMSALAEMERELIVERTLAGLAAARAQGRLGGRPRAITKHEQEQ
Gin GRFFFHVMGALAEMERELIIERTMAGLAAARNKGRIGGRPPKLTKAEWEQ
Cin GRFFFMVMGALAEMERELIVERTRAGLDAARAEGRIGGRRPKYQEETQQR
Pin GRFFFHVMGALAEMERELIVERTKAGLETARAQGRIGGRRPKLTPEQWAQ
TnpR GQMVVTILSAMAQAERRRILERTNEGRQEAKLKGIKFGRRRTVDRNV----
Hin ISRLLEKGHPRQQLAIIFGIGVSTLYRYFPASSIKKRMN
Gin AGRLLAQGIPRKQVALIYDVALSTLYKKHPAKRAHIENDDRIN
Cin MRRLLEKGLPRKQVAIIYDVAVSTLYKKFPASSFQS
Pin AGRLIAAGTPRQKVAIIYDVGVSTLYKRFPAGDK
TnpR VLTLHQKGTGATEIAHQLSIARSTVYKILEDERAS

The nucleotide sequence of the various cross-over sites has also been compared, see Figure 7. This reveals an equally strong homology with a consensus inverted repeat of the following sequence:-

- A - G T T T - - G A - A A

The high degree of homology between these systems is further demonstrated by their ability to complement one another. The *pin* system of *E. coli* was initially recognised by its ability to complement a *gin* mutation carried on a plasmid (van de Putte *et al.*, 1984). Given the strong homology and complementarity of these systems it is assumed that inversion will occur by the same mechanism in any other related systems which may be discovered.

Curran *et al.* (1984) exploited this complementarity and constructed a plasmid to test for *hin*-like invertase activity in a number of Gram-negative bacteria. This group cloned the invertible region of *S. typhimurium* (H2 'OFF') into the broad host range plasmid RP4. This recombinant plasmid, pMD8, did not confer motility when conjugated into a non-motile *E. coli* strain. However, P1 lysogens of the non-motile strain gave rise to motile colonies, the invertase encoded by the phage inverting the *hin* segment into the H2 'ON' configuration in a number of cases.

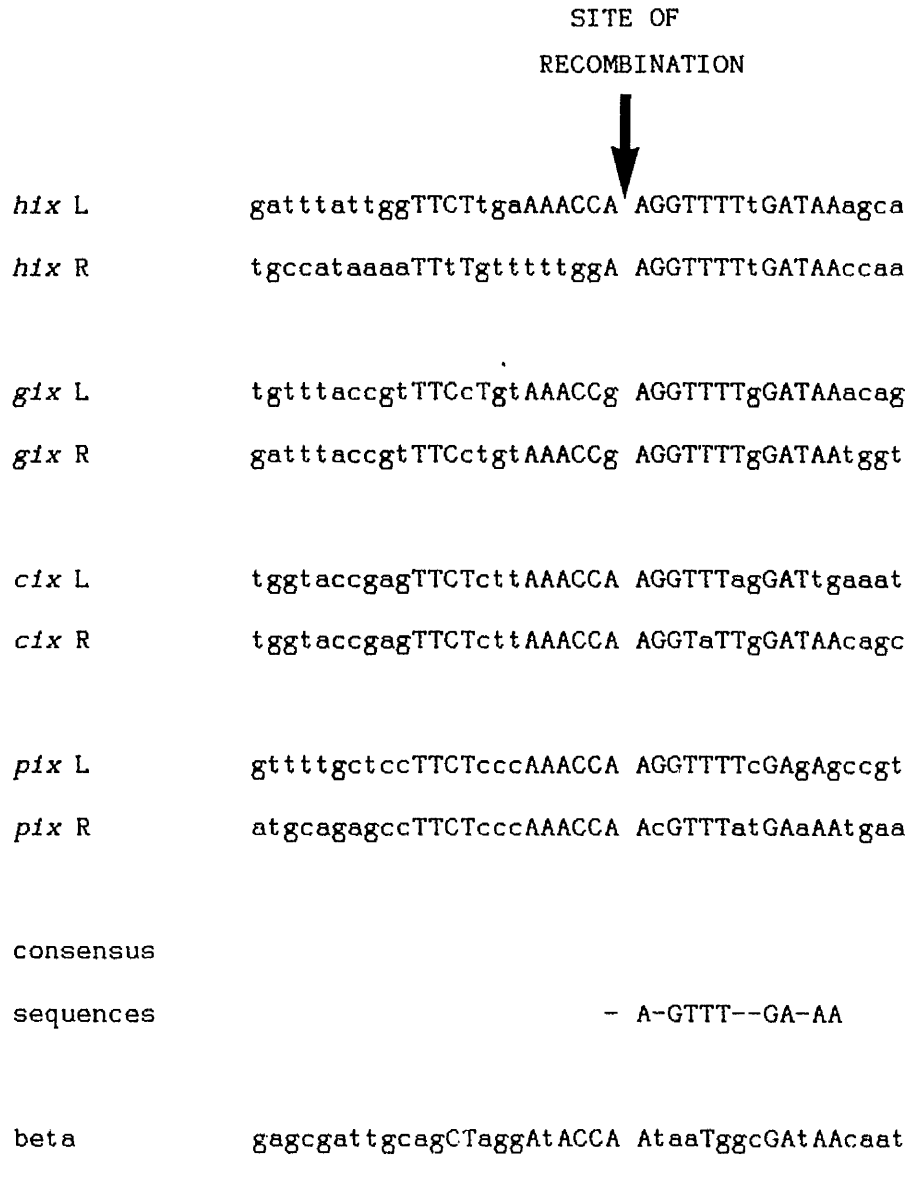
pMD8 was then conjugated into a variety of bacterial strains and grown overnight in the new hosts. The plasmid was subsequently reconjugated into the non-motile *E. coli* strain and transconjugants tested for motility. Plasmid pMD8 grown up in several strains of *E. coli* K-12 and *E. coli* C was found to

FIGURE 7. COMPARISON OF THE NUCLEOTIDE SEQUENCES OF THE RECOMBINATION SITES OF VARIOUS INVERTIBLE DNA SYSTEMS.

Conserved nucleotides in capitals.

The sequence labelled beta is found at the boundaries of the invertible region found in *M. bovis*.

Sequences are derived from the same sources as Figure 6, except beta (Marrs *et al.*, 1988).


SITE OF
RECOMBINATION
hix L gatttattggTTCTtgaAAACCA AGGTTTTtGATAAagca
hix R tgccataaaaTTtTgtttttggA AGGTTTTtGATAAccaa
gix L tgtttaccgtTTCcTgtAAACCg AGGTTTTgGATAAacag
gix R gatttaccgtTTCctgtAAACCg AGGTTTTgGATAAtggt
cix L tggtaccgagTTCTcttAAACCA AGGTTTagGATtgaaat
cix R tggtaccgagTTCTcttAAACCA AGGTaTTgGATAAcagc
pix L gttttgctccTTCTcccAAACCA AGGTTTTcGAgAgccgt
pix R atgcagagccTTCTcccAAACCA AcGTTTatGAaAAtgaa
consensus
sequences - A-GTTT--GA-AA
beta gagcgattgcagCTaggAtACCA AtaaTggcGAtAAcaat

have inverted, as it also did when grown in strains of *Proteus morganii*.

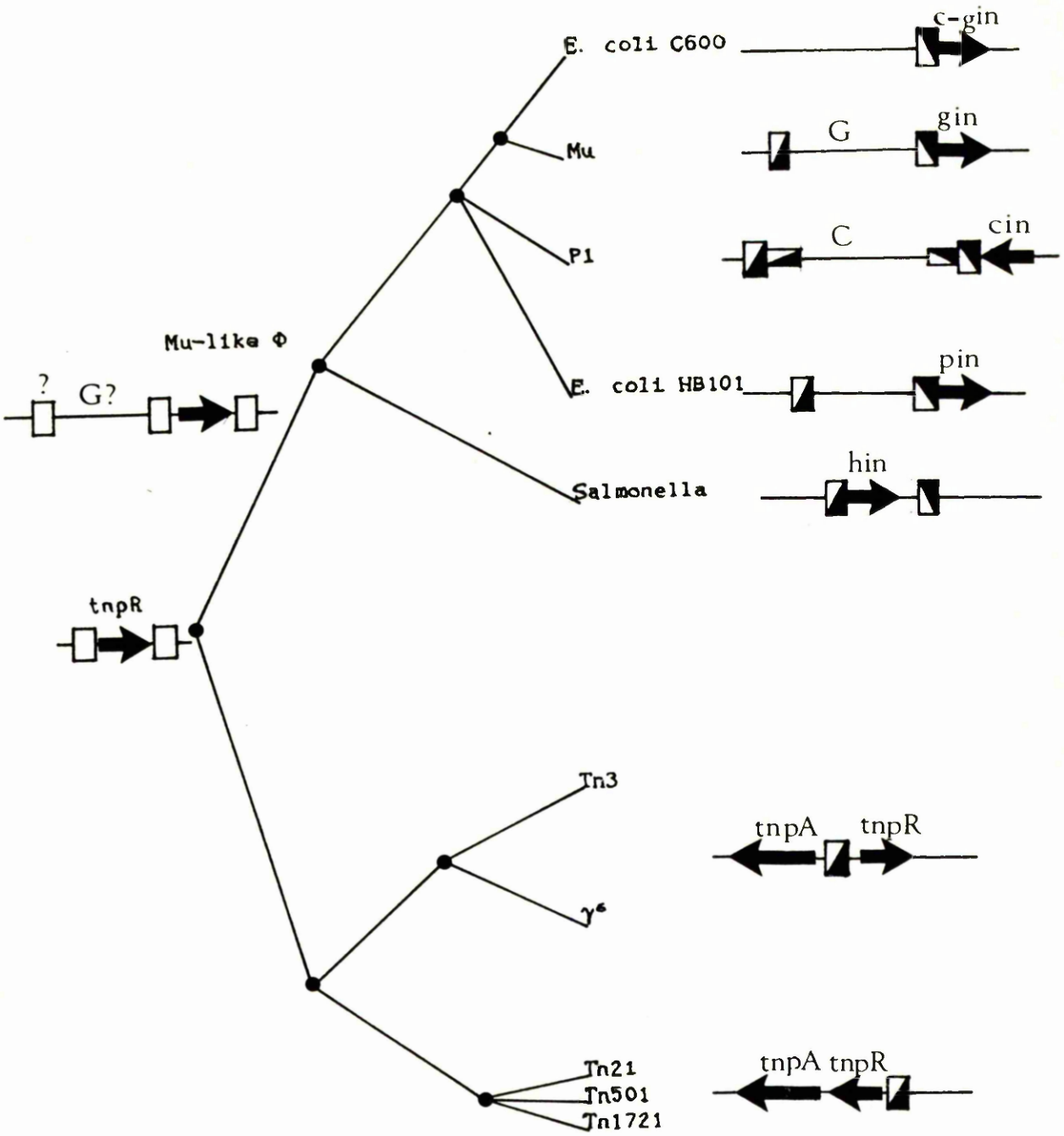
The evolution of the *hin*-like systems has been studied by Kamp *et al.* (1984) who analysed amino acid and nucleotide sequence data from the various systems. This group proposed the evolutionary tree shown in Figure 8, and suggested that all the *hin*-like systems evolved from a Mu-like ancestral phage. This ancestral phage would have possessed an invertible recombinase gene, deletion of one of its cross-over sites would have fixed it in one orientation and capture of a pseudo-crossover site would give rise to invertible segments of DNA. The ancestral phage would, presumably, be able to infect several species of bacteria and thus be disseminated throughout a range of species. Integration of this ancestral phage near the *S. typhimurium* flagellin gene followed by fusion of a phage promoter to the flagellin gene and deletion of the majority of the prophage would give rise to the *hin* system. A precedent for this method of evolution exists as Kamp *et al.* (1984) have found a cryptic *gin* gene and a portion of the G segment within the genome of *E. coli* C600.

1.6.1.6. Non *hin*-like invertible DNA in *E. coli*.

A system has been described in *E. coli* involving an invertible segment of DNA, separate from the *hin*-like system located on e14, which is responsible for fimbrial variation. The expression of type 1 fimbriae in *E. coli* is regulated by a 314bp invertible DNA segment which contains the promoter for the fimbrial subunit gene *fim* A (Abraham *et al.*, 1985). In a manner analogous to the *S. typhimurium* invertible system the

FIGURE 8. EVOLUTIONARY TREE FOR THE INVERTASE FAMILY OF PROTEINS.

Diagram adapted from Kamp *et al.* 1984.


E. coli C600
c-gin
Mu
G
gin
P1
C
cin
Mu-like Φ
?
G?
E. coli HB101
pin
Salmonella
hin
tnpR
Tn3
tnpA
tnpR
γδ
Tn21
Tn501
Tn1721
tnpA tnpR

orientation of the promoter determines the expression of the fimbriae. The 314bp segment has been sequenced (Dorman and Higgins, 1987) and is bounded by two 9bp inverted repeats. The orientation of the segment is controlled by the FimB and FimE proteins (Klemm, 1986), products of the closely linked genes *fim* B and *fim* E, and requires the host protein known as integration host factor (Dorman and Higgins, 1987). Both the Fim B and Fim E proteins are required for efficient bi-directional inversion (Pallesen *et al.*, 1989). The amino acid sequences of these two recombinases share no apparent homology with the family of Hin-like invertases and are not complementary (Freitag *et al.*, 1985). However, homology is found between the C-terminal sequences of Fim B and Fim E and a number of integrase-like phage recombinases (P2Int, P1Int, λInt) (Dorman and Higgins, 1987). In this family of proteins one region is perfectly conserved and may act as the active site. Argos *et al.* (1986) suggest that a tyrosine residue present in this region in all the proteins is responsible for forming a transient covalent linkage to DNA during strand breakage and religation.

1.6.1.7. Invertible DNA in *Moraxella bovis*.

The pili of *Moraxella bovis*, a cattle pathogen, undergo a variation between two pilin types, α and β. The change between the two is mediated by the inversion of a 2kb DNA segment (Marrs *et al.*, 1988). The amino-terminal portions of both the α and β pilin subunits are encoded by the same locus, the differing amino-terminal portions are encoded by two different gene fragments within the invertible segment. In one

orientation the α-specific carboxy region is spliced into the expression site, after inversion the β-specific region is in a position to be expressed. Homology exists between this system and the left inverted repeat of the *hin* system (Marrs *et al.*, 1988), see Figure 7. *M. bovis* also undergoes a phase variation between piliated and non-piliated cells; the mechanism behind this switch is unknown.

1.6.2. Variation in *Neisseria gonorrhoeae*.

Members of the genus Neisseria are Gram-negative diplococci that colonize the mucosal surfaces of mammals; the genus contains both pathogenic and commensal species. *N. gonorrhoeae* is a major cause of venereal disease in humans.

Two major surface proteins involved in the disease process are the pili and the opacity protein. Both of these proteins undergo phase and antigenic variation independently.

Gonococcal pili are composed of repeating subunit proteins and mediate attachment to cell surfaces. The pilin subunits are composed of a constant amino-terminal region and a variable carboxy-terminal region (Meyer *et al.*, 1984; Hagblom *et al.*, 1985). The *N. gonorrhoeae* strain, $MS11_{MS}$, studied by Meyer *et al.* (1984) carries two loci on the chromosome *pil*E1 and *pil*E2, located approximately 20Kb apart, which can encode functional pili. Subsequent studies of clinical isolates have shown that most strains possess only a single expression site, *pil*E1 (Nicolson *et al.*, 1987; Swanson, 1987). Many other regions of the chromosome contain homologous sequences which do not encode pili (Meyer *et al.*, 1984; Swanson *et al.*, 1986). During phase variation, from piliated to non-piliated cells, there is a

deletion in one or both of the expression sites. A further control system represses expression of pilin from the expression site that has not been deleted (Segal *et al.*, 1985). The deletions occur between repeated sequences within the coding region and upstream of the gene. Pilus phase variation is closely linked to antigenic variation and the non-piliated state may be an obligatory intermediate in the process of antigenic variation.

Pilus antigenic variation is due to the placement of varying numbers of cassettes which code for portions of the variable region within the expression sites (Hagblom *et al.*, 1985). These coding regions may come from the silent pilin sequences (Segal *et al.*, 1986) or from transformed DNA released by autolysed gonococci (Seifert *et al.*, 1988).

The opacity protein, OPII, is another major gonococcal adhesin which can undergo phase and antigenic variation. The genome of *N. gonorrhoeae* contains at least nine loci which can produce variant OP molecules (Stern *et al.*, 1984); all of these loci are constitutively transcribed. Translation to functional protein is controlled by the presence of pentanucleotide repeats known as control repeats. These repeats are located in the region of the DNA which codes for the hydrophobic portion of the leader peptide. The number of repeats determines whether the coding sequence for mature PII is in or out of frame and, consequently, whether the OP is expressed or not (Stern *et al.*, 1986). The number of the repeats is also thought to account for antigenic variation.

The enzymatic machinery responsible for the various recombination events occurring during pilus and OP phase and antigenic variation is not yet known. However, Saunders (1986) suggests that if a mechanism similar to the *hin*-like system is used the enzymes must have a 'fairly lax specificity' to explain the diversity of events and the occurrence of apparently unfavourable rearrangements.

1.6.3. Rearrangements during Anabaena differentiation.

Genomic rearrangement is also involved in the developmental cycle of the cyanobacterium Anabaena. During nitrogen deprivation a number of Anabaena cells in a given population will differentiate into heterocysts. This change is accompanied by at least two genomic rearrangements. One involves the excision of an 11kb circular DNA molecule from within the 3' end of a gene encoding a nitrogenase (Golden *et al.*, 1985). The second rearrangement involves a conservative site-specific recombination event between two 11bp direct repeats (Golden *et al.*, 1985). The repeat sequences identified as the breakpoints in both these rearrangements share no homology with the target sites of either λ integrase or Hin protein.

1.6.4. Variation of Borrelia VMP protein.

Members of the genus Borrelia are spirochetes known to cause relapsing fever in humans and several animal species (Felsenfeld, 1965). The disease is characterised by a repeated cycle of feverish and convalescent periods. Antigenically distinct spirochetes can be isolated from animals experimentally infected with Borrelia of a single serotype (Schuhardt and Wilkerson, 1951). The most extensively studied

member of the genus is *Borrelia hermsii*, which causes relapsing fever in mice. A single organism of this species can give rise to at least 24 different serotypes (Stoenner *et al.*, 1982). The frequency of this serotype variation is approximately 10^{-4}-10^{-3}. The change in serotype has been correlated with changes in a major 40Kd surface protein known as the variable major protein, VMP. The serotype specificity is due to differences in the primary structure of the protein.

Meier *et al.* (1985) found that differential expression of VMP genes was accompanied by a DNA rearrangement. This group presented data which suggests that each different VMP gene exists as a silent copy in the borrelial genome and expression of a particular VMP gene is the result of duplication and placement of this gene into an expression site. The VMP genes may be located on a multicopy linear plasmid (Plasterk *et al.*, 1985). The silent VMP genes stored on the linear plasmids are complete copies of a structural gene lacking any upstream regulatory sequences. Site-specific recombination occurs between the the genes on the storage plasmid and an expression plasmid. This results in placement of an intact VMP gene downstream of the appropriate regulatory sequences in the expression site. The enzymatic machinery and DNA chemistry of this system has not been elucidated.

AIM OF RESEARCH.

Bordetella pertussis produces a range of virulence factors which are implicated in the disease process. Through the process of phase variation the expression of these virulence factors is halted by a genetic event. Within this range of virulence factors the bacterium produces two distinct fimbriae, and the variation in their expression is controlled by a different system from the one controlling phase variation.

A variety of genetic control systems responsible for antigenic variation in a wide range of bacterial species have been characterised, and many of these control systems involve DNA rearrangements.

The aim of this study was to examine DNA from phase and serotype variants of *B. pertussis* to find out if any DNA rearrangements were occurring in association with the observed variations.

A previous study had revealed homology between the *hin* region of *S. typhimurium* and a region within the *B. pertussis* genome, is this homologous region responsible for either phase or serotype variation?

2. MATERIALS AND METHODS.

2.1. BACTERIAL STRAINS AND PLASMIDS.

2.1.1. *B. pertussis* strains.

The fimbrial serotypes of all *B. pertussis* strains were tested by slide agglutination (section 2.5.2) when the cultures were first opened and then regularly throughout the study.

The following *B. pertussis* strains were used in this study:-

1. Taberman: This strain has a 1,0,3 serotype. Isolated pernasally before the death of an infant. Obtained from Professor G.T. Stewart, Ruchill Hospital, Glasgow.

2. Tohama: This strain has a 1,2,0 serotype. Obtained from the culture collection of the Microbiology Department, Glasgow University.

3. Wellcome 28: This strain has a 1,2,3 serotype. Obtained from Dr. P. Novotny, Wellcome Biotechnology, Beckenham, Kent.

4. L84 I (NCTC 11089): This strain is a virulent isolate of *B. pertussis*, originally isolated from a nasopharyngeal swab in 1950 (Ackers and Dolby, 1972). Obtained from the culture collection of the Microbiology Department, Glasgow University.

5. L84 IV (NCTC 10902): This strain is an avirulent variant of L84 I (Ackers and Dolby, 1972). The properties of strains L84 I and L84IV are described by Parton (1988). Obtained from the culture collection of the Microbiology Department, Glasgow University.

6. BP 347: A transposon-induced pleiotropic mutant of strain Tohama deficient in haemolysin, ACT, AGGs, FHA, PT and dermonecrotising toxin production (Weiss *et al.*, 1983). Obtained from Dr. A.A. Weiss, Virginia Commonwealth University, USA.

2.1.2. *B.pertussis* serotype variants.

1. 106V: This strain has a 1,0,0 serotype and is afimbriate, but is not a mouse avirulent strain. The strain was derived from a parent strain with a 1,0,3 serotype (Bronne-Shanbury and Dolby, 1976). Obtained from the culture collection of the Microbiology Department, Glasgow University.

2. ARG Strains: *B.pertussis* strains bearing the ARG prefix are strains isolated from the lungs of infant mice three weeks after infection with the parental strains. The fimbrial serotype of the strains and details of the parent strains are given in Table 1. Obtained from Dr. A. Robinson, PHLS, Porton Down.

2.1.3. *B.parapertussis.*

B.parapertussis strain NCTC 10520 was used throughout this study. Obtained from the culture collection of the Microbiology Department, Glasgow University.

2.1.4. *E.coli* strains.

1. *E. coli* DH1. *rec*A1 *end*A1 *gyr*A96 Thi$^-$ *hsd*R17 *sup*E44.

2. *E. coli* HB101. *rec*A13 F$^-$*ara*14 λ^- *gal*K2 *lac*Y1 *pro*A2 *rsp*L20 *xyl*-5 *sup*E44.

3. *E. coli* C600. *thi*-1 *leu*B6 *lac*Y1 *ton*A21 λ^- *sup*E44.

TABLE 1: THE FIMBRIAL SEROTYPES OF *B. pertussis* ARG

STRAINS AND THEIR PARENT STRAINS.

STRAIN	FIMBRIAL SEROTYPE	PARENT STRAIN	PARENT FIMBRIAL SEROTYPE
ARG 40	2,0	Wellcome 28	2,3
ARG 41	0,3	Wellcome 28	2,3
ARG 43	0,3	Tohama	2,0
ARG 44	0,3	Tohama	2,0

4. *E. coli* K 12.

All the above strains obtained from the culture collection of the Microbiology Department, Glasgow University.

5. *E. coli* WA 3782 (Iida, 1984). hsd_K *rec*A *met pin*. The strain is negative for DNA inversion function and has been used in several inversion assays (Huber *et al.*, 1985; Kahmann *et al.*, 1985). Obtained from Dr. H. Hübner, Basel University.

6. *E. coli* RZ211. Δ(*pro-lac*) *ara str*A *rec*A56 att::λ*fla*-406 off. The original strain RZ211 (Johnson *et al.*, 1982) did not contain the λ phage insertion. The phage λ*fla*-406 off contains the *lac*Z gene under the control of the *hin* invertible DNA segment (Bruist and Simon, 1984), and has inserted lysogenically into the chromosome of strain RZ211. The phage is trapped in the 'off' orientation due to a deletion in the *hin* gene it carries. In this 'off' state the phage does not transcribe the *lac*Z gene. In the presence of an exogenously provided invertase protein the *hin* region inverts and permits transcription of the *lac*Z gene. Inversion also causes the phage to express the H2 gene it carries and putative inversion events can be tested for by the presence of H2 flagellin in the cell. This strain was obtained from Dr. M. Simon, California Institute of Technology, California, USA.

2.2. PLASMIDS.

2.2.1. *B. pertussis* strain Tohama gene library.

The construction of this library from *B. pertussis* strain Tohama chromosomal DNA is described by Brownlie *et al.* (1986). Each clone contains approximately 30Kb *B. pertussis* DNA cloned

into the single *Eco* R1 site of the cosmid vector pLAFR1 (Freidman *et al.*, 1982). Plasmids from this library used:-

2.2.1.1. pRMB1 contains the adenylate cyclase locus and IS481v1.

2.2.1.2. pRMB2 contains the *bvg* locus.

Both plasmids described by Brownlie *et al.* (1988).

2.2.2. Plasmids from other sources.

2.2.2.1. pRK2013: helper plasmid for three way conjugations (Figurski and Helenski, 1979). Obtained from the culture collection of the Microbiology Department, Glasgow University.

2.2.2.2. pIL22: contains the *fim* 2 gene from *B. pertussis* strain Wellcome 28. Construction of this plasmid is detailed in Livey *et al.* (1987). Obtained from I. Livey, PHLS, Porton Down.

2.2.2.3. pKK1001B: contains 8.8kb *S. typhimurium* DNA cloned into pBR322 (Dr. K. Kutsukake, personal communication). The cloned DNA includes the entire invertible DNA segment of the *hin* system, the H2 gene and the rh1 gene. Obtained from Dr. K. Kutsukake, University of Tokyo.

2.2.2.4. pSH1313: an indicator plasmid constructed by Huber *et al.* (1985). The plasmid contains genes encoding kanamycin resistance and chloramphenicol resistance within a short discrete invertible DNA segment. The segment is bounded by two inverted repeat sequences from the *cin* invertible region of bacteriophage P1. Expression of these genes is controlled by a single promoter outwith the invertible region, see Figure 9 for diagram. In the 'ground' state the plasmid encodes

FIGURE 9. DIAGRAM OF pSH1313.

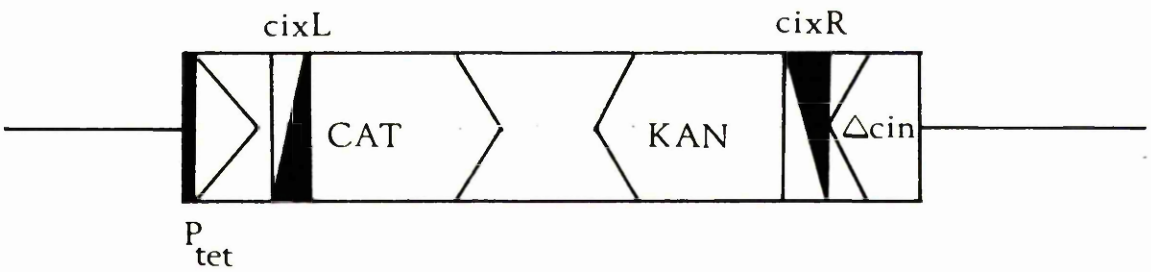
$P_{t\rightarrow t}$ = PROMOTER.

CAT = GENE ENCODING CHLORAMPHENICOL RESISTANCE.

KAN = GENE ENCODING KANAMYCIN RESISTANCE.

cixR and *cixL* = INVERTED REPEAT RECOMBINATION SITES FROM BACTERIOPHAGE P1 *cin* SYSTEM.

Δc1n = *cin* GENE FROM BACTERIOPHAGE P1 CARRYING A LARGE DELETION.


cixL
cixR
CAT
KAN
Δcin
P
tet

chloramphenicol resistance. In the presence of an active invertase the DNA segment containing the resistance genes is inverted and the plasmid then encodes kanamycin resistance. Obtained from Dr H. Hübner, Basel University.

2.3. MEDIA AND GROWTH OF BACTERIA.

For details of media composition and preparation see Appendix 1.

Growth and manipulation of *E. coli* containing recombinant plasmids was done under category I containment according to ACGM guidelines (ACGM/HSE note 8, June 1988). *B. pertussis* containing recombinant plasmids was grown and manipulated under category II containment.

2.3.1. Growth of *B. pertussis*.

B. pertussis was grown on solid Bordet-Gengou (BG) medium (Gibco-BRL) containing 20%(v/v) defibrinated horse blood (InterMed) for 72 h at 36°C in a moist atmosphere. Alternatively, *B. pertussis* was grown on cyclodextrin solid media (CSM) containing 0.002%(w/v) Congo red dye.

In liquid culture, *B. pertussis* was grown in Stainer and Scholte medium (Stainer and Scholte, 1971), with or without the growth stimulant heptakis (2,6-O-dimethyl) β-cyclodextrin, in 100ml volumes in dimpled 250ml flasks. Cultures were incubated on an orbital incubator at 80rpm at 37°C.

2.3.2. Growth of *E. coli*.

E. coli was grown on nutrient agar (Oxoid) or on McConkey agar containing crystal violet (Difco). Liquid cultures were grown in nutrient broth number 2 (Oxoid) or L-broth. All

cultures were incubated at 37°C; liquid cultures were incubated in 10ml or 100ml volumes on an orbital shaker at 80rpm.

2.3.3. Antibiotics.

Antibiotics were made up in distilled water, except tetracycline (made up in ethanol), filter sterilised and stored at -20°C. They were included in media at the final concentration listed below.

Cephalexin (Ceph)	50µg/ml
Chloramphenicol (Cm)	50µg/ml
Kanamycin sulphate (Km)	50µg/ml
Tetracycline (Tc)	25µg/ml

2.4. MICROBIOLOGICAL TECHNIQUES.

2.4.1. Plate conjugations.

B. pertussis strains were grown on solid BG medium for 72h. Growth from these plates was scraped from the plate using sterile loops, streaked onto a fresh BG plate and incubated overnight.

The donor *E. coli* strain and the helper strain HB101[pRK2013] were incubated overnight in 10ml of nutrient broth containing appropriate antibiotics. The two cultures were then mixed into 100ml of sterile saline and an excess pipetted onto the lawn culture of *B. pertussis* on the surface of the BG plates. After gentle mixing, the excess *E. coli* suspension was removed and the plates incubated overnight. Growth was scraped

from these plates and suspended in 10ml of sterile saline. A 10^{-2} dilution of this suspension was made and 100µl aliquots of the dilution plated onto the selective plates. The selective plates contained cephalexin (final concentration 50µg/ml) to select against *E. coli* and appropriate antibiotics to select for transconjugants. Plates were incubated for 7-10 days, or until single colonies of *B. pertussis* were isolated. Colonies were then restreaked to single colonies on selective plates to eliminate contamination with *E. coli*.

2.4.2. Selection of *B. pertussis* phase variants.

Virulent strains of *B. pertussis* were grown for 72h on BG plates. A large inoculum of these cells was used to inoculate 100ml of Stainer and Scholte medium and grown for 48h. Several 100µl aliquots of this culture were removed and plated onto BG plates containing erythromycin at a final concentration of 0.25µg/ml. A further 10ml of the culture was used to inoculate 100ml of pre-incubated Stainer and Scholte medium. This culture was incubated overnight. Serial dilutions of the turbid culture were then plated onto BG plates containing erythromycin (0.25µg/ml final concentration). These plates were incubated and examined for single non-haemolytic colonies.

2.4.3. Assays for *B. pertussis* virulence factors.

2.4.3.1. Haemolysin.

Haemolysin was assayed visually on BG sandwich plates. These plates have a lower layer of translucent BG agar without added blood, covered by a thin layer of BG agar containing 20%(v/v) defibrinated horse blood. Virulent strains of *B.*

pertussis produce small clear areas of haemolysis on these plates.

2.4.3.2. Filamentous haemagglutinin.

B. pertussis was scraped from BG plates and resuspended in sterile phosphate-buffered saline, pH7.2 (PBS), to a concentration of 10 opacity units. The opacity of the suspension was checked against the WHO 5th International Reference Preparation of Opacity (Perkins *et al.*, 1973). 50µl aliquots of this suspension were added to equal volumes of 2%(v/v) washed horse erythrocytes in U-shaped wells of plastic microtitre trays. After mixing, the trays were incubated for 2h at room temperature before visual assay of haemagglutination. This assay does not appear to measure haemagglutination caused by PT (Parton, 1988).

2.4.3.3. Dermonecrotising toxin.

B. pertussis strains were grown in Stainer and Scholte liquid medium for 48h at 37°C. Culture were harvested by centrifugation at 10 000g for 30min at 4°C. Packed cells were resuspended in saline and frozen. Whilst frozen the cells were disrupted by three passages through an X-press (Life Sciences Laboratories, Sarum Road, Luton). After thawing, the suspension was centrifuged at 100 000g for 30min at 4°C. The supernate of the cell extract was distributed in small amounts and stored at -20°C.

The presence of dermonecrotising toxin in these samples was tested in 3-4-week-old male HaM/ICR mice (Charles River UK Ltd, Manston Road, Margate, Kent). After ether anasthesia, groups

of five or ten mice were given injections of 0.5ml of a suitable dilution of the extract sub-cutaneously in the dorsal region. After 24hrs the animals were killed with CO_2 and the diameters of the haemorrhagic reactions within the skin measured.

2.4.3.4. X-bands.

The presence of the major phase I (X-mode) specific proteins (X-bands) of ≃ 30Kd was determined by SDS-polyacrylamide gele electrophoresis of whole-cell suspensions. *B. pertussis* strains were grown in Stainer and Scholte medium for 48hrs at 37°C and harvested as above. The harvested cells were washed once in sterile saline and then resuspended to ≃2mg protein ml^{-1}. The suspension was then mixed with an equal volume of 125mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.002% bromophenol blue, and heated at 100°C for 5min. The solubilized samples (25µl) were applied to each track of the gel, which consisted of 10% (w/v) in the separating gel and 5% in the stacking gel. The protein profiles also served to confirm the identity of the samples as *B. pertussis*.

2.4.4. Transformation of *E. coli*.

The transformation procedure used throughout this study is based on the method of Lederberg and Cohen (1974).

A fresh overnight culture of the recipient *E. coli* strain was diluted into L-broth and grown until the culture reached an optical density of 0.5-0.6 at 650nm. The cells were then placed on ice for twenty min, recovered by centrifugation (10 000g at

4°C) and resuspended in half the initial culture volume of ice-cold 0.1M magnesium chloride. The cells were immediately re-pelleted and resuspended in approximately 1/20th of the initial culture volume of ice-cold 0.1M calcium chloride. The cells were then left on ice for ≃ 40 min. Approximately 0.5-1µg of DNA was then added to 0.2ml of the cells and left on ice for 30 min. The cells were then heat shocked at 42°C for 2 min and returned to ice for a further 30 min. 1ml of L-broth was then added to the suspension and incubated at 37°C for one hour before plating out 100µl aliquots of the suspension onto selective plates containing appropriate antibiotics.

2.5. IMMUNOLOGICAL TECHNIQUES.

2.5.1. Antibodies used in this study.

The agglutinating antibodies used in this study were rabbit polyclonal absorbedtype-specific antibodies obtained from Dr. N.W. Preston, University of Manchester.

The mouse monoclonal antibody raised against AGG-1 was supplied by Dr. A. Robinson, PHLS, Porton Down.

2.5.2. Slide agglutination tests.

A loopful of *B. pertussis* (approximately 5µl) was scraped from a BG plate and suspended on a clean, grease-free glass slide in two loopfuls of sterile saline. A loopful of the appropriate antiserum was then mixed with the suspension and left for several minutes. A positive agglutination gave large 'granules' of bacteria, whereas a negative result did not alter the suspension. For negative controls the antisera was omitted and a third loopful of sterile saline added. This negative

control procedure allowed any autoagglutination of bacteria to be detected.

2.5.3. Immunoblotting.

Several loopfuls of *B. pertussis* or *B. parapertussis* were scraped from BG plates and resuspended in 2ml of sterile saline to give a fairly turbid suspension. Nitrocellulose membranes (Schleicher and Schull) were pre-wetted in saline for five minutes. The wetted membranes were then placed into the Dot Blot apparatus (Bio-Rad) and the unit assembled as per manufacturers instructions. The wells were then filled with the cell suspensions and surplus wells covered with tape. A vacuum was applied until the cells were firmly attached to the membrane. If lysis of the cells was required the membrane was placed above a dish of chloroform for 10 min. The membrane was then blocked overnight in 5%(w/v) BSA in PBS. After blocking, the filter was given three 15 min washes in PBS with gentle agitation. This was followed by a 2h incubation in a 1 in 10 dilution of the test antibody in BSA 1%(w/v) in PBS. Again, this was followed by three 15 min washes in PBS with gentle agitation. The membrane was then incubated for 2 hours in a 1/3000 dilution of anti-mouse IgG conjugated with horse-radish peroxidase in 1%(w/v) BSA made up in PBS. After a further three 15 min washes in PBS with gentle agitation the membrane was incubated for 30 min in freshly-prepared 4-chloro-1-naphthol solution (see Appendix A.1.7). The membrane was then rinsed in distilled water to terminate the reaction.

All incubations and washes were carried out at room temperature in glass dishes.

2.6. DNA MANIPULATION TECHNIQUES.

The composition of all solutions and buffers not described in the text below is given in Appendix 2.

2.6.1. *B. pertussis* chromosomal DNA preparation.

B. pertussis strains were grown on BG plates for 72h. A large inoculum was taken and used to inoculate 200ml of Stainer and Scholte medium. Liquid cultures were grown for 36-48h until turbid, but still in exponential phase. Cells were harvested at 10,000g for 10 min at 4°C and washed once in TE (Appendix A.2.1) containing 100mM NaCl. The cells were then resuspended in 50ml of chromosomal lysis buffer (Appendix A.2.2) on ice. 50µl of freshly prepared lysozyme (Sigma, 40mg/ml in TE) was added to the suspension and left at room temperature for 5 mins. The following were then added in the given order:- 10µl of proteinase K (Sigma, 20mg/ml in distilled H_2O), 100µl RNase A (BRL, made up at 10mg/ml in distilled H_2O, then boiled for 15 minutes), 400µl of 0.5mM EDTA and 250µl of 10%(w/v) sodium lauroyl sarcosinate (Sigma). The mixture was left on ice until lysis was complete. The lysate was then incubated overnight at 50°C.

If the lysate was too viscous for easy handling after incubation, it was diluted further with lysis buffer. An equal volume of 1:1 TE-saturated phenol (Appendix A.2.3) and chloroform was added to the lysate. The mixture was then emulsified gently and centrifuged at 5,000g for 5 min. The upper phase was removed, avoiding the interphase material. The phenol/chloroform extraction was repeated twice and followed by an extraction with chloroform alone. After the extractions,

1/10th volume 5M NaCl was added and mixed by inversion. Two volumes of ice-cold ethanol were added, mixed and left at -20°C for 1h. After precipitation the DNA was spooled out using a bent pasteur pipette and redissolved in 5ml TE buffer. The DNA solution was then placed in prepared dialysis tubing and dialysed at 4°C against 4l TE for 72h with four changes of TE.

2.6.2. Plasmid preparation.

This protocol, based upon the method of Birnboim and Doly (1979), was routinely used for preparation of plasmid DNA. The given protocol is suitable for a culture volume of 100ml, which yields ≈200µg plasmid DNA; the procedure can be scaled up or down according to the amount of DNA required.

The *E. coli* strain harbouring the plasmid was grown in 100ml of nutrient broth containing the appropriate antibiotic. Cells were harvested at 10,000g for 10 min at 4°C. The pellet was resuspended in 2ml of ice-cold plasmid lysis buffer (Appendix A.2.4) and left for five min at room temperature. Ice-cold 0.2M NaOH containing 1%(w/v) SDS was freshly prepared from stock solutions (2N NaOH, 20%(w/v) SDS), 4ml added to the lysate and the mixture left on ice for 10 min. Acidified potassium acetate solution (Appendix A.2.5)was freshly prepared and 3ml added to the mixture which was then left for a further 10 min on ice. The suspension was centrifuged at 10,000g for 10 min at 4°C. The supernate was then filtered through tissue paper to remove any remaining proteinaceous debris. Approximately 0.6 volumes of isopropanol were then added and the mixture left for 15 min at room temperature, then centrifuged at 10,000g for 10 min. The pellet was gently washed

once with 70%(v/v) alcohol and then freeze-dried for 15 min. The dried pellet was resuspended in 0.5ml TE and, if necessary, the buffer was heated to 65°C to aid dissolving the pellet.

After resuspension of the pellet 200µl of TE-saturated phenol and 200µl of chloroform were added and mixed gently, the mixture was again centrifuged at 10,000g for 10 min. The upper phase was removed to a fresh tube, avoiding the material at the interphase. The phenol/chloroform extraction was repeated, and then followed by an extraction with 400µl of chloroform alone. An equal volume of 5M ammonium acetate was added to the supernate and left on ice for 15 min, then spun again. This step removes the majority of RNA in the solution. The supernate was decanted into a fresh tube and 2 volumes of isopropanol added. The mix was then left on ice for 15 min, spun at 10,000g for 15 min and the supernate removed. The pellet was rinsed in 70%(v/v) alcohol and freeze-dried for 10 min. The dried pellet was resuspended in 200µl TE, again being heated to 65°C if needed.

2.6.3. Concentration and purity of DNA.

DNA concentrations were estimated both spectrophotometrically and visually. The optical density of DNA solutions was measured at both 260nm and 280nm. Long chain duplex DNA solutions at 50µg/ml have an optical density at 260nm of approximately 1. The ratio between the 260nm reading and the 280nm reading for a pure DNA solution is ≈1.7. Alternatively, the concentration of a DNA sample was estimated visually on agarose gels. A known volume of the sample to be tested was run into a 0.5% agarose gel along with samples of λ

DNA (BRL) at known concentrations. After staining with ethidium bromide the intensities of the various bands could be compared to give an estimation of the concentration of the sample.

2.6.4. Digestion of DNA with restriction endonucleases.

The restriction enzymes and incubation buffers used in this study are detailed in Table 2. The methylation sensitive enzymes used are detailed in Table 3.

Digests were routinely performed in a volume of 20µl. Approximately 1µg of DNA was used in each digest with 1-8 units of enzyme. All enzymes were used with the reaction buffers supplied by the manufacturers. Digestions were incubated at 37°C for at least 4h, or overnight. Digests of chromosomal DNA also included 40mM spermidine.

Simultaneous double digests were prepared using a volume of 40µl and twice the amount of DNA. *Bam*HI/*Eco*RI digests were performed in REact™3, *Cla* I/*Stu* I double digests in REact™1.

Instances where the isoschizomers *Cla* I and *Bsc* I were used indisriminately at different stages of the study are shown as *Cla* I (*Bsc* I).

After digestion 5µl loading buffer (Appendix A.2.7) was added to the sample prior to electrophoresis.

2.6.5. Size markers for electrophoresis.

2µg of λ DNA was digested with *Hin*dIII at 37°C for 3h. The ensuing fragments were then either used directly on agarose gels or labelled with ^{32}P d-ATP (see section 2.6.6.) before use.

TABLE 2: RESTRICTION ENZYMES USED IN THIS STUDY.

ENZYME	CLEAVAGE SITE	BUFFER*	SUPPLIER
Apa I	$^{5'}$GGGCCC$^{3'}$	REact™4	BRL
Bam H1	$^{5'}$GGATCC$^{3'}$	REact™3	BRL
Bsc I	$^{5'}$ATCGAT$^{3'}$	Bsc Buffer	NBL
Cla I	$^{5'}$ATCGAT$^{3'}$	REact™1	BRL
Eco R1	$^{5'}$GAATTC$^{3'}$	REact™3	BRL
Hin dIII	$^{5'}$AAGCTT$^{3'}$	REact™2	BRL
Kpn I	$^{5'}$GGTACC$^{3'}$	REact™4	BRL
Pst I	$^{5'}$CTGCAG$^{3'}$	REact™2	BRL
Sph I	$^{5'}$GCATGC$^{3'}$	REact™6	BRL
Stu I	$^{5'}$AGGCCT$^{3'}$	REact™2	BRL

* Composition of buffers is given in Appendix 3.
NBL = Northumbria Biologics Limited, Cramlington, Northumbria.

TABLE 3: METHYLATION SENSITIVE RESTRICTION ENDONUCLEASES USED IN THIS STUDY.

ENZYME	RECOGNITION SEQUENCE	METHYLATION SENSITIVITY	BUFFER*
Mbo I	$^{5'}$GATC$^{3'}$	No cleavage if A residue methylated, cleavage if C residue methylated.	REact™2
Sau 3A	$^{5'}$GATC$^{3'}$	No cleavage if C residue methylated, cleavage if A residue methylated.	REact™4
Dpn I	$^{5'}$G^{Me}ATC$^{3'}$	Will cleave only if A residue methylated.	REact™4
Alu I	$^{5'}$AGCT$^{3'}$	No cleavage if G or A residue methylated.	M buffer
Cfo I	$^{5'}$GCGC$^{3'}$	No cleavage if either C residue methylated.	M buffer
Hpa II	$^{5'}$CCGG$^{3'}$	No cleavage if internal C residue methylated.	REact™8
Msp I	$^{5'}$CCGG$^{3'}$	Cleavage irrespective of methylation of internal C residue.	REact™1

All enzymes obtained from BRL, except Alu I and Cfo I obtained from Northumbria Biologicals Limited.

* See Appendix 3 for details of buffer composition.

Hae III fragments of ΦX174 DNA (BRL) were used as size markers for low molecular weight fragments.

The size of the fragments generated in the above procedures are given in Appendix 5.

2.6.6. Radioactive labelling of size markers.

500ng of λ-*Hin* dIII digest fragments were resuspended in 50μl of 1 X Core Buffer (BRL). To this was added 10μl of Å solution, 1μl of α-^{32}P-dATP (300 Ci/mmol; Amersham Corp.) and 1μl of the Klenow fragment of *E. coli* DNA polymerase (BRL). The reaction was incubated for ≃15 min at room temperature and then loaded onto agarose gels after the addition of loading buffer.

2.6.7. Agarose gel electrophoresis.

Horizontal gel systems (Pharmacia) were used throughout this study. Type II-A medium EEO agarose (Sigma) was used in analytical gels, Low gelling temperature agarose (Seaplaque) was used in preparative gels. Analytical gels were prepared and run using 0.5 X TBE; preparative gels using 1 X TAE.

Gels were routinely run at ≃15V/cm. After running, gels were stained for 15 minutes in ethidium bromide (final concentration 1mg/ml), then destained in distilled water for an equal length of time. DNA fragments were visualised under ultraviolet illumination. Photographs were taken through a red filter, using Polaroid type 667 film.

The size of fragments were calculated by using a graph of the mobility of the markers against the $\log_{10}$ of their length in base pairs as a calibration curve for that gel. Restriction

fragment maps were made using the technique of Fitch *et al.* (1983) which employs a branch and bound technique.

2.6.8. Polyacrylamide gel electrophoresis.

5% polyacrylamide gels were used in this study. 16.6ml of a 30%(w/v) acrylamide solution were added to 71.3 ml of distilled H_2O, 10ml of 10 X TBE and 2.1ml of freshly prepared 3%(w/v) ammonium persulphate solution. This mixture was deaerated using a side-arm flask. The solution was mixed gently and during mixing 30µl of TEMED (N,N,N',N'-tetramethylethylene diamine) was added. The solution was immediately poured between two grease-free glass plates sealed together with tape. The comb was inserted at the top of the gel, which was left for several hours until polymerisation was complete. The reservoirs of the electrophoresis tank were filled with 1 X TBE and the gel placed in position. The wells were filled with sample in loading buffer, and the gel run at 15V/cm for 3h, or until the gel front reached the bottom of the gel. After running, one glass plate was prised carefully from the gel and the gel stained and visualised as described for agarose gels.

2.6.9. Southern blotting.

DNA fragments were transferred from agarose gels onto nylon membranes using a vacuum blotting system (LKB 2016 Vacugene). Conditions employed were dependent on the membrane being used.

Zeta-probe membranes (Bio-Rad) were pre-wetted in distilled water and laid on the apparatus. The apparatus was assembled according to the manufacturer's instructions. The vacuum was switched on and stabilised at 50cm H_2O.

Approximately 30ml of 0.25M HCl was pipetted over the surface of the gel and left for 4 min, after this time the excess was removed and the gel submerged in 0.4N NaOH. The gel was left under vacuum for 50 min. After transfer the gel was lifted off and re-stained in ethidium bromide to check the degree of transfer. The membrane was then rinsed in 2 X SSC, left to air dry and then placed immediately in pre-hybridisation solution.

Hybond-N (Amersham) and nitrocellulose membranes were pre-wetted in 2 X SSC and placed in the apparatus as above. Approximately 30ml of 0.25M HCl was pipetted over the gel and left for 4 min. After removal of excess HCl, an equal volume of denaturing buffer was pipetted over the gel and left for 3 min, after which any excess was removed. An equal volume of neutralising buffer was then pipetted over the gel, left a further 3 min and removed. The gel was submerged in 20 X SSC for the 50 min transfer period. The membranes were rinsed in 2 X SSC after transfer.

Hybond-N membranes were then wrapped in Saran Wrap (Dow Chemical Company) and the side of the membrane with the bound DNA was exposed to UV light for 5 min. The membrane was then either used immediately or stored at 4°C.

Nitrocellulose membranes were baked in a vacuum oven at 80°C for 2h and then used immediately.

2.6.10. Hybridisation conditions.

Hybridisation was carried out under two sets of conditions:- i) high stringency, and ii) reduced stringency.

1. High stringency conditions.

Membranes were placed into heat-sealable polythene bags, 20ml of pre-hybridisation buffer added and all air bubbles were removed from the bag before it was sealed. The membrane was incubated with this solution in a shaking water bath at 42°C overnight with gentle agitation. After incubation the pre-hybridisation buffer was discarded and 20ml of hybridisation buffer added to the bag. The radio-labelled probe was then added to the bag, the air bubbles removed and the bag sealed. The membrane was again incubated overnight at 42°C.

After incubation the membrane was removed from the bag and rinsed twice in 2 X SSC at room temperature, twice in 2 X SSC, 0.1%(w/v) SDS at 65°C for 30 min with agitation and once in 0.1 X SSC, 0.1%(w/v) SDS at 65°C for 15 min with agitation.

2. Reduced stringency conditions.

The manipulation of the membranes was identical under both sets of conditions. However, different pre-hybridisation and hybridisation buffers, and washing conditions were employed. The composition of these buffers, the conditions they were used in and details of the washing procedures are given in the relevant Results section.

After washing, membranes hybridised under both sets of conditions were blotted dry and immediately wrapped in Saran Wrap. The wrapped membrane was then placed in an autoradiography cassette loaded with Kodak X-OMATR X-ray film and intensifying screens. Autoradiographs were exposed at -70°C or room temperature for varying periods of time. After

exposure the film was developed in Kodak DX80 developer and fixed using Kodak FX40 diluted fixing solution.

2.6.11. Preparation of radioactively labelled probes.

Probes were labelled using the random hexanucleotide primer method of Feinberg and Vogelstein (1984).

1-2μg of plasmid DNA was cleaved with the appropriate restriction endonuclease(s). The products were separated electrophoretically on 1%(w/v) low melting temperature agarose gel made up and run in 1 X TAE. After staining to visualise the fragments the required band was excised and weighed. The gel slice was placed in a microfuge tube and 3ml of distilled H_2O added per gram of gel. The two were boiled together for 7 min and then stored at -20°C.

When required the gel was reboiled for 5 min and incubated at 37°C for a further 15 min. The following were added to a fresh microfuge tube in the given order:- i) H_2O (to give a final reaction volume of 50μl), ii) 10μl of oligo-labelling buffer (OLB), iii) 2μl of bovine serum albumin (Sigma, 10mg/ml), iv) 10 - 30μl of the boiled DNA in low melting temperature (LMT) agarose (volume added being dependent on the concentration of DNA within the agarose), v) 5μl of α-^{32}P-dATP (300μCi/mmol; Amersham), and vi) 2 units of Klenow fragment of *E. coli* DNA polymerase (BRL). The reaction was left overnight at room temperature, and terminated by the addition of 200μl of STOP buffer.

2.6.12. Biotin-labelled probes.

1. Labelling of probes with biotin.

The labelling reaction is essentially as described in 2.6.11. except that the α-^{32}P-dATP is replaced by the same concentration of biotin-7-dATP (BRL).

2. Use of biotin-labelled probes.

The filters used for hybridisation with biotin-labelled probes are prepared as described previously (section 2.6.9). The hybridisation conditions used were also as described (section 2.6.10). The concentration of formamide in the hybridisation mixture was decreased empirically due to the slight decrease in the melting temperature of the biotin-labelled probe/target duplex as compared to the ^{32}P-labelled probe/target duplex.

3. Development of filter after hybridisation.

The filter was removed from the hybridisation bag and rinsed for 1 min in 0.1M Tris-HCl (pH 7.5), 0.15M NaCl (buffer A). The filter was then incubated in 3% (w/v) bovine serum albumin (BSA) (Fraction V) (Sigma) made up in buffer A at 65°C for 1 hour. After incubation the filters were blotted dry and then baked in a vacuum oven at 80°C for 20 mins. Filters were then rehydrated in the 3% (w/v) BSA solution.

The following steps were performed in polypropylene or glass vessels. The streptavidin-alkaline phosphatase (SA-AP) conjugate (BRL) was diluted to 1.0µg/ml in buffer A. Approximately 7.0ml of SA-AP conjugate were prepared per 100cm^2 of filter. The filters were then incubated in this solution for

10 mins with gentle agitation and occasionally pipetting. After incubation the used SA-AP conjugate was decanted and the incubation repeated using fresh SA-AP conjugate. After this second incubation the filters were washed in buffer A, using a volume at least 30 times that of SA-AP conjugate used in the previous incubation. Filters were washed for 15 mins and the wash repeated using fresh buffer A.

The dye solution was prepared, immediately prior to use, by adding 33µl of NBT solution (BRL) to 7.5ml of 0.1M Tris-HCl (pH9.5), 0.1M NaCl, 50mM $MgCl_2$, mixed gently, and 25µl BCIP solution (BRL) added. Approximately 7.5ml of this dye solution were prepared per 100cm^2 of filter. The filter was incubated in this dye solution in a dim light or darkness until a signal was observed. Filters were then washed briefly in 20mM Tris-HCl(pH7.5), 0.5mM EDTA and then baked in a vacuum oven at 80°C for 2 minutes.

2.6.13. Synthesis and labelling of oligonucleotide probes.

Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer at PHLS CAMR, Porton Down, using the phosphoramidite coupling procedure. By-products of the reaction were removed by gel exclusion chomatography using a Pharmacia P-10 (Sepahdex G25) column pre-equilibrated with 10mM ammonium acetate elution buffer. The concentration of the oligonucleotide was estimated spectrophotometrically.

Labelling of oligonucleotide probes was performed using the method of Woods (1984). 15µl of fresh γ-^{32}P-dATP (3000Ci/mmol; Amersham) and 200-300ng of oligonucleotide were dried under vacuum and resuspended in 7µl of H_2O. 1µl of 10 X T4

polynucleotide kinase buffer and 1µl of T4 polynucleotide kinase (BRL) were then added and mixed. The reaction was incubated at 37°C for 30 min and terminated by heating to 65°C for 5 min.

2.6.14. Hybridisation with oligonucleotide probes.

Restriction digests of *B. pertussis* chromosomal DNA were transferred to nylon membranes as described. The membranes were then placed in a heat sealed polythene bag with 20ml of oligonucleotide pre-hybridisation buffer and incubated in a shaking water bath at 37°C for 3h. After this time the bag was drained and 20ml of oligonucleotide hybridisation buffer containing ^{32}P-labelled probe added. The membrane was then incubated overnight at varying temperatures. Following incubation the membrane was removed from the bag and rinsed several times at room temperature in 6 X SSC, 0.05%(w/v) sodium pyrophosphate (SSC/PP). The membrane was then washed in SSC/PP for 1h at 30°C, followed by a 10 min wash in SSC/PP at various temperatures, the temperatures used for each different experiment are given in the relevant Results section. Membranes were then autoradiographed as described.

2.6.15. Colony hybridisations.

Clones from the *B. pertussis* Tohama gene library were grown in sterile 96 well microtitre plates overnight in nutrient broth containing tetracycline. They were then transferred using a 48 prong replicator onto nitrocellulose filters placed on the surface of nutrient agar plates. The plates were incubated overnight at 37°C. The membranes were then placed on sheets of 3MM paper (Whatman) saturated with denaturing solution for 3

minutes. After denaturing the membranes were transferred onto 3MM paper saturated with neutralisation buffer for 5 min. Membranes were then placed on paper saturated with 3 X SSC for 3 min. The membranes were again transferred onto dry 3MM paper and allowed to air dry for 30 min. The membranes were then stacked between sheets of dry 3MM paper and baked in a vacuum oven at 80°C for 2h. Subsequently, the membranes were hybridised with either oligonucleotide or plasmid DNA probes using the methods detailed previously.

2.6.16. Two-dimensional S1 nuclease heteroduplex mapping.

The method is based on the protocol of Yee and Inouye (1984).

At least 100µg of *B. pertussis* DNA was used in each experiment. The DNA samples were concentrated down to approximately 100µl using alcohol precipitation and butanol concentration. Samples from parent and variant pairs were both digested with a four base-pair recognition restriction enzyme, usually *Sau* 3A. After overnight digestion at 37°C the reaction was stopped by the addition of EDTA to a final concentration of 10mM. Digestion was checked by removing ≃1µg of both digests and running them on a 1%(w/v) agarose mini-gel (Pharmacia). The remaining DNA was extracted twice with chloroform/isoamyl alcohol (24:1) then precipitated with an equal volume of isopropanol after the addition of 1/10 volume of 5M sodium acetate. After pelleting by centrifugation and freeze-drying for fifteen minutes the pellet was resuspended in 100µl of TE. The DNA was then re-precipitated with 2.5 volumes of ethanol after the addition of 1/20 volume of 3M sodium acetate. The

pellet was then resuspended in 90µl of 50%(v/v) deionised formamide/100mM Tris.HCl (pH8.5)/10mM EDTA. A mixture of 30µl from each of the paired DNA samples was used to give a heteroduplex solution, the remaining 60µl of each sample were homoduplex solutions. All three samples were heat denatured for 5 min in a boiling water bath and left at room temperature for ≈20h to renature.

After renaturation, loading buffer was added to all three samples and they were then added to the 1.6cm wells of a 5% polyacrylamide gel (17cm long by 0.25cm thick) prepared in TBE and electrophoresed at 15V/cm until the bromophemol blue in the running buffer had reached the end of the gel. Each lane containing the DNA was then cut from the gel and trimmed to 11.5cm long. These strips were then sealed in polythene bags and incubated for two 2h washes in 50ml of S1 nuclease reaction buffer. The strips were then soaked for 24h in 10ml each of S1 nuclease buffer containing S1 nuclease (BRL) at 70 units/ml for 24h with occasional agitation at 4°C. The S1 nuclease reaction was stopped by soaking the strips in 50ml of 2 X TBE for 1½h followed by three soakings in 0.2 X TBE for a minimum of 2h each at room temperature. The gel strips were then loaded on to the top of a second 5% acrylamide gel made up in 0.2 X TBE. Due to swelling of the strips during the soaking procedures these second gels were prepared 25% thicker. The gel strips were sealed onto the surface of the second gels with 0.7%(w/v) agarose made up in 0.2 X TBE. The second gel was electrophoresed at 11V/cm until a sample of bromophenol blue added to the surface of the second gel reached the bottom. The

gels were removed from one glass plate and stained with ethidium bromide (final concentration 0.4μg/ml) and visualised as previously described.

3. RESULTS.

3.1. ISOLATION AND TESTING OF Vir⁻ VARIANTS ISOGENIC WITH THEIR PARENTAL *B. pertussis* STRAINS.

B. pertussis strains Taberman and Tohama were grown in Stainer and Scholte liquid medium and plated onto selective BG plates containing erythromycin (final concentration 0.25µg/ml) as described in section 2.4.2. Several erythromycin resistant non-haemolytic colonies of both strains were picked from the selective BG plates. The exact frequency of the variants was not calculated but was estimated to be within the limits quoted by Weiss and Falkow (1984). Parallel experiments in which *B. pertussis* grown in Stainer and Scholte medium were plated onto BG plates without erythromycin did not give rise to any non-haemolytic colonies.

Following removal from the erythromycin-containing selective BG plates, variant strains were then grown only on normal BG plates. In the course of this subculturing, up to 12 passages in some cases, no revertant haemolytic colonies were detected. The colonies of the non-haemolytic, variant strains were more fluid and emulsified more easily in sterile saline than colonies from the parent strains.

Several of the virulence-associated properties of these non-haemolytic strains were tested and compared to those of several control strains. The results of these comparisons are shown in Table 4. Both strain Taberman and strain Tohama shared the same characteristics as strain L84I, the control virulent strain. The non-haemolytic variant strains of Taberman and Tohama had the same properties as strain BP347, an avirulent

TABLE 4: COMPARISON OF SEVERAL VIRULENCE-ASSOCIATED FACTORS OF VIRULENT, AVIRULENT AND ERYTHROMYCIN RESISTANT STRAINS OF *B. pertussis*.

STRAIN	FHA ACTIVITY	HLY ACTIVITY	GROWTH ON NA	CONGO RED BINDING
L84I	+	+	—	+
L84IV	—	—	+	—
BP347	—	—	—	—
Tohama	+	+	—	+
Tohama ery^r	—	—	—	—
Taberman	+	+	—	+
Taberman ery^r	—	—	—	—

FHA activity was tested by haemagglutination of horse erythrocytes in microtitre trays (section 2.4.3.2) and haemolysin activity was assayed visually on BG agar sandwich plates (section 2.4.3.1). To confirm lack of growth on nutrient agar the strains were streaked onto nutrient agar plates and incubated for 7 days at 37°C in moist conditions, growth was checked visually. The ability of the cells to take up Congo red dye was tested by growing the cells on cyclodextrin medium containing 0.002%(w/v) Congo red. The cells were incubated on this medium for 3 days at 37°C and uptake of Congo red checked visually.

strain containing a Tn5 insertion within the *bvg* locus, but did not grow on nutrient agar as L84IV, a Phase IV control strain, did.

Whole-cell suspensions of both parent and variant strains were analysed using SDS-PAGE gels. These gels revealed that the avirulent and Phase IV control strains plus the variant strains were deficient in the 28 and 30Kd virulence-associated X-bands, which were present in both the virulent control strains and the parent strains (R. Parton, personal communication). In addition, the variant strains did not express any dermonecrotic toxin activity when tested sub-cutaneously in mice (H. Frampton, personal communication).

Conjugation of the cosmid clone pRMB2 (Brownlie *et al.*, 1988) containing the *bvg* locus into the variant strains was sufficient to restore expression of all the virulence-associated factors tested.

Cosmid pRMB2 was cured by three passages of the cosmid-containing strains on BG plates unsupplemented by tetracycline. Loss of the cosmid was confirmed by replica plating onto BG plates ± tetracycline (25μg/ml). All colonies which had been passaged three times had lost the cosmid and did not grow on BG plates containing tetracycline. When tested, the cured strains had lost the ability to express any of the virulence-associated factors tested.

Several of the virulence-associated factors produced by the strains containing pRMB2 were assayed. When grown on BG sandwich plates containing tetracycline (25μg/ml) the haemolytic zone surrounding the cosmid-containing colonies was

noticeably larger than the zone surrounding the wild-type colonies. The cosmid-bearing strains grew only half as well as the parental wild-type strain in liquid culture but did produce three times as much dermonecrotizing toxin per gram of bacterial protein as the wild-type (H. Frampton, personal communication). This increase in haemolytic zone and dermonecrotizing toxin activity was also observed when pRMB2 was conjugated into the parent virulent strains.

In view of these analyses the erythromycin resistant, non-haemolytic strains were deemed to fulfil the criteria required of avirulent (Vir$^-$)strains using the definition of Weiss and Falkow (1984). These avirulent variant strains were assumed to be isogenic with the parent strains, apart from the genetic event responsible for the observed phase variation.

3.2. ANALYSIS OF CHROMOSOMAL DNA FROM ISOGENIC VIR$^+$ AND VIR$^-$ *B. pertussis* STRAINS.

3.2.1. Analysis using restriction endonucleases.

Chromosomal DNA prepared from the isogenic virulent and avirulent strains of *B. pertussis* described above were digested to the same degree by all restriction endonucleases listed in Table 2 except *Hin* dIII, which did not digest *B. pertussis* DNA from any source. In stained agarose electrophoresis gels of chromosomal digests there was no apparent difference in restriction patterns between DNA from virulent and avirulent strains (data not shown). The inclusion of cyclodextrin into the Stainer and Scholte growth medium of *B. pertussis* strains

used for DNA preparation had no effect on the digestibility of the DNA produced.

Chromosomal DNA from the isogenic virulent and avirulent strains were digested with restriction enzymes which are affected by the methylation status of specific residues within their recognition sequence (see Table 3). The results of these digests were run on 2% agarose gels and 5% polyacrylamide gels, see Figure 10.

The enzymes *Mbo* I, *Sau* 3A and *Dpn* I are isoschizomers, they recognise and digest the same sites. However, the effect of methylation is different for each enzyme (see Table 3 for details). Chromosomal DNA from the virulent and avirulent strains was digested to an equal degree by both *Mbo* I and *Sau* 3A, although the digests of the paired DNA samples produced using the enzyme *Mbo* I appeared to have slightly different restriction patterns. Several bands, of approximately 2-3kb, appear to be missing from digests of DNA from avirulent strains. This difference is not apparent in the patterns obtained from Sau 3A digestion. The enzyme *Dpn* I will not digest DNA unless the adenine residue within the recognition site is methylated, *Dpn* I did not digest any DNA samples from virulent or avirulent strains.

There was no gross difference in the restriction pattern of the paired DNA samples digested with *Alu* I or *Cfo* I.

The restriction enzymes *Msp* I and *Hpa* II are isoschizomers, again differing in the reaction to methylation within their recognition and cleavage sequence (Table 3). Chromosomal DNA from both virulent and avirulent strains was fully digested by

FIGURE 10. DIGESTION OF *B. pertussis* CHROMOSOMAL DNA WITH METHYLATION-SENSITIVE RESTRICTION ENDONUCLEASES.

Photograph A shows digested *B. pertussis* chromosomal DNA running in a 2% agarose gel:

Lane 1 contains Tohama DNA digested with *Mbo* I.

Lane 2 contains Tohama DNA digested with *Dpn* I.

Lane 3 contains Tohama Vir$^-$ DNA digested with *Mbo* I.

Lane 4 contains Tohama Vir$^-$ DNA digested with *Dpn* I.

Lane 5 contains Taberman DNA digested with *Mbo* I.

Lane 6 contains Taberman DNA digested with *Dpn* I.

Lane 7 contains Taberman Vir$^-$ DNA digested with *Mbo* I.

Lane 8 contains Taberman Vir$^-$ DNA digested with *Dpn* I.

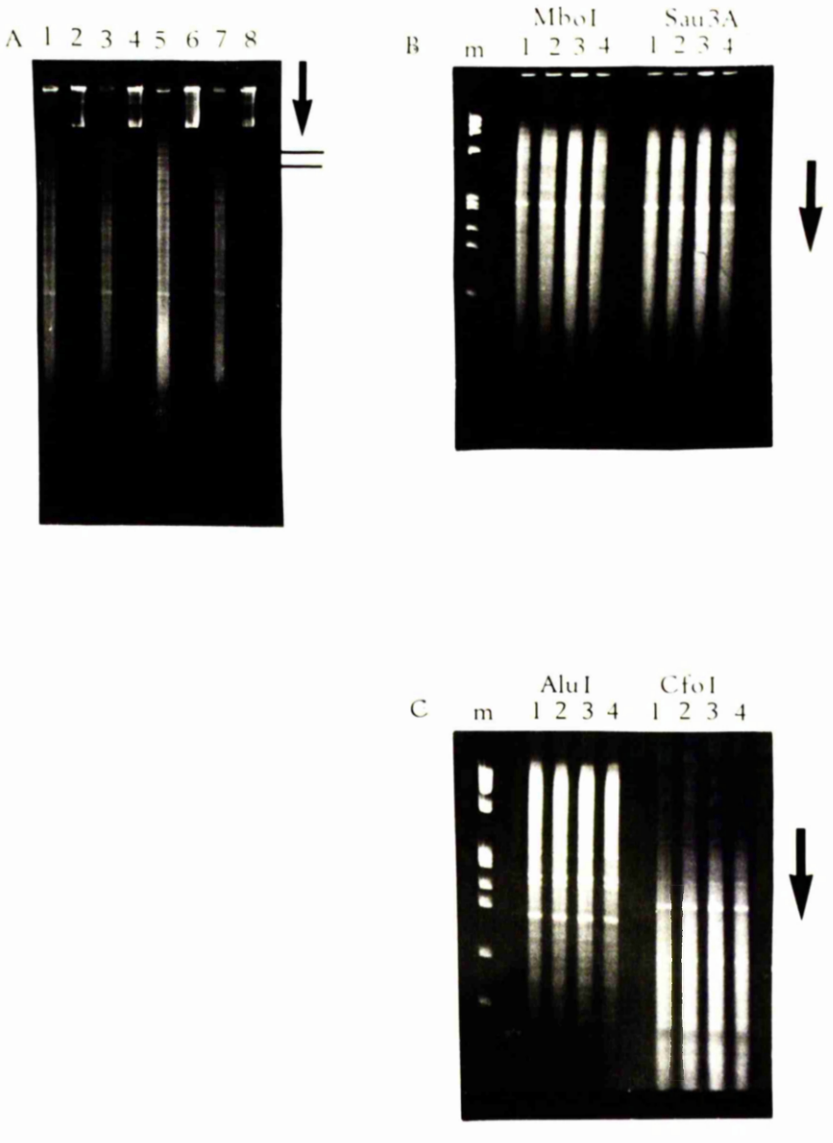
The two smaller arrows indicate the position of the ≃1.5kb bands missing from *Mbo* I digests of the Vir$^-$ DNA samples.

Photographs B and C show digested *B. pertussis* chromosomal DNA running in a 5% polyacrylamide gel.

In both photographs, lane 1 contains Tohama DNA, lane 2 Tohama Vir$^-$ DNA, lane 3 Taberman DNA and lane 4 Taberman Vir$^-$ DNA. The enzyme used to digest the samples is given above each quartet of lanes.

The molecular weight markers used in photographs B and C are ΦX174 *Hae* III fragments, the size of these fragments is given in Appendix 5.

In all the photographs the large arrow indicates the direction of migration.


A 1 2 3 4 5 6 7 8
B m MboI 1 2 3 4 Sau3A 1 2 3 4
C m AluI 1 2 3 4 CfoI 1 2 3 4

Hpa II , whereas none of the DNA samples were digested by *Msp* I.

Chromosomal DNA was prepared from strains of *B. pertussis* grown in media that caused antigenic modulation. The virulence factors produced by these variant strains were tested and the absence of expression confirmed that the strains were C-mode cells (D. McGillivray, personal communication). The DNA from the X-mode parental and the C-mode variant strains exhibited the same digestion patterns as DNA from the Vir^+ parental and Vir^- variant strains respectively.

3.2.2. Analysis using 2-dimensional S1 nuclease heteroduplex mapping.

Control experiments performed on chromosomal DNA prepared from *E. coli* strain HB101 gave results similar to those described by Yee and Inouye (1984). However, the technique proved to be virtually useless for studying the DNA of *B. pertussis*. The region of the second dimension gel in which any putative rearrangement involved in phase variation would be seen was totally obscured by a mass of DNA.

3.2.3. Analysis using radioactively-labelled probes.

3.2.3.1. The *bvg* locus.

The plasmid pRMB2 (Brownlie *et al.*, 1988) was radioactively labelled and hybridised to chromosomal digests of parent and variant DNA samples. *Eco* RI digests probed with pRMB2 gave rise to the hybridisation pattern described by McGillivray *et al.* (1989). This pattern consists of six *Eco* RI fragments with the following sizes:- 10.0kb, 5.1kb, 4.7kb, 2.7kb, 2.5kb and 1.1kb.

All the variant DNA samples tested gave this pattern except BP347 in which the 2.7kb fragment is replaced by an 8.4kb fragment which contains Tn5 (data not shown).

3.2.3.2. The *fim* 2 gene locus.

The *fim* 2 gene isolated from a *B. pertussis* Wellcome 28 gene library by Livey *et al.* (1987) is bracketed by a pair of *Sph* I cleavage sites. The plasmid pIL22, containing the *fim* 2 locus, was digested with SphI and the 0.85kb fragment containing the *fim* 2 gene isolated and used as a probe. This fragment also contains part of the repeated sequence IS481v2 (McPheat *et al.*, 1989).

DNA from the parent and variant strains of *B. pertussis* and *B. parapertussis* strain 10520, was double-digested with *Bam* HI and *Eco* RI, to yield small fragments which can be subsequently cloned, and blotted onto a nylon membrane. The *fim* 2 probe hybridised to this blot under high stringency conditions (final wash, 0.1 X SSC at 65°C for 30 minutes). The results of this hybridisation are shown in Figure 11.

The *fim* 2 gene probe hybridised to at least 40 bands in the *B. pertussis* lanes. There are differences in the pattern of these bands between the DNA samples tested, particularly in the region of the low molecular weight fragments. The pattern of bands in the DNA derived from the two pairs of parent and variant strains are distinct from each other and from the pattern found in L84I DNA. Differences can also be seen between the paired DNA samples but the number of bands seen in these

FIGURE 11. PROBING OF *B. pertussis* AND *B. parapertussis* DNA WITH THE *fim* 2 GENE PROBE.

The figure shows *B. pertussis* and *B. parapertussis* chromosomal DNA which had been double-digested with *Eco*RI and *Bam*HI, run on a 0.8% agarose gel and then Southern-blotted as described (section 2.6.9). The blotted DNA was hybridised with the 0.85kb *Sph* I fragment of pIL22 containing the *fim* 2 gene and part of IS481v2. The final wash for this experiment was carried out in 0.1 X SSC at 65°C for 30 minutes.

The autoradiograph was exposed to the filter for five days at -70°C prior to developing.

Key to figure:-

m = position of λ *Hin*dIII fragments.

1 = L84 I DNA.

2 = Taberman DNA.

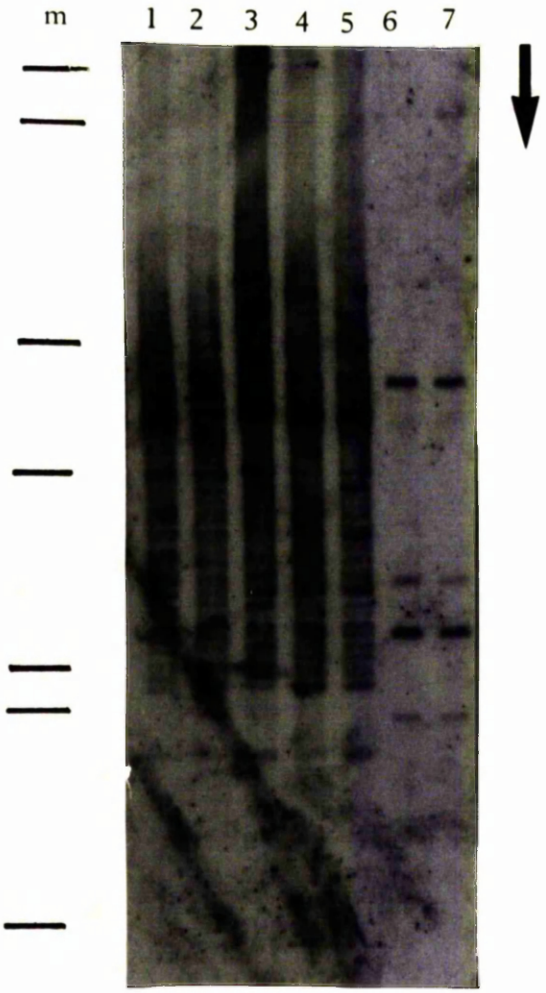
3 = avirulent Taberman DNA.

4 = Tohama DNA.

5 = avirulent Tohama DNA.

6 = *B. parapertussis* 10520 DNA.

7 = *B. parapertussis* 59521 DNA.


m
1 2 3 4 5 6 7

patterns does not allow any significance to be attached to these differences.

The probe hybridised to 4 bands in the *B. parapertussis* lanes and to 4 similar bands in a double digest of *B. bronchiseptica* chromosomal DNA (data not shown). In an experiment performed under identical conditions the *B. pertussis* results were repeated but there was no hybridisation observed to *Bam* HI/*Eco* RI double digests of DNA prepared from several strains of *B. avium* (data not shown).

3.3. ISOLATION OF *fim* GENES FROM *B. pertussis* STRAIN TABERMAN.

3.3.1. Probing of *B. pertussis* strain Taberman gene library.

A gene library prepared using DNA from *B. pertussis* strain Taberman (fimbrial serotype 0,3) (Brownlie *et al.* 1986) was grown on nitrocellulose filters, as described, and probed with a radioactively-labelled oligonucleotide.

The oligonucleotide was formulated by Livey *et al.* (1987) and was homologous to the amino terminal sequence of the *B. pertussis fim* 2 gene. They used this oligonucleotide probe to isolate the *fim* 2 gene from a *B. pertussis* gene library constructed using DNA from strain Wellcome 28 (fimbrial serotype 2,3). The conditions used by Livey *et al.* (1987) to isolate the *fim* 2 gene involved an overnight hybridisation at 65°C and a one hour final wash in SSC/PP also at 65°C. These conditions were specific only for the *fim* 2 gene.

The sequence of this oligonucleotide probe was as follows:-

5'-GTGTCGGTGATGGTGCCGGTGATGACGATGGTGCCGTCGTC-3'

This probe was radio-labelled and hybridised to the gene library overnight at 50°C with a final wash at 50°C for one hour in 6 X SSC/0.05% (w/v) sodium pyrophosphate. Nine of the 520 cosmid clones in the Taberman gene library hybridised with the probe under these reduced stringency conditions.

3.3.2. Analysis of the putative *fim* gene-containing clones.

3.3.2.1. Analysis using restriction endonucleases.

The nine cosmids which hybridised to the oligonucleotide probe were analysed using *Cla* I (*Bsc* I) and *Sal* I. *Cla* I (*Bsc* I) was used to check for the presence of the 0.6kb fragment homologous with the *hin*-containing probe (section 3.4.3). *Sal* I was used to identify the signal fragments for the various genes identified by Mooi *et al.* (1987). The size of the insert in each cosmid was found by estimating the total size of the cosmid and subtracting 21.6kb (the size of pLAFR1), the insert sizes are given in Table 5. Digestion of the cosmids revealed that the nine cosmids could be divided into three distinct groups sharing a number of common bands.

The *Cla* I (*Bsc* I) and *Sal* I bands shared by the clones are summarised in Table 6. In all the restriction digests performed cosmids pDA126 and pDA128 shared the same restriction patterns and were therefore deemed to be identical cosmids containing the same piece of DNA, thereafter only pDA126 was used.

As can be seen from Table 6 the eight clones can be divided into three groups. The clones in each group presumably contain the same *fim* gene, which is present on the shared fragments. The number of shared *Sal* I fragments makes identification of the *fim* gene carried by each clone difficult. The clones were therefore probed with a *fim* gene containing probe.

3.3.2.2. Hybridisation with *fim* 2 gene probe.

The 0.85kb *Sph* I fragment of *B. pertussis* DNA containing the *fim* 2 gene and a part of IS481v2 described in Section

TABLE 5: INSERT SIZES IN THE NINE COSMIDS CONTAINING PUTATIVE *fim* GENES.

COSMIDS	INSERT SIZE.
pDA126	21.2kb
pDA128	21.2kb
pDA163	19.0kb
pDA192	25.0kb
pDA204	18.6kb
pDA216	22.5kb
pDA319	23.3kb
pDA395	18.6kb
pDA446	24.0kb

TABLE 6: *Cla* I (*Bsc* I) AND *Sal* I FRAGMENTS SHARED BY THE COSMIDS CONTAINING PUTATIVE *fim* GENES.

Cla I (*Bsc* I) FRAGMENTS

GROUP 1		GROUP 2			GROUP 3		
pDA126	pDA395	pDA216	pDA319	pDA446	pDA163	pDA192	pDA204
4.6kb	4.6kb	6.4kb	6.4kb		4.7kb		4.7kb
2.4kb	2.4kb	3.7kb	3.7kb	3.7kb	3.9kb	3.9kb	3.9kb
0.83kb	0.83kb					3.0kb	3.0kb
						1.32kb	1.32kb
					0.8kb		0.8kb

Sal I FRAGMENTS

2.3kb	2.3kb	2.7kb	2.7kb	2.7kb		5.0kb	5.0kb
2.0kb	2.0kb	2.3kb		2.3kb	2.0kb	2.0kb	2.0kb
1.6kb	1.6kb	1.8kb	1.8kb	1.8kb		1.8kb	1.8kb
1.5kb	1.5kb	1.65kb	1.65kb		1.5kb	1.5kb	1.5kb
0.9kb	0.9kb	1.45kb	1.45kb		1.4kb	1.4kb	
0.8kb	0.8kb	1.3kb	1.3kb	1.3kb	1.3kb	1.3kb	1.3kb
		1.25kb	1.25kb	1.25kb		1.0kb	1.0kb
		1.1kb	1.1kb	1.1kb	0.95kb	0.95kb	0.95kb
		1.07kb	1.07kb	1.07kb			
		1.04kb	1.04kb	1.04kb			

3.1.3.2., was radio-labelled and used to probe *Sal* I digests of each of the eight clones. These hybridisations were carried out at reduced stringency conditions, final wash 1 X SSC, 0.1%w/v SDS at 50°C for 1 hour. The probe hybridised to three bands in pDA126 and pDA395 and to two bands in the other digests, see Figure 12. The bands divided the eight cosmids into the groups detailed above. The probe hybridised to a 2.7kb, a 1.6kb and a 0.9kb band in the cosmids pDA126 and pDA395, to 0.5kb and 0.4kb bands in cosmids pDA163, pDA192 and pDA204, and to 1.65kb and 1.4kb bands in pDA216, pDA319 and pDA446. Hybridisation was noticeably stronger to pDA216, pDA319 and pDA446.

Mooi *et al.* (1987) have reported that each *fim* gene is carried on a distinct *Sal* I fragment. These fragments are:- a 0.9kb fragment containing the *fim* 3 gene, a 1.7kb fragment carrying the *fim* 2 gene and a 3.9kb fragment containing the *fim* X gene. The data from this hybridisation experiment suggests that the group I cosmids contain a *fim* 3 gene and that the group 2 cosmids contain a *fim* 2 gene. The group 3 cosmids do not contain a fragment corresponding to the *fim* X gene from *B. pertussis* strain 165 sequenced by Pedroni *et al.* (1988) but appear to contain a different *fim*-like gene.

3.3.2.3. Testing of *E. coli* strains.

All *E. coli* strains carrying the cosmids were tested using polyclonal anti-sera to AGGs 1, 2 and 3. None of the *E. coli* strains gave positive reactions in slide agglutination tests (section 2.5.2) using the polyclonal anti-sera raised against AGGs 1, 2 or 3.

FIGURE 12. HYBRIDISATION OF THE *fim* 2 PROBE TO THE COSMIDS CONTAINING PUTATIVE *fim* GENES.

The figure shows *Sal* I-digested cosmid DNA, run on a 0.8% agarose gel and Southern-blotted as described (section 2.6.9). The blotted DNA was then hybridised with the *fim* 2 probe (the 0.85kb *Sph* I fragment of pIL22) under reduced stringency conditions, final wash in 1 X SSC, 0.1% (w/v) SDS at 50°C for 1h. These autoradiographs were exposed for 3 days at -70°C. The Figure is a composite of several filters performed under identical conditions.

Key to Figure:-

A pDA126

B pDA395

C pDA216

D pDA319

E pDA446

F pDA163

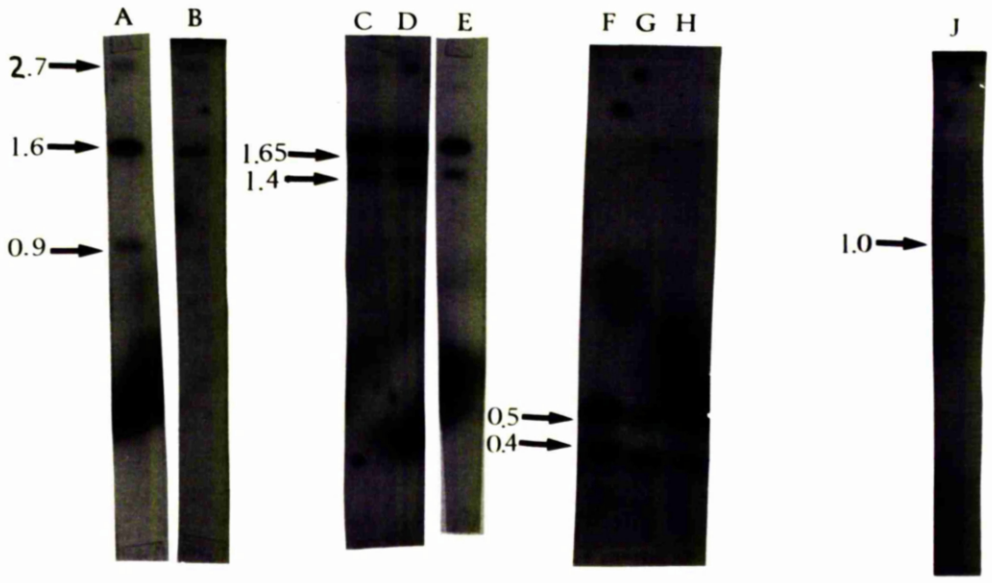
G pDA192

H pDA204

J pDI116

The members of the same cosmid groups have been placed together in this composite.

The small arrows indicate the position of the *Sal* I fragments that hybridise with the probe under these conditions, the size of the fragments is given alongside them.


A
B
C
D
E
F
G
H
J
2.7
1.6
0.9
1.65
1.4
0.5
0.4
1.0

3.3.2.4. Behaviour of the *fim* gene containing-cosmids in *B. parapertussis*.

B. parapertussis strain 10520 did not agglutinate when tested with polyclonal anti-sera raised against *B. pertussis* AGGs 2 or 3. However, partial agglutination was observed when *B. parapertussis* 10520 was tested with the polyclonal antibody raised against *B. pertussis* AGG 1.

The eight clones were conjugated into *B. parapertussis* 10520 and tested with the polyclonal anti-sera raised against AGGs 2 and 3.

To test for expression of AGG 1, all the cosmid-bearing strains and *B. parapertussis* 10520 were dot-blotted onto nitrocellulose filters and tested with a monoclonal antibody raised against *B. pertussis* AGG 1 (section 2.5.3). The results of these tests are shown in Table 7.

Only *B. parapertussis* strains containing pDA126 and pDA395 expressed AGG 3. After the cosmids were cured, by three passages on BG plates without tetracycline, the strains did not react with the anti-sera raised against AGG 3. This indicates that the expressed AGG 3 gene was carried on the cosmid.

None of the strains tested produced any detectable AGG 2.

5. Behaviour of AGG 3-encoding plasmids in AGG 3$^-$ *B. pertussis* strains.

Plasmid pDA126, which encoded AGG 3 in *B. parapertussis* 10520, was conjugated into the following *B. pertussis* strains:-

1. BP 347, fimbrial serotype 0,0.
2. *B. pertussis* strain Tohama, fimbrial serotype 2,0.

TABLE 7: RESULTS OF TESTING *B. parapertussis* 10520 STRAINS CONTAINING COSMIDS BEARING PUTATIVE AGG GENES.

COSMID	McAb AGG 1	PcAb AGG 1	PcAb AGG2	PcAb AGG3
pDA126	—	+/—	—	+
pDA163	—	+/—	—	—
pDA192	—	+/—	—	—
pDA204	—	+/—	—	—
pDA216	—	+/—	—	—
pDA319	—	+/—	—	—
pDA395	—	+/—	—	+
pDA446	—	+/—	—	—

The strains were tested with the monoclonal antibody raised against AGG 1 (McAb AGG 1) by immunoblotting as described in section 2.5.3.

The strains were tested using the polyclonal antibody raised against the three AGGs (PcAb AGG *X*) by slide agglutination (section 2.5.2).

KEY: - indicates no reaction,
+ indicates a positive reaction,
+/- indicates a partial reaction.

3. *B. pertussis* strain ARG 40, fimbrial serotype 2,0.

4. *B. pertussis* strain 106V, fimbrial serotype 0,0.

These strains were tested by slide agglutination using the polyclonal anti-sera raised against AGGs 2 and 3, the results of these tests are summarised in Table 8. AGG 3 was expressed in all the AGG 3$^-$ strains except strain BP347.

After loss of the cosmid, via three passages on BG plates without tetracycline, the strains were again tested with the agglutinating anti-sera raised against AGGs 2 and 3. None of these strains expressed AGG 3. Thus, none of the strains had regained expression of AGG 3 from a chromosomal locus after curing of the cosmid.

B. pertussis strains 106V and 106V[pDA126] were grown on BG plates, the latter supplemented with tetracycline (25µg/ml). Bacteria were lifted gently from the plates and emulsified in sterile saline. After emulsification the bacteria were negatively stained with ammonium molybdate and prepared for the electron microscope. Electron-micrographs of representative samples of both strains are shown in Figures 13 and 14. Assembled fimbrial structures can be seen extending from the surface of the bacteria containing pDA126. The electron-micrographs show a representative sample of the cells of each strain. Strain 106V[pDA126] gave a far greater degree of cell 'clumping' when being emulsified in sterile saline than strain 106V, which dispersed into single cells fairly readily. After loss of pDA126 the strain was once again afimbriate (data not shown).

TABLE 8: FIMBRIAL SEROTYPES OF *B. pertussis* AGG 3⁻ STRAINS CARRYING PLASMID pDA126.

B. pertussis STRAIN	ORIGINAL SEROTYPE	SEROTYPE PLUS pDA126	SEROTYPE AFTER LOSS OF pDA126
BP347	0, 0	0, 0	0, 0
Tohama	2, 0	2, 3	2, 0
ARG 40	2, 0	2, 3	2, 0
106V	0, 0	0, 3	0, 0

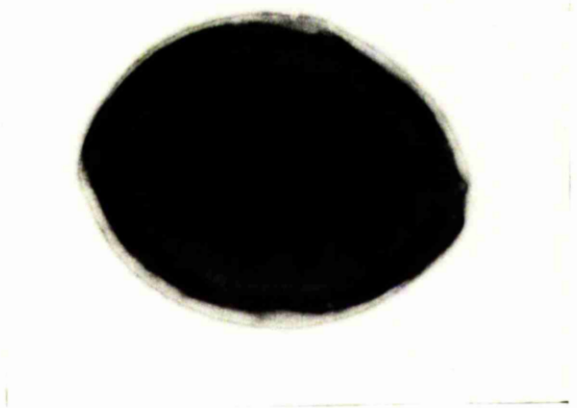
The fimbrial serotypes of these strains were tested with the polyclonal antibodies raised against AGG 2 and AGG 3 by slide agglutination.

The cosmid was cured by three passages of the strain on BG plates unsupplemented with tetracycline. Loss of the cosmid was confirmed by re-streaking the strains after the final passage on BG plates containing tetracycline.

FIGURE 13. ELECTRON-MICROGRAPHS OF *B. pertussis* STRAIN 106V.

Cells have been stained with ammonium molybdate.

Final magnification of these electron-micrographs is 102, 000 X.



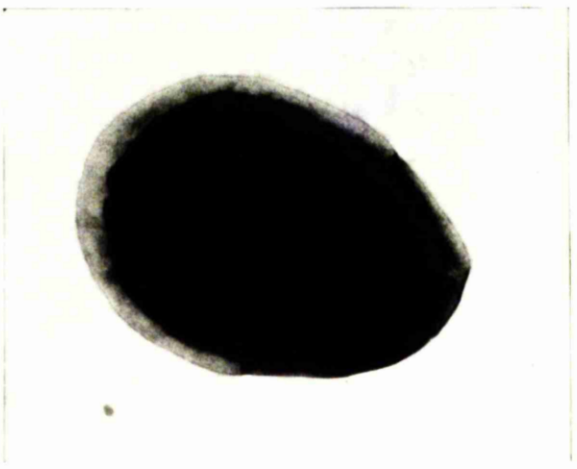
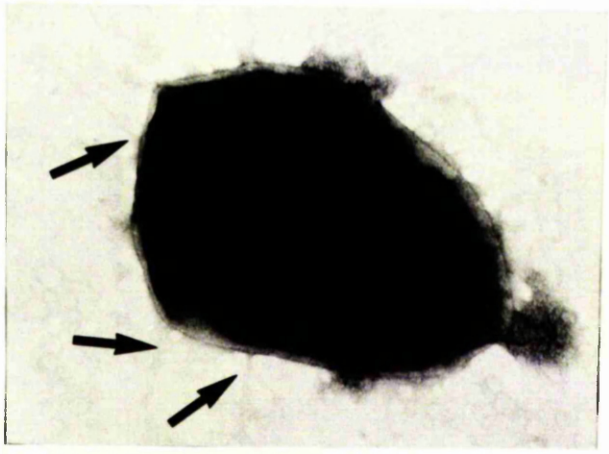


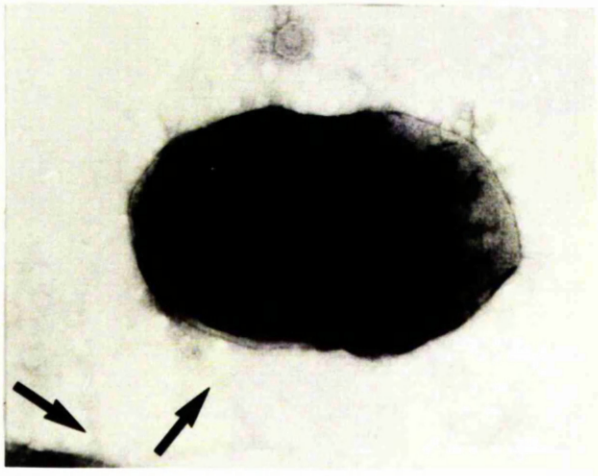
FIGURE 14. ELECTRON-MICROGRAPHS OF *B. pertussis* STRAIN 106V[pDA126].

Cells have been stained with ammonium molybdate.

Final magnification of these electron-micrographs is 102, 000 X.

The arrows indicate some of the visible fimbrial structures.





3.3.3. Probing of serovariant chromosomal DNA with *fim* 2 probe.

Chromosomal DNA from the *B. pertussis* strains Tohama, Taberman, ARG 40, ARG 41, ARG 43 and ARG 44 was digested with *Eco* RI and *Cla* I (*Bsc* I) and blotted onto nylon membranes. These filters were then hybridised with the 0.85kb Sph I fragment of pIL22 described earlier. These experiments gave rise to multibanded patterns similar to those shown in Figure 11. Visual analysis of these results could not differentiate between the banding patterns of any of the strains, despite their differing serotypes (data not shown).

3.4. DETECTION OF SEQUENCES HOMOLOGOUS TO THE INVERTIBLE *hin* SEQUENCE OF *Salmonella typhimurium*

3.4.1. Probing of *B. pertussis* chromosomal DNA with a *hin* containing probe.

The plasmid pKK1001B contains the entire invertible region of *S. typhimurium* within an 8.8kb insert of *S. typhimurium* DNA cloned into *Eco*RI/*Bam*HI sites of pBR322. This plasmid did not bind to *B. pertussis* DNA under the high stringency conditions defined in section 2.6.10.1. Under the high stringency conditions the probe did bind to *Sau* 3A digested-pKK1001B. The stringency of the conditions was then systematically reduced until hybridisation was achieved. Significant hybridisation occurred using the following conditions: the probe was hybridised in the standard hybridisation buffer described in section 2.6.10 except that the formamide concentration was reduced to 20% (v/v), and the SSC concentration was raised to 6 X SSC. The filter was incubated in this hybridisation buffer at 42°C overnight. The filter was then washed twice in 2 X SSC, 0.1% SDS at room temperature for 30mins, followed by washing twice in 2 X SSC, 0.1% SDS at 55°C for 1 hour. The filter shown in Figure 15 was then exposed at -70°C for 4 hours.

The filter illustrated contains *Eco*RI-digested chromosomal DNA isolated from the Taberman and Tohama parent and variant strains described previously and *Sau* 3A-digested pKK1001B. The probe demonstrated a similar hybridisation pattern to *B. pertussis* DNA as that reported by Foxall *et al.* (1990). The probe hybridised to two high molecular weight *Eco*RI fragments (≃15kb) in each sample. However, the degree of hybridisation

FIGURE 15. PROBING OF *B. pertussis* DNA WITH pKK1001B.

The figure shows *B. pertussis* chromosomal DNA digested with *Eco*RI and plasmid pKK1001B DNA digested with *Sau* 3A, run on a 0.8% agarose gel and Southern-blotted as described (section 2.6.9). The blotted DNA was hybridised with radio-labelled pKK1001B under low stringency conditions. The hybridisation buffer contained 20% (v/v) formamide and 6 X SSC, and the filter was incubated in this buffer at 42°C overnight. The final wash was performed in 2 X SSC, 0.1% (w/v) SDS at 55°C for 1h. The autoradiograph was then exposed at -70°C for 4h.

Key to Figure:-

m = position of λ *Hin*dIII fragments.

1 = Taberman DNA.

2 = avirulent Taberman DNA.

3 = Tohama DNA.

4 = avirulent Tohama DNA.

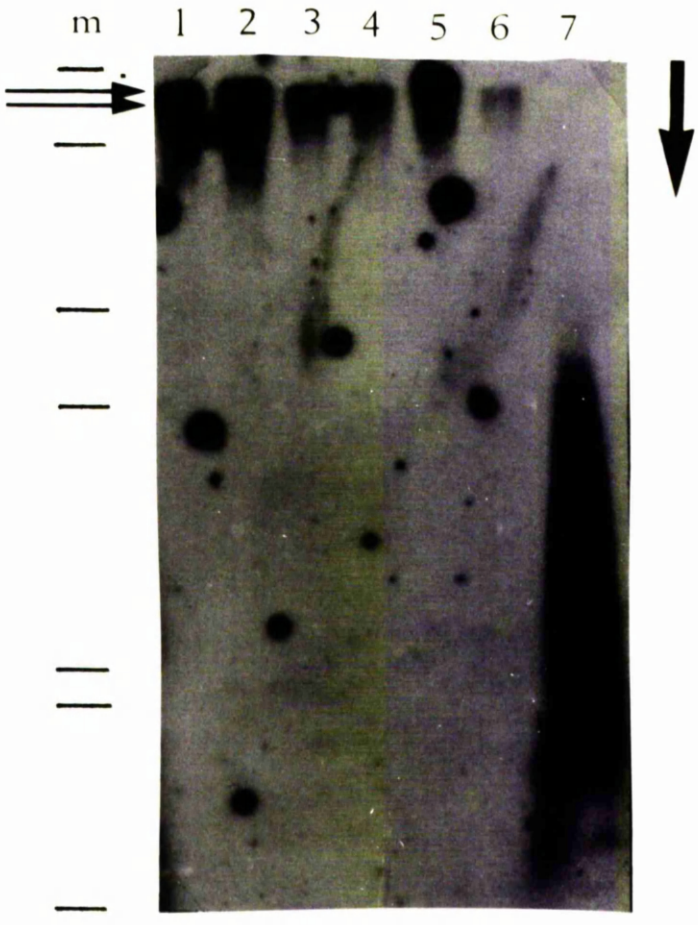
5 = L84 I DNA.

6 = L84 IV DNA.

7 = pKK1001B.

The large arrow indicates the direction of migration.

The smaller arrows indicate the position of the two high molecular weight bands the probe hybridises to under these conditions.


m
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2
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7

varies between the Taberman and the Tohama strains. The probe consistently hybridised with greater intensity to chromosomal DNA derived from the Taberman strains than to the Tohama derived DNA. There is also a difference between the degree of hybridisation to the L84I and L84IV-derived DNA, hybridisation is stronger to the L84I DNA. This discrepancy in the binding of the probe is not related to the concentration of sample DNA as these were equilibrated prior to digestion. The DNA samples which display the greatest degree of homology also show the probe hybridising to a number of lower molecular weight fragments. After initial exposure the filter was rewashed in 2 X SSC, 0.1% SDS at 65°C for 2hrs and reexposed at -70°C. In order to achieve a comparable signal the exposure time was increased to 36hrs. This exposure still retained the circular areas of non-specific hybridisation, but had a greater general background due to the increased exposure time. The high stringency wash removed the probe from several of the lower molecular weight bands in the Taberman DNA samples. The amount of probe hybridised to the DNA was greatly decreased, although the relative proportions of the hybridisation signals was retained.

3.4.2. Creation of a *hin*-specific probe.

The nucleotide sequence of the invertible DNA region of *S. typhimurium* contained in pKK1001B has been published (Zeig and Simon, 1980) and is shown in Figure 3. In the given sequence the *hin* gene begins at position 99 and ends at position 671, the *hix* R sequence runs from position 12 to

position 37, and the *hix* L sequence from position 1005 to position 1030.

This sequence was analysed using a computer program (DNAStar) which identifies restriction enzyme cleavage sites within DNA sequences.

The *S. typhimurium hin* sequence contains the following restriction sites:-

i) *Asp* 700 @ position 775.
ii) *Bgl* I @ position 474.
iii) *Cla* I @ position 144.
iv) *Nar* I @ position 514.
v) *Sac* I @ position 352.
vi) *Sau* 3A @ position 527.
vii) *Spe* I @ position 168.
viii) *Stu* I @ position 822.
ix) *Taq* I @ positions 145, 460, 963.

No cleavage sites were found within this sequence for 54 other commonly-used restriction enzymes.

The 671bp fragment bounded by the *Cla* I site at position 144 and the *Stu* I site at position 822 contains the largest possible amount of the *hin* gene coupled with the minimum of redundant DNA. This 671bp sequence contains 527bp of the *hin* gene, including the dorsal domain of the recombinational enhancer sequence, and 144bp of non-coding DNA. The probe did not contain either of the *hix* sites, the H2 gene promoter or any of the H2 gene.

pKK1001B was digested with *Cla* I and *Stu* I together in a single step using the buffer REact™1 (See Appendix 3 for

details of reaction buffers). The recommended restriction buffers for the two enzymes are different and thus the activities of each of the enzymes was investigated in different reaction buffers. Digests of *E. coli* chromosomal DNA were prepared with *Stu* I using the recommended buffer REact™2, the alternative buffer REact™1 and M buffer (Appendix 3). Digests of *E. coli* DNA were also set up with *Cla* I using the recommended REact™1, REact™2 and M buffer. These digests were then incubated at 37°C for four hours, 20µl removed and frozen at -20°C. The remaining 20µl of each digest was then incubated overnight at 37°C. The products of these digests were run on a 0.7% agarose gel and compared. Both enzymes digested *E. coli* DNA in their recommended buffers, *Stu* I also digested the DNA fully in REact™1. The digests using M buffer and the *Cla* I digests using REact2 were unsatisfactory. A double digest of *E. coli* DNA with *Stu* I and *Cla* I was set up and incubated at 37°C overnight. A similar 40µl digest was prepared using *Cla* I and REact™1 and digested at 37°C for eight hours. The digest was then heated at 65°C for 10 minutes, phenol/chloroform extracted, and the DNA alcohol-precipitated. After freeze drying the DNA was resuspended in REact™2 and digested with *Stu* I overnight. This sample and the *Cla* I/*Stu* I double digest were then compared. There was no difference between the digestion pattern of either sample, although the yield of the DNA which had been precipitated prior to *Stu* I digestion was significantly lower.

Due to the small size of the required band, 671bp, approximately 2-2.5µg of pKK1001B was digested overnight with

Cla I and *Stu* I using REact™ 1. The product of this digestion was run in a 1.5% (w/v) low melting temperature ultra-pure agarose gel using ΦX174 *Hae* III fragments as size markers, see Figure 16. After staining with ethidium bromide the gel was inspected and the required fragment excised to be used as a probe.

3.4.3. Hybridisations using the *hin*-specific probe.

The *hin*-specific probe was radioactively labelled using the hexanucleotide primer method (section 2.6.11) and hybridised under high stringency conditions to chromosomal digests of *B. pertussis* chromosomal DNA and *Sau* 3A – digested pKK1001B. Hybridisation occurred between the probe and the digested pKK1001B but not between the probe and *B. pertussis* DNA. The experiment was repeated using the low stringency conditions described above (20% v/v formamide, 6 X SSC, etc.). Hybridisation was then achieved between the probe and the two high molecular weight *Eco* RI fragments described above in DNA isolated from Taberman strains. Again the probe hybridised with greater intensity to the Taberman DNA samples than to the Tohama samples. The probe also hybridised to the plasmid RP4::Mu under these conditions, indicating that these conditions allowed hybridisation between the *hin*-containing probe and the *gin* gene of phage Mu. As with the earlier experiment the background observed on filters hybridised under these conditions was very high and interfered with interpretation of the data.

The probe was then hybridised under the same conditions to *B. pertussis* chromosomal DNA, which had been digested

FIGURE 16. PRODUCTION OF THE *hin*-SPECIFIC PROBE.

The figure shows pKK1001B DNA which has been double-digested with *Cla* I and *Stu* I and then run in a 1.5% low melting temperature agarose gel.

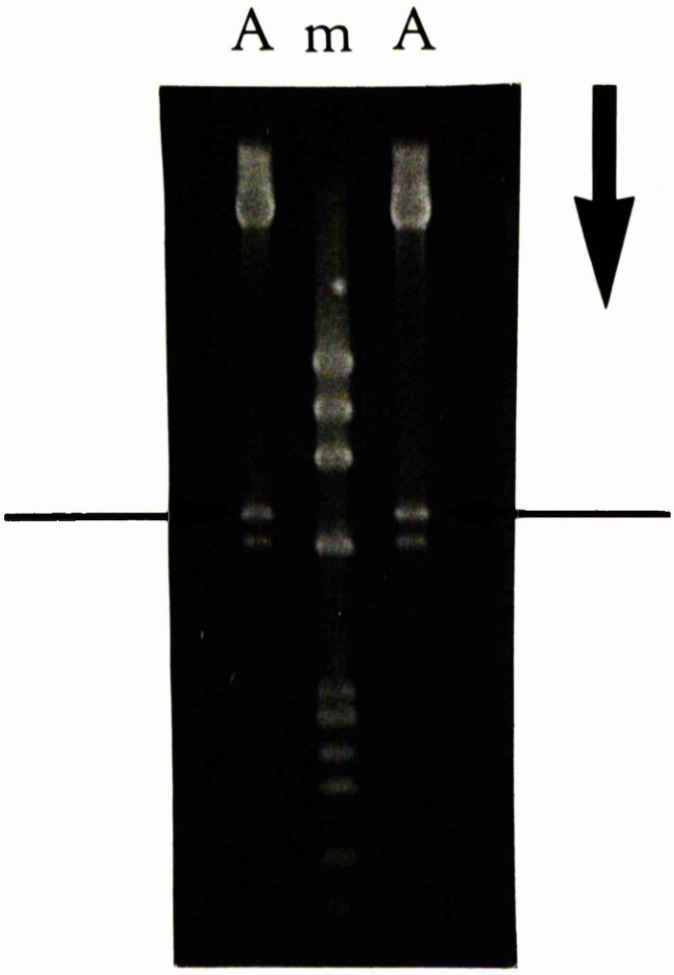
Key to Figure:-

m = ΦX174 *Hae* III fragments (see Appendix 5 for size of the markers).

A = *Cla* I and *Stu* I double digest of pKK1001B.

The small arrows indicate the position of the *hin*-specific 671bp fragment, which was cut from the gel and used as a probe.

The large arrow indicates direction of migration.


A m A

separately with *Cla* I (*Bsc* I) and *Stu* I. Representative results of these experiments are shown in Figure 17.

The probe hybridised to a 0.6kb *Cla* I (*Bsc* I) fragment and to 3.0kb and 3.2kb *Stu* I fragments of *B. pertussis* DNA. Once again the probe hybridised more strongly to Taberman DNA than to Tohama DNA.

Under these conditions the probe did not hybridise with *B. parapertussis* DNA.

The probe hybridised to chromosomal DNA isolated from *B. bronchiseptica* and *B. avium* DNA under these conditions. The probe hybridised to a 0.9kb *Cla* I (*Bsc* I) fragment and a 0.5kb *Stu* I fragment in *B. bronchiseptica* DNA. The intensity of hybridisation to *B. bronchiseptica* DNA was lower than that observed with *B. pertussis* Tohama DNA.

B. avium DNA proved refractory to digestion with *Cla* I (*Bsc* I), but the probe hybridised to the undigested chromosomal DNA transferred onto the filter. The probe hybridised to a 3.8kb and a 4.0kb *Stu* I fragment; the degree of hybridisation was greater than that observed for *B. pertussis* DNA.

The high amount of background observed using these conditions interfered with interpretation of the data and made reproduction of many of the blots pointless.

The hybridisation and washing conditions were then systematically altered to find a method which gave reproducible hybridisation with a minimum of background. The percentage of formamide in the hybridisation solution was varied between 0% (v/v) and 60% (v/v), using the washing conditions described

FIGURE 17. PROBING OF *B. pertussis* DNA WITH THE *hin*-SPECIFIC PROBE UNDER LOW STRINGENCY CONDITIONS.

The figure shows *B. pertussis* chromosomal DNA which has been digested separately with *Cla* I and *Stu* I, run in a 0.8% agarose gel and Southern-blotted as described (section 2.6.9). The blotted DNA was then hybridised with the 671bp *hin*-specific probe under low stringency conditions. The hybridisation buffer contained 20% (v/v) formamide and 6 X SSC, and the filter incubated in this buffer at 42°C overnight. The final wash was performed in 2 X SSC, 0.1% (w/v) SDS at 55°C for 1h. This autoradiograph was exposed for 3 days at -70°C.

Key to Figure:-

m = position of λ *Hin* dIII fragments.

1 = *Cla* I (*Bsc* I) digested Taberman DNA.

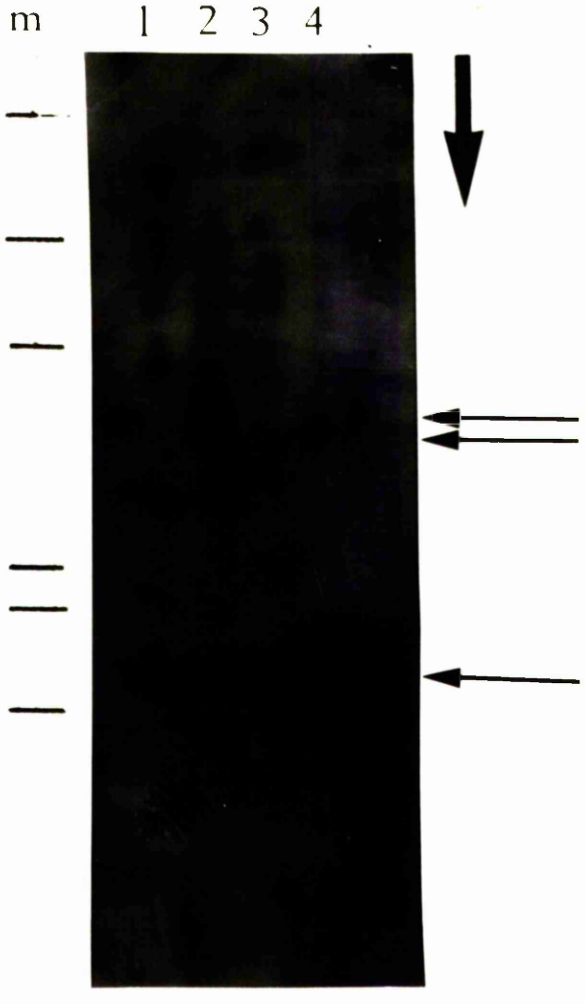
2 = *Stu* I digested Taberman DNA.

3 = *Cla* I (*Bsc* I) digested Tohama DNA.

4 = *Stu* I digested Tohama DNA.

The small arrows indicate the position of the 0.6kb *Cla* I (*Bsc* I) fragment and 3.0kb and 3.2kb *Stu* I fragments which hybridise with the probe.

The large arrow indicates the direction of migration.


m
1
2
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4

above. The alteration of formamide concentration in the hybridisation mixture did not reduce the background, but did affect hybridisation of the probe. The optimum hybridisation signal was obtained using a formamide concentration of 33% (v/v) at a constant incubation temperature of 42°C. The concentration of SSC in the hybridisation mixture was also varied using a constant 33% (v/v) formamide. Optimum hybridisation was obtained at 6.6 X SSC at 42°C, again the background was not greatly reduced. The remainder of the hybridisation mixture was left as described previously.

The concentration of SSC in the washing buffer, the temperature of the washes and their duration were also systematically altered. The optimum conditions for washing were found to be:-

i) twice in 5 X SSC, 0.1% SDS at 50°C for 30 mins,

ii) once in 1 X SSC, 0.1% SDS at 50°C for 120mins.

After washing, the film was exposed to the ^{32}P bound to the filters at -70°C for varying lengths of time until optimum exposure was reached. The autoradiograph shown in Figure 18 was exposed for 14 days before developing. In this blot the probe is shown to have hybridised cleanly to a 0.6kb *Cla* I(*Bsc* I) fragment and to 3.5kb and 3.8kb *Stu* I fragments. The autoradiograph has been extensively cropped due to the large signal from the λ markers and the positive control lane. As in the previous experiments the probe hybridised more strongly to Taberman DNA than to Tohama DNA. These conditions also gave clear hybridisation to 3.8kb and 4.0kb *Stu* I fragments in *B. avium* DNA (Figure 19).

FIGURE 18. PROBING OF *B. pertussis* DNA WITH THE *hin*-SPECIFIC PROBE UNDER OPTIMUM STRINGENCY CONDITIONS.

The figure shows *B. pertussis* chromosomal DNA which has been digested separately with *Cla* I (*Bsc* I) and *Stu* I, run in a 1% agarose gel and Southern-blotted as described (section 2.6.9). The blotted DNA was then hybridised with the 671bp *hin*-specific probe under the optimum stringency conditions. The hybridisation buffer contained 33% (v/v) formamide and 6.6 X SSC, and the filter was incubated in this buffer at 42°C overnight. The washing protocol used for this blot involved two washes in 5 X SSC, 0.1% (w/v) SDS at 50°C for 30min, followed by a final wash in 1 X SSC, 0.1% (w/v) SDS at 50°C for 2h. This autoradiograph was exposed at -70°C for 14 days.

Key to Figure:-

1 = *Cla* I (*Bsc* I) digested Taberman DNA.

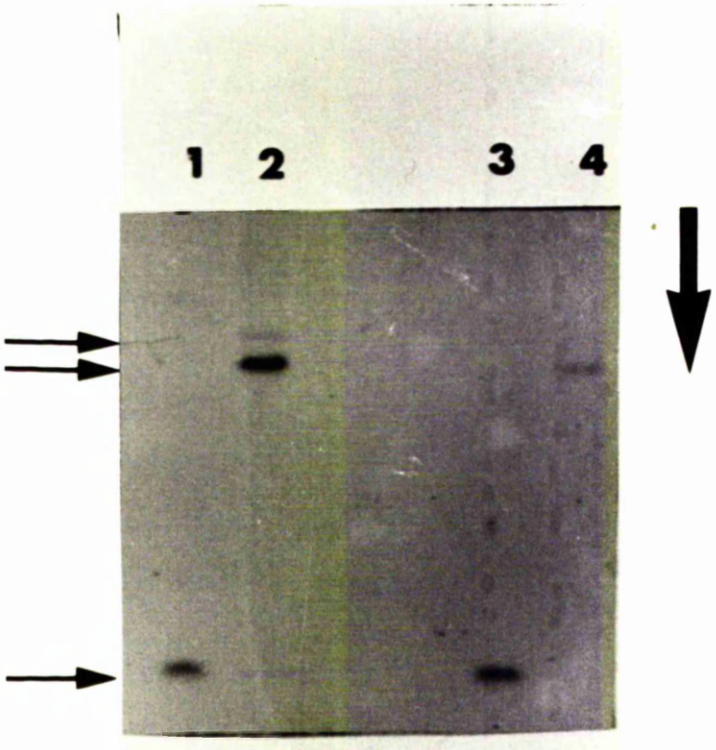
2 = *Stu* I digested Taberman DNA.

3 = *Cla* I (*Bsc* I) digested Tohama DNA.

4 = *Stu* I digested Tohama DNA.

The small arrows indicate the position of the 0.6kb *Cla* I (*Bsc* I) fragment and 3.0kb and 3.2kb *Stu* I fragments which hybridise with the probe under these conditions.

The large arrow indicates the direction of migration.


1
2
3
4

Throughout these experiments one of the major factors governing the signal to noise ratio of the exposed autoradiographs was the age of the ^{32}P-α-dATP used in the labelling reaction. Using fresh isotope, less than 7 days old, the exposure time was restricted to approximately 14 days and the background was minimal. However, as the age of the isotope increased the exposure time was increased and, consequently, the background increased dramatically. Labelling of probes with biotin is a simple procedure and the activity of the label does not decrease drastically with age. Labelling of probes with biotin does not alter the hybridisation kinetics of the labelled probe but does slightly decrease the melting temperature of the probe-target hybrid.

Control experiments using the hybridisation of biotin-labelled pBR322 to *Eco*RI digested pBR322 and biotin-labelled λ to *Bam*HI digested λ DNA showed that the standard techniques and manipulations used in this study gave positive hybridisation results.

The 671bp *hin*-specific probe was labelled with Biotin-7-dATP (BRL) using the hexanucleotide primer technique and hybridised to *B. pertussis* DNA using the conditions detailed above. These experiments gave no hybridisation signals using reduced stringency conditions. When the conditions were altered to allow for the reduced T_M of the hybrid, by decreasing the formamide concentration to 25%(v/v) with a final wash temperature of 45°C, hybridisation was still not observed.

The *B. pertussis* Taberman gene library constructed by Brownlie *et al.* (1986) was grown as described (section 2.6.15)

FIGURE 19. PROBING OF *B. avium* DNA WITH THE *hin*-SPECIFIC PROBE UNDER OPTIMUM STRINGENCY CONDITIONS.

The figure shows *B. avium* DNA (prepared using the same method as *B. pertussis* DNA, section 2.6.1) digested with *Stu* I, run in a 0.8% agarose gel and Southern-blotted as described (section 2.6.9). The filter was then incubated with the 671bp *hin*-specific probe under the optimum stringency conditions. The hybridisation buffer contained 33% (v/v) formamide and 6.6 X SSC, and the filter was incubated in this buffer at 42°C overnight. The washing protocol for this filter consisted of two washes in 5 X SSC, 0.1 (w/v) SDS at 50°C for 30min, followed by a final wash in 1 X SSC, 0.1% (w/v) SDS at 50°C for 2h. This autoradiograph was exposed at -70°C for 3 days.

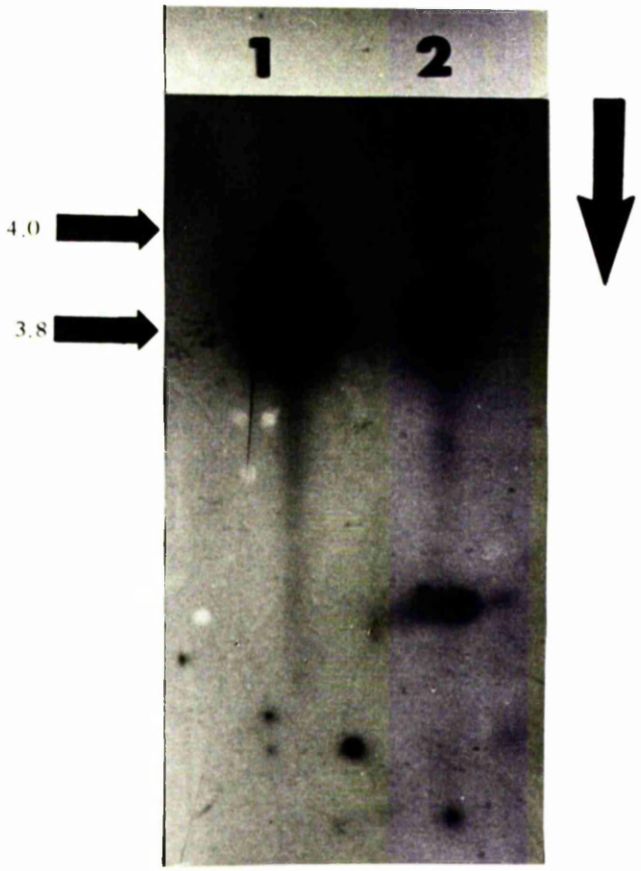
Key to Figure:-

1 = *B. avium* 4041 DNA.

2 = *B. avium* 4049 DNA.

The small arrows indicate the position of the 4.0kb and 3.8kb *Stu* I fragments which hybridise with the probe under these conditions.

The large arrow indicates the direction of migration.


1
2
4.0
3.8

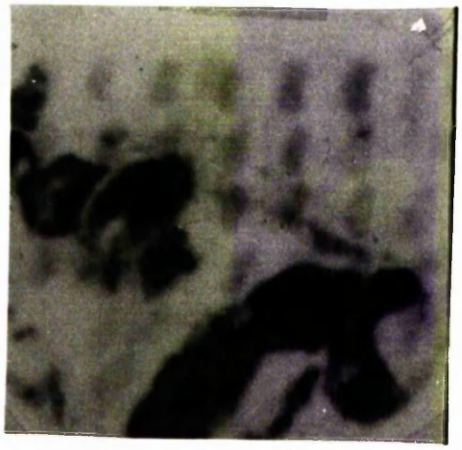
on circular nylon membranes (Pall Biodyne). After growth the cells were denatured and the chromosomal DNA bound to the membrane. The filters were then placed into hybridisation bags and hybridised using the ^{32}P-α-dATP labelled 671bp *hin*-specific probe. From the 520 clones in the gene library one gave a strong positive signal (Figure 20). This clone was isolated from the library, designated pDI116 and characterized (section 3.5).

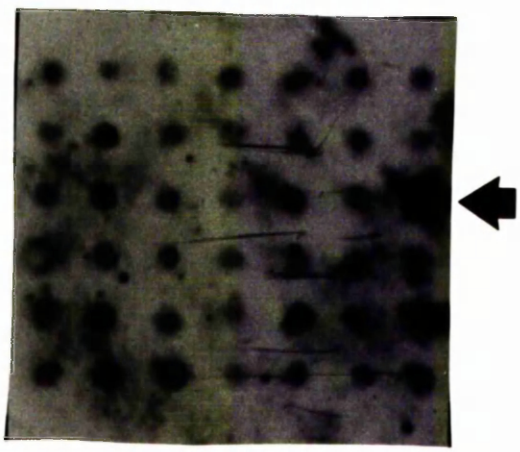
Hybridisation under the conditions described previously was found to be membrane-specific. These conditions were originally created for use with DNA immobilised onto Zeta-Probe (Bio-Rad) nylon membranes, and were also succesful using Gene Screen Plus (DuPont) nylon membranes. However, no hybridisation was observed using these conditions when the DNA was immobilised onto Hybond-N (Amersham) nylon membranes. From visual inspection of DNA transfer and the strength of radio-labelled markers transferred along with the chromosomal DNA it appeared that Hybond-N membranes bound the DNA as effectively as the other membranes.

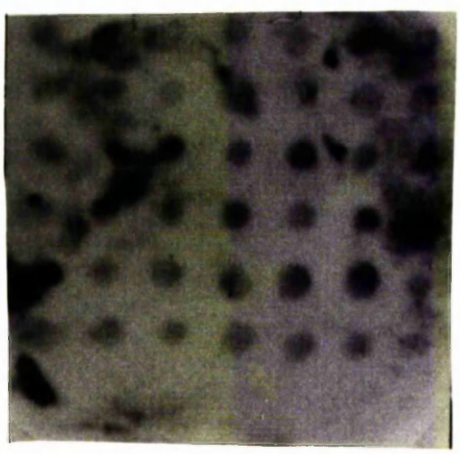
FIGURE 20. PROBING OF *B. pertussis* TABERMAN GENE LIBRARY WITH THE 671bp *hin*-SPECIFIC PROBE.

The Figure shows representatives of the *B. pertussis* strain Taberman gene library which have been hybridised with the 671bp *hin*-specific probe as described (section 2.6.15). Hybridisation was performed under the optimum stringency conditions. The hybridisation buffer contains 33% (v/v) formamide and 6.6 X SSC and the filters were incubated at 42°C overnight in this buffer. The washing protocol for these filters involved two washes in 5 X SSC, 0.1% (w/v) SDS at 50°C for 30min, followed by a final wash in 1 X SSC, 0.1% (w/v) SDS at 50°C for 2h. These aoutoradiographs were exposed at -70°C for 10 days.

The cosmid clone which hybridised with the probe under these conditions is shown by an arrow, this cosmid was designated pDI116. The other colonies are representative negatives from the entire gene library.







3.5. CHARACTERISATION OF pDI116.

3.5.1. Genetic characterisation of pDI116.

pDI116 contains an insert of 22kb. This insert of chromosomal DNA was analysed using *Eco*RI, *Cla* I (*Bsc* I) and *Xho* I. These enzymes were chosen for ease of cloning any relevant fragments identified into the broad host range plasmids pKT230, pKT231 (Bagdasarian *et al.*, 1981) and pMMB34 (Frey *et al.*, 1983).

From the information a restriction fragment map of the insert was generated, this map is presented in Figure 21. The 0.6kb *Cla* I (*Bsc* I) fragment which hybridises to the *hin*-specific probe is found within a 15kb *Eco*RI fragment. This agrees with the observations made from the chromosomal DNA experiments. Several candidate fragments for sub-cloning are apparent; these include the 9kb *Eco*RI/*Xho* I fragment which contains the 0.6kb *Cla* I fragment.

3.5.2. Hybridisation with the *fim* 2 gene probe.

The 0.85kb *Sph* I fragment of pIL22 containing both the *fim* 2 gene and a part of IS481v2 was radio-labelled and hybridised to *Sal* I digested pDI116. The probe hybridised to only a single 1.0kb *Sal* I fragment, see Figure 12.

This fragment is different from those indicative of the *fim* genes and the repeat sequences associated with them (section 3.3.2.2). This suggests that pDI116 is not linked to the *fim* genes, but is linked with a repeat sequence similar to IS48v2.

FIGURE 21. RESTRICTION FRAGMENT MAP OF pDI116.

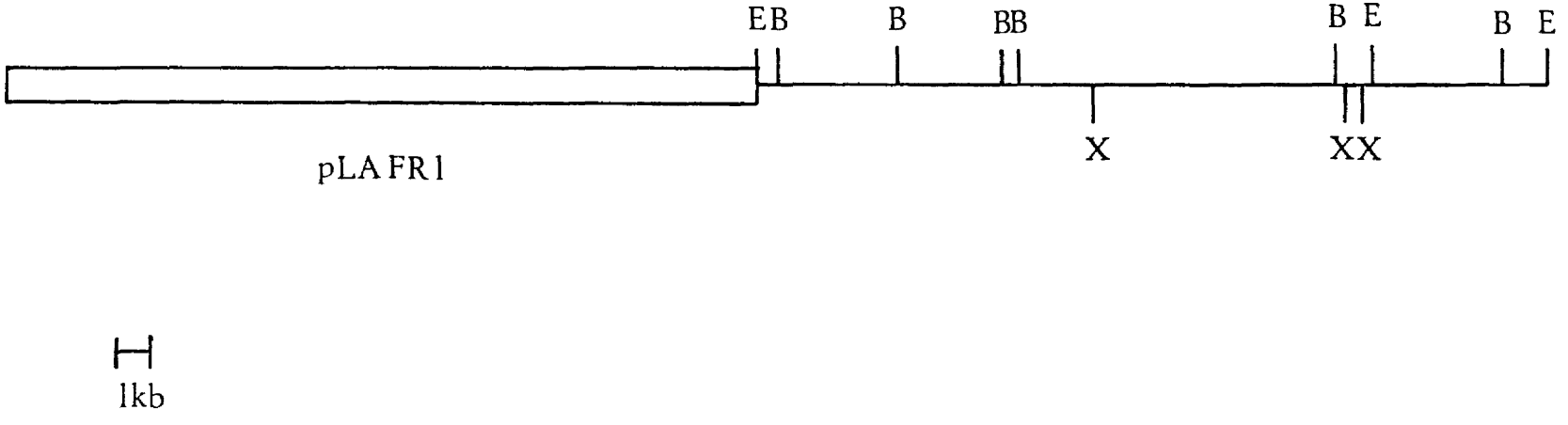
This map was generated from double-digest data using the Branch and Bound technique of Fitch *et al.* (1983).

Key to Figure:-

E = *Eco*RI sites.

B = *Cla* I (*Bsc* I) sites.

X = *Xho* I sites.


EB
B
BB
B E
B E
X
XX
pLA FR1
1kb

3.5.3. Assays for the production of invertase by pDI116.

3.5.3.1. Preliminary test of invertase production using pSH1313.

The indicator plasmid pSH1313 (section 2.2.2.4) was transformed into the *cin*$^+$ *E. coli* strain K12 and *cin*$^-$ *E. coli* strain WA3782. Four 10ml cultures of each strain were grown overnight in nutrient broth containing ampicillin, to maintain pSH1313 within the cells, on an orbital shaker at 37°C. After incubation triplicate serial dilutions were made from each culture. Duplicate 100µl aliquots of the undiluted culture, the 10^{-1} dilution and the 10^{-2} dilution were spread onto nutrient agar plates containing kanamycin (final concentration 50µg/ml) and incubated overnight (16h). Duplicate 100µl aliquots of the 10^{-5} and 10^{-6} dilutions were also plated onto nutrient agar plates containing chloramphenicol (final concentration 50µg/ml) and incubated overnight (16hrs). After incubation the numbers of colonies on each plate were counted. The ratio of kanamycin resistant colonies to chloramphenicol colonies was used as the relative frequency of inversion, a method used previously by Kahmann *et al.* (1985) and Hübner and Arber (1989). The results of this preliminary experiment are given in Table 9. This experiment proved that pSH1313 was functioning correctly in *cin*$^+$ and *cin*$^-$ strains and that the plasmids which had inverted could be detected. The experiment also served to confirm that *E. coli* strain WA3782 was *cin*$^-$ and would be able to serve as a neutral environment for conducting experiments on pDI116. The large difference between the relative inversion frequency in

TABLE 9: RESULTS OF THE PRELIMINARY EXPERIMENT USING pSH1313.

STRAIN	RELATIVE FREQUENCY OF INVERSION EVENTS.
K12[SH1313]	7.80×10^{-5}
WA3782[SH1313]	2.06×10^{-6}

the positive and negative background negated the need to score for the reverse process.

3.5.3.2. First experiment using pSH1313 in conjunction with other plasmids.

pSH1313 was transformed into *E. coli* strains K12 and WA3782 as above. After colony purification strain WA3782[pSH1313] was separately supertransformed with i) pDI116, ii)pLAFR1 and iii) pDA126, which is non-homologous with the *hin*-specific probe and encodes AGG 3.

The strains carrying pSH1313 were grown in nutrient broth plus ampicillin, whilst the supertransformed strains were grown in nutrient broth plus ampicillin and tetracycline. Five 10ml cultures of each strain were grown overnight (16h) at 37°C in randomised positions on an orbital shaker. After growth duplicate serial dilutions were made from each of the five cultures and duplicate 100µl aliquots of these dilutions plated out onto nutrient agar plates containing either kanamycin or chloramphenicol as described above. All the nutrient agar plates used for growth of the supertransformed strains also contained tetracycline. After overnight growth, the numbers of colonies on the plates were counted and the relative frequency of the inversion events calculated as described above. The standard deviation of these frequencies was also calculated. The result of this experiment is shown in Table 10. These relative frequencies were recalculated as percentages of relative inversion, the mean of the positive control serving as 100%, and the results are given in Table 11.

TABLE 10: RESULTS OF THE FIRST EXPERIMENT USING pSH1313.

STRAIN	RELATIVE FREQUENCY OF INVERSION EVENTS. (mean ± SD)
K12[SH1313]	$5.90 \times 10^{-5} \pm 2.2 \times 10^{-5}$, $n = 5$.
WA3782[SH1313]	$3.61 \times 10^{-7} \pm 1.0 \times 10^{-7}$, $n = 5$.
WA3782[SH1313, pDI116]	$3.66 \times 10^{-7} \pm 2.17 \times 10^{-7}$, $n = 5$.
WA3782[SH1313, pLAFR1]	$1.56 \times 10^{-7} \pm 6.6 \times 10^{-8}$, $n = 5$.
WA3782[SH1313, pDI126]	$1.90 \times 10^{-7} \pm 8.4 \times 10^{-8}$, $n = 5$.

TABLE 11: RESULTS OF THE FIRST EXPERIMENT USING pSH1313 CONVERTED TO RELATIVE PERCENTAGES.

STRAIN	RELATIVE PERCENTAGE OF INVERSION EVENTS. (mean ± SD)
K12[SH1313]	100% ± 37%.
WA3782[SH1313]	0.6% ± 0.1%.
WA3782[SH1313, pDI116]	0.6% ± 0.3%.
WA3782[SH1313, pLAFR1]	0.3% ± 0.1%.
WA3782[SH1313, pDI126]	0.3% ± 0.1%.

3.5.3.3. Second experiment using pSH1313 in conjunction with other plasmids.

For this experiment fresh strains were constructed by transformation from plasmid stocks stored at -20°C. *E. coli* strains K12 and WA3782 were again transformed with pSH1313. After colony purification, strain WA3782[pSH1313] was supertransformed with pDI116. The manipulations of the previous experiment were repeated, although in this case 10 cultures of WA3782[pSH1313, pDI116] were used. Once more the relative frequencies of the inversion events were calculated and the standard deviation of the results determined, these results are given in Table 12. These relative frequencies were recalculated as percentages of relative inversion as above, and these results are given in Table 13. The results of the experiments involving pSH1313 suggest that pDI116 does not encode a functional invertase in *E. coli*, this may be due to a requirement for the BvgA positive effector for promoter function. Also, the presence of both pSH1313 and one of the large cosmid clones in strain WA3782 affected the growth pattern of the strains. This excess genetic load may have been exerting selection pressure against the plasmids, which in turn may have led to unexpected recombination events, such as deletions. Attempts were thus made to transfer pSH1313 into *B. pertussis* strain Taberman in order to assay any invertase protein produced *in vivo*.

The first method tried involved electroporation of *B. pertussis* cells with 50ng of pSH1313 using a number of different field strengths, no transformants were obtained. A

TABLE 12: RESULTS OF THE SECOND EXPERIMENT USING pSH1313.

STRAIN	RELATIVE FREQUENCY OF INVERSION EVENTS. (mean ± SD)
K12[SH1313]	$9.70 \times 10^{-6} \pm 2.0 \times 10^{-6}$, $n = 5$.
WA3782[SH1313]	$2.62 \times 10^{-6} \pm 4.8 \times 10^{-7}$, $n = 5$.
WA3782[SH1313, pDI116]	$6.90 \times 10^{-7} \pm 1.3 \times 10^{-7}$, $n = 10$.

TABLE 13: RESULTS OF THE SECOND EXPERIMENT USING pSH1313 CONVERTED TO RELATIVE PERCENTAGES.

STRAIN	RELATIVE PERCENTAGE OF INVERSION EVENTS. (mean ± SD)
K12[SH1313]	100% ± 2.0%.
WA3782[SH1313]	3% ± 0.4%.
WA3782[SH1313, pDI116]	0.7% ± 0.1%.

study by Zealey *et al.* (1988) confirmed that extremely high field strengths (25kV/cm) were required for succesful electroporation, and that the Bio-Rad Gene Pulser, which was used in this study, was unsuitable for electroporation of *B. pertussis*.

Plasmid pSH1313 does not possess the transfer genes necessary for activation by pRK2013 and cannot be conjugated into *B. pertussis* as described (section 2.4.1). The plasmid pGJ28 which can supply these transfer functions in *trans* (D. Sherratt, personal communication), was supertransformed into *E. coli* strain WA3782[pSH1313], and used as the donor strain in a plate conjugation with *B. pertussis* strain Taberman. No ampicillin resistant transconjugants were obtained by this procedure. This failure is probably due to the combination of the low frequency of conjugation of *B. pertussis* and the low frequency with which pGJ28 *trans* activates pSH1313.

3.5.3.4. First experiment using *E. coli* strain RZ211.

When grown on MacConkey agar or nutrient agar containing X-gal (final concentration 40μg/ml), *E. coli* RZ211 grew as pale colonies which were indicative of a lac^- phenotype.

pDA116 and pLAFR1 were separately transformed into strain RZ211 and grown on nutrient agar plates plus tetracycline. Five 10ml nutrient broth cultures of each transformed strain plus strain RZ211 were grown overnight (16h) in randomised positions on an orbital shaker at 37°C. After incubation serial dilutions of the cultures were made. Duplicate 100μl aliquots of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread onto MacConkey agar plates and further duplicate 100μl aliquots spread onto

nutrient agar plates containing X-gal (40μg/ml). The plates were then incubated overnight at 37°C and the colonies examined for *lac*$^+$ phenotypes. No blue coloured colonies, indicating a switch to the *lac*$^+$ phenotype, were found . This experiment was repeated four times with the same result. The experiment was also performed with overnight incubation temperatures of 30°C, but this did not affect the result of the experiment.

3.5.3.5. Second experiment using *E. coli* strain RZ211.

E. coli strain RZ211 is able to grow on minimal agar supplemented with proline (final concentration 20μg/ml) and 0.2% (w/v) glucose. The strain did not grow on minimal agar supplemented with proline (20μg/ml) and 0.2% (w/v) lactose. The two transformed strains described above, and strain RZ211, were grown, in duplicate, in 100ml volumes of minimal medium supplemented with proline (20μg/ml)and 0.2% (w/v) glucose for 24h at 37°C. The cultures were then harvested by centrifugation (10,000g for 10 min at 4°C) and resuspended in 10ml of sterile saline. In each case, 5ml of the saline suspension was used to inoculate a further 100ml of minimal medium supplemented with proline (20μg/ml) and 0.2% (w/v) glucose, whilst the remaining 5ml was used to inoculate 100ml of minimal medium supplemented with proline (20μg/ml), 0.01% (w/v) glucose and 0.2% (w/v) lactose. These cultures were then incubated for 24h at 37°C. After incubation all cultures in minimal broth supplemented with proline (20μg/ml)and 0.2% (w/v)glucose had grown well whereas those cultures grown in minimal broth supplemented with proline (20μg/ml), 0.01% (w/v) glucose and 0.2% (w/v) lactose had grown only slightly. Serial dilutions of all cultures were

made and, as before, plated onto MacConkey agar and nutrient agar containing X-gal(40μg/ml). All colonies were pale indicating a *lac*$^-$ phenotypes. As with the pSH1313 experiments the results of the RZ211 experiments suggest that pDI116 does not encode a functional invertase in *E. coli*.

3.6. CONSTRUCTION AND USE OF OLIGONUCLEOTIDE PROBES FOR THE CONSENSUS INVERTASE RECOMBINATION SITES.

Oligonucleotide probes were constructed which were homologous to the consensus *dix* sites previously published. The sequences of these *dix* sites is given in Figure 7, as is the homologous sequence discovered in the genome of *M. bovis*. The *M. bovis* sequence had not been described when these oligonucleotide probes were synthesized.

All of the oligonucleotide probes synthesised were based around the consensus sequence of the *hin*-like recombination sites, which is: -A-GTTT--GA-AA. The ambiguous residues were decided using the table of recombination sites given in Figure 7, taking into account the preferred codon usage of *B. pertussis* (Glaser *et al.*, 1988). In most cases this meant using a G if it was known to occur in that position in any of the other related sequences.

The first probe synthesised had the following sequence:-

$^{5'}$TTT GGA AGG TTT TTG ATA A$^{3'}$

The sequence is based on that of *hix*R; the recombination site that is thought to be least related to the other members of the family. This oligonucleotide was a single sequence probe and was synthesised at a concentration of 232μg/ml.

An alternative mixed sequence probe was also constructed using different residues around the consensus sequence.

This probe had the sequence:-

$^{5'}$AAA CCA AGG TTT ATG AAA AAG$^{3'}$
G C T T T

This sequence is based on *hix*R and the other recombination sites. The main sequence is that of *hix*R and the other residues were added if they occurred in that position in any of the known *hin*-like systems. This probe was synthesised at a concentration of 237μg/ml and consisted of 32 different sequences.

Cla I (*Bsc* I)-digested *B. pertussis* DNA was immobilised onto Zeta-Probe (Bio-Rad) nylon membranes. These membranes were then placed into sealed bags containing the standard oligonucleotide pre-hybridisation buffer (section A.2.18) and manipulated as described (section 2.6.14). The optimum concentration for an oligonucleotide probe in a hybridisation experiment ranges from 1 to 5ng/ml of each sequence (Zeff and Geliebter, 1987). The hybridisation experiments were all carried out in a constant 20ml volume of hybridisation buffer. A 2μl aliquot of the first oligonucleotide was diluted ten-fold and 1μl of this dilution dried down with the γ-^{32}P-dATP and labelled. After dilution in hybridisation buffer this gave a final probe concentration of 1.2ng/ml. For the second oligonucleotide 3μl of the undiluted solution was dried down and labelled with the γ-^{32}P-dATP. This procedure gave a final concentration for each sequence in the mixed-sequence probe of 1.1ng/ml. Each of the two probes was then incubated with the filters in separate experiments at 50°C, 37°C, 25°C and 15°C. Each of the filters was then washed in SSC/PP at a final wash

temperature 15°C below the incubation temperature. In no experiment was hybridisation observed between either of the probes and *B. pertussis* DNA.

A new probe was then synthesized with the following sequence: -

$$\begin{array}{l} {}^{5'}\text{ACG TTT AGG AAA AAG }{}^{3'} \\ \quad\ \text{G} \qquad\ \text{TT} \quad \text{T} \quad \text{T} \end{array}$$

The sequence of this probe is more closely based around the consensus sequence than the previous two probes. The sequence is most closely based on *gixR* and *pixR*, the other residues added in accordance with their presence in the known sequences. This probe was synthesised at a concentration of 210µg/ml, and consisted of 32 different sequences.

A 4µl aliquot of this solution was dried down and labelled with the γ-^{32}P-dATP. This gave a final concentration of each of the sequences in the probe of 1.3ng/ml. The hybridisation temperatures used for this probe were 50°C, 37°C and 30°C. This final incubation temperature coupled with a final wash in SSC/PP at 37°C for 10 minutes permitted hybridisation between the probe and chromosomal DNA of *B. pertussis*, see Figure 22.

The probe hybridised to 14.5kb and 12.5kb *Cla* I (*Bsc* I) fragments in the DNA from strain Taberman DNA and 18.6kb and 16.5 kb *Cla* I (*Bsc* I) fragments of DNA from strain Tohama.

*Eco*RI digests of chromosomal DNA from a number of avirulent *B. pertussis* strains carrying transposon insertions within the *bvg* locus blotted onto a Hybond-N (Amersham) nylon membrane

FIGURE 22. HYBRIDISATION OF OLIGONUCLEOTIDE PROBE TO *B. pertussis* DNA.

The figure shows *B. pertussis* chromosomal DNA digested with *Cla* I (*Bsc* I) and unlabelled λ DNA digested with *Hin*dIII which has been run in a 0.8% agarose gel and Southern-blotted as described (section 2.6.9). The filter was incubated in the standard oligonucleotide pre-hybridisation buffer (Appendix 2, A.2.18) for 6h at 37°C. The oligonucleotide probe was then hybridised to this filter in the standard oligonucleotide hybridisation buffer (Appendix 2, A.2.19) overnight at 30°C. After incubation the filter was washed in SSC/PP several times at room temperature and once at 37°C for 15min. This autoradiograph was exposed for 14 days at -70°C.

Key to Figure:-

m = position of λ *Hin*dIII markers.

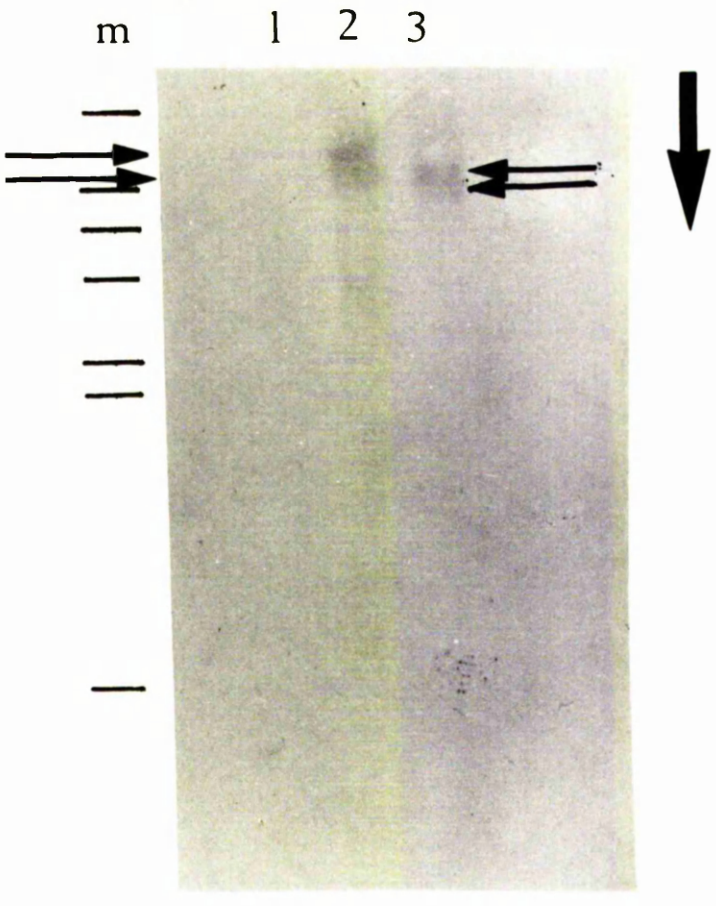
1 = λ *Hin*dIII fragments, unlabelled.

2 = *Cla* I (*Bsc* I) digested Tohama DNA.

3 = Cla I (*Bsc* I) digested Taberman DNA.

The small arrows indicate the position of the 18.6kb and 16.5kb *Cla* I (*Bsc* I) fragments in Tohama DNA, and the 14.5kb and 12.5kb *Cla* I (*Bsc* I) fragments in Taberman DNA the probe hybridises to under these conditions.

The large arrow indicates the direction of migration.


m
1
2
3

were probed with Tn5. This hybridisation highlighted the component *Eco*RI bands of the *bvg* locus in each strain which carried a Tn5 insertion responsible for the loss of virulence (M.J. Ward, personal communication). The Tn5 probe was stripped from this filter by incubation in 0.4N NaOH at 42°C for 30 mins, followed by a further 30 min incubation in 0.1 X SSC, 0.1%(w/v)SDS, 0.2M Tris-HCl, pH7.5, at 42°C with constant vigorous agitation. After stripping, the filter was exposed overnight at -70°C to check it was clean. The filter was then hybridised with the third oligonucleotide probe at 30°C with a final wash at 37°C. After exposure for 40 days at -70°C the probe was found to have hybridised to the same 15kb *Eco*RI fragment in all the strains. This fragment was not one of those highlighted by the Tn5 probe. However, after stripping and re-probing, the already weak signal from the oligonucleotide probe was virtully swamped by the background signal and reproduction of the filter was pointless.

This result suggests that sequences homologous to the *hin*-like recombination sites exist within the chromosome of *B. pertussis* but are unlikely to be associated with the *bvg* locus.

4. DISCUSSION.

4.1. PHASE VARIANTS AND DNA MODIFICATION.

The erythromycin-resistant non-haemolytic colonies isolated during the selection process appeared to fulfil the criteria demanded of avirulent strains by Weiss and Falkow (1984). During this study no colonies were observed which had reverted to virulent phase, despite the continuos subculturing of the avirulent strains. All *B. pertussis* strains used in this study were grown on BG agar supplemented with 10% (v/v) defibrinated horse blood. The only revertants reported in the literature occurred within variant strains grown on BG agar supplemented with 15% (v/v) sheep blood (Weiss and Falkow, 1984; Stibitz *et al.*, 1988). It is possible that some component of defibrinated sheep blood stimulates reversion, or, conversely, that some component of defibrinated horse blood may supress reversion.

The enrichment/selection procedure using increased resistance to erythromycin as a marker was more effective and less time consuming than the differential method which involved plating virulent *B. pertussis* onto cyclodextrin solid medium plates containing congo red (0.002%(w/v)) and isolating spontaneous phase variants which did not take up the dye.

The mutation responsible for phase variation in several strains of *B. pertussis* has been located and occurs within the *bvg* locus responsible for the production of a positive effector which stimulates the expression of genes encoding several virulence-associated factors. The cosmid pRMB2 contains a copy of the unmutated *bvg* locus and is able to complement strains carrying mutations in the *bvg* locus (Brownlie *et al.*, 1988;

McGillivray *et al.*, 1989). The recombinant plasmid pRMB2 is composed of 26kb of *B. pertussis* DNA cloned into the cosmid vector pLAFR1 and is present in low copy number in *B. pertussis* (R.M. Brownlie, personal communication). Introducing pRMB2 into the avirulent strains not only complemented the expression of the virulence-associated factors tested but increased the expression of these factors to a level above that of the virulent parent strains. It is possible that these strains may be useful in the production of components for a defined acellular vaccine, or could be used as vaccine strains themselves. It is thought that one of the major factors responsible for the adverse reactions to pertussis vaccines is the presence of lipooligosaccharide endotoxin in the final preparation. If these strains containing multiple copies of the *bvg* locus overproduce immunogenic proteins but retain otherwise normal biosynthetic metabolism, the number of bacteria needed to achieve a potent vaccine may be reduced, with a posssible concomitant reduction in reactogenicity. The reason for the reduced growth of these strains carrying pRMB2 may be explained not only by the extra genetic load but by observations made by Lee *et al.* (1989). Lee *et al.* placed multiple copies of the PT operon into strains of *B. pertussis* and found that strong selective pressure was exerted to reduce the amount of PT produced by each bacterium. They suggested that the excess PT was disrupting the internal biochemistry of the bacterium. If the PT genes in a strain carrying multiple copies of the *bvg* locus were mutagenised in a directed manner, as the results of Burnette *et al.* (1989) suggest may be possible, to give a non-functional immunogenic protein, the unwanted effect on the

growth of the bacteria may be removed and normal growth patterns recovered.

Evidence has beeen presented which suggests a 'hierachy' in the process of phase variation (Goldman *et al.*, 1984). In contrast to the one-stage virulent to avirulent switch suggested by Weiss and Falkow (1984) it has been proposed that phase variation may occur in an ordered manner, with the sequential loss of virulence factors in a given order. Goldman *et al.* (1984) isolated four distinct groups of *B. pertussis* variants:- i) Group A, equivalent to fully virulent strains, ii) Group B, haemolysin$^-$, but producing PT and FHA, iii) Group C, haemolysin$^-$, PT$^-$, producing only FHA, and iv) Group D, equivalent to fully avirulent strains. These patterns of expression appeared to be stably inherited.

All the avirulent strains tested in this study had lost expression of haemolysin, dermonecrotizing toxin and FHA, and consequently did not belong to any of the intermediate stages described by Goldman *et al.* (1984). No other group has reported this 'hierachy' of loss of virulence factors and the data generated by this study suports the 'one-step' model of phase variation, at least for those avirulent strains selected via increased resistance to erythromycin. The low basal expression levels of several virulence factors observed in some avirulent strains may simply reflect the strength of the promoters without the benefit of the positive effector BvgA. The apparently inheritable pattern of expression described by Goldman *et al.* (1984) may be the result of mutations in the promoter regions of various virulence factors which restore

function These hypothetical back mutations may be preferentially selected by the media used in those particular experiments.

Goldman *et al.* (1987) also studied the chromosomal DNA of a stable strain from each of the four groups they had defined. They found that DNA from virulent, Group A, strains was refractory to digestion by the restriction endonucleases *Eco*RI, *Sac* I, *Pst* I, *Kpn* I and *Msp* I. The DNA from avirulent, Group D, strains was digested by all of these enzymes. These results are contradictory to the results obtained in this and other studies. Digestion of chromosomal DNA from virulent strains of *B. pertussis* with *Eco*RI is performed routinely and the restriction pattern was the same as that of DNA from an avirulent strain digested by *Eco*RI. The DNA prepared from virulent strains in this study was also digested by *Sac* I, *Pst* I and *Kpn* I.

The enzyme *Msp* I is not affected by methylation of the recognition site (see Table 3 for details). However, Goldman *et al.* (1987) found that DNA from virulent, Group A, strains was not digested by *Msp* I, whereas avirulent, Group D, DNA was digested by the enzyme. However, they did not check the digestion patterns of their various DNA samples with *Hpa* II, an isoschizomer of *Msp* I, which is sensitive to methylation of the internal C residue. In this respect the results reported here are paradoxical; the enzyme affected by methylation at the cleavage site, *Hpa* II, digests the DNA samples from virulent

and avirulent strains, whereas the enzyme unaffected by methylation, *Msp* I, does not digest DNA from either source.

Goldman *et al.* (1987) were able to detect raised levels of 6-methyl adenine in virulent strains as compared to avirulent strains, but were not able to reproduce evidence of raised levels of 5-methyl cytosine in virulent strains. The only evidence available from the experiments performed during this study suggests that a small amount of methylation may be occuring to adenine residues within the cleavage site of *Mbo* I. Methylation of guanine residues within the cleavage sequence recognised by *Hpa* II and *Msp* I may explain the anomalous results generated by these enzymes.

The reasons for the discrepancies between the results of this study and that of Goldman *et al.* (1987) is possibly due to the method by which the *B. pertussis* strains were grown prior to isolation of chromosomal DNA. The DNA used in this study was prepared from *B. pertussis* grown in the synthetic, chemically defined liquid Stainer and Scholte medium whilst the bacteria used by Goldman *et al.* (1987) were grown on BG agar supplemented with 15% (v/v) sheep blood. After the harvesting of the bacteria, the DNA isolation procedures used were very similar. Given the specificity of *B.pertussis in vivo* growth and the evidence that expression of virulence factors is governed by the chemical composition of the external environment it is possible that the growth conditions are responsible for these apparent differences in DNA modification.

The inability of *Hin*dIII to digest *B. pertussis* DNA is to be expected since the bacterium is known to produce a restriction enzyme, *Bpe* I, with the same sequence specificity as *Hin*dIII (Greenaway, 1980). Interestingly, *B. pertussis* DNA cloned into *E. coli* was also found to be refractory to *Hin*dIII digestion, suggesting that *B. pertussis* may have purged all *Hin*dIII/*Bpe* I sites from its chromosome. This hypothesis could easily be tested by checking the various published *B. pertussis* nucleotide sequences for the presence of *Hin*dIII cleavage sites.

The 2-dimensional S1 heteroduplex mapping technique first described by Yee and Inouye (1982) has been used to detect differences between two isogenic DNA samples which differ in only one, or a few, small locations. The technique can pick up small differences caused either by genetic rearrangement or differential methylation.

The first step in this technique involves digesting the chromosomal DNA isolated from the parent and variant strains with a restriction enzyme that recognizes a 4bp cleavage site. This digestion generates a large number of small DNA fragments. One-third of each of these homoduplex mixtures is removed and both are mixed in a separate tube. All three DNA samples are then boiled to dissociate the DNA strands and allowed to reanneal slowly. The mixed DNA sample will now contain a proportion of heteroduplex DNA fragments, containing a strand from each of the two strains. The three samples are then separated electrophoretically in a 5% polyacrylamide gel. After separation, each of the three DNA-containing lanes of the gel

are removed and incubated with S1 nuclease. This enzyme only digests single-stranded DNA and does not affect double-stranded DNA. The homoduplex fragments may contain single-stranded DNA loops for a number of reasons: snapback of fragments containing two, or more, repeated sequences; reannealment of repeat sequences on different fragments may leave single stranded overhangs at either end. The heteroduplex fragments may contain the same single stranded loops as the homoduplex fragments but may also contain a region of single-stranded DNA caused by a rearrangement in one strand. A deletion in the DNA from one strand would result in a loop of single-stranded DNA in the other strand only in the heteroduplex fragments. An inversion in only one strand would result in several DNA loops depending on the location with respect to the restriction fragment.

When run on a second dimension polyacrylamide gel the majority of the fragments form a single diagonal streak. The fragments which have had single-stranded loops digested by S1 nuclease migrate faster and form 'blobs' underneath the streak. Analysis of these faster migrating fragments from the three samples should allow the novel area(s) in the heteroduplex sample to be found. This DNA can be cut from the gel, purified and used to probe chromosomal digests or the relevant gene library to isolate the loci of interest.

In control experiments involving *E. coli* chromosomal DNA model results were obtained, consisting of a long diagonal streak of DNA on the second dimensional gel and a collection of various small fragments of DNA migrating beneath the streak. In the case of the *B. pertussis* DNA the area beneath the streak

was an undefinable mass of fragments. This mass was presumably due to the number of different insertion sequences which have been reported within the *B. pertussis* chromosome.

Probing of the paired variant and parent strains with pRMB2 did not reveal any differences between the two. When these same DNA samples were probed with the *fim* 2 gene probe containing a portion of IS481v1 a complex pattern of bands was observed. However, scrutiny of this pattern also revealed no differences between the paired DNA samples, an observation confirmed by McPheat *et al.* (1989).

The data from this investigation into DNA from isogenic virulent and avirulent *B. pertussis* strains strongly suggests that no large-scale DNA rearrangements are involved in the phase variation of strains selected via increased resistance to erythromycin. This does not mean that small-scale DNA rearrangements either below the resolving power of Southern blotting techniques or involving areas we have not probed do not take place. The *B. bronchiseptica* chromosome contains a locus similar in function and structure to the *bvg* locus of *B. pertussis*. Monack *et al.* (1989) have found that the *vir* locus from some spontaneous *B. bronchiseptica* phase variants can contain small deletions ranging in size from 50-bp to 500bp. McGillivray *et al.* (1989) have also reported a deletion around the *bvg* locus in a *B. pertussis* phase variant. An avirulent Tn5 mutant of *B. pertussis* has been characterised. The Tn5 insertion in this mutant is not within the *bvg* locus, so it is possible that this is a spontaneous avirulent strain. Characterization of the *bvg* locus of this strain has revealed a

deletion of approximately 100bp in a 2.7kb *Eco* RI fragment encoding BvgA (M.J. Ward, personal communication). If these observations concerning spontaneous phase variants in *B. pertussis* are confirmed it may be possible that the selection of *B. pertussis* avirulent variants via increased resistance to antibiotics, erythromycin in this case and rifampicin and streptomycin in the strains investigated by Stibitz *et al.* (1989), preferentially selects strains with the frame-shift mutation in the *bvg* locus. A proportion of spontaneous avirulent mutants may therefore arise by deletions in the *bvg* locus. The deletion of a portion of the *bvg* locus would be a non-reversible genetic event which would be of little adaptive value to *B. pertussis* as a pathogen.

The results of the investigation involving methylation-sensitive pairs of restriction endonucleases would suggest that large-scale methylation of *B. pertussis* DNA is not involved in the Vir^+ to Vir^- switch. The major point of interest raised by this stage of the study was the loss of a band in the *Mbo* I digests of DNA isolated from the Vir^- strains. This implies that at least one *Mbo* I site has been either created or made available for digestion during the switching process. This phenomenon could be investigated further by excising the relevant band from *Mbo* I digests of Vir^+ DNA and using it to probe both Vir^- DNA and the gene library to isolate the involved locus for further study. This may reveal a level of DNA modification involved in the switching process that is too subtle to be picked up using Southern blotting techniques.

4.2. THE *fim* GENES OF *B. pertussis* STRAIN TABERMAN.

The oligonucleotide used to probe the *B. pertussis* Taberman gene library for fimbrial subunit genes has previously been used to probe a *B. pertussis* strain Wellcome 28 (fimbrial serotype 2,3) gene library (Livey *et al.*, 1987). The sequence of the probe was taken from the amino acid sequence of the first 14 amino-terminal residues of the fimbrial serotype 2 subunit (M_r 22 500) from *B. pertussis* strain Tohama (fimbrial serotype 2,0). Livey *et al.* found that oligonucleotide probes constructed for use with *B. pertussis* DNA had to take account of the preferred codon usage of the organism. Simply, this requires substitution of a G residue at every ambiguous site, since there is a strong bias against codons ending with A or T (Glaser *et al.*, 1988). Experiments by Livey *et al.* (1987) revealed that this probe was highly specific for *B. pertussis* DNA and they successfully hybridised this oligonucleotide probe to the *B. pertussis* Wellcome 28 gene library using the methods described earlier (section 3.6). To isolate the *fim* 2 gene hybridisation was performed at 65°C overnight and several washes at room temperature were followed by a 1h wash at 65°C. These conditions allowed hybridisation to occur only between the probe and cosmids containing the *fim* 2 gene.

A comparison of the amino-terminal amino acid sequences of the serotype 2, serotype 3 fimbrial subunit proteins (Cowell *et al.*, 1987) and the proposed product of the *fim* X gene (Pedroni *et al.*, 1988) reveals extensive homology and this homology is reflected in the nucleotide sequences of the three genes (Livey *et al.*, 1987; Pedroni *et al.*, 1988; Mooi *et al.*, 1990). At the

time of the experiment the nucleotide sequence of only the *fim* 2 and the *fim* X genes were known. Comparison of these sequences with that of the oligonucleotide probe demonstrated a 15% mis-match between the two sequences; extrapolation of the *fim* 3 nucleotide sequence from the amino-acid sequence predicted a 13% mis-match between the gene and the probe. A 1% mis-match between a probe and a target sequence results in a 1°C reduction in the T_M of the hybrid sequence. Thus, the hybridisation was carried out at 50°C overnight, with the final wash also being performed at 50°C. These conditions should theoretically have permitted hybridisation between the probe and the amino-terminal portions of the three fimbrial subunit genes. The *B. pertussis* strain Taberman (fimbrial serotype 0,3) gene library (Brownlie *et al.*, 1986) was then probed under these conditions. The nine cosmids which hybridised to the probe were analysed to reveal which fimbrial subunit genes they contained. The results of the enzyme analysis grouped the nine cosmids into three groups each of which contains a different putative *fim* gene.

The group 1 cosmids contained pDA126 and pDA128, which were copies of the same cosmid, and pDA395. These cosmids contained restriction fragments characteristic of the *fim* 3 locus. The *fim* 2 probe hybridised to the 0.9kb *Sal* I fragment which contains the *fim* 3 gene sequenced by Mooi *et al.* (1990). These two cosmids were thus deemed to contain a copy of the *fim* 3 gene from strain Taberman, a strain which expresses AGG 3.

The group 3 cosmids contained restriction fragments characteristic of the *fim* 2 locus. The *fim* 2 probe hybridised

to the 1.7kb *Sal* I fragment indicative of the *fim* 2 gene (Mooi *et al.*, 1987). The probe hybridised more strongly to the bands from these three cosmids than to the other five. This data suggests that the group 3 cosmids contain the silent *fim* 2 gene from strain Taberman, a strain which does not express AGG 2.

The group 2 cosmids did not contain any restriction fragments characteristic of the *fim* X locus described by Pedroni *et al.* (1988). The *fim* 2 probe hybridised to 0.5kb and 0.4kb *Sal* I fragments and not to the 3.9kb *Sal* I fragment characteristic of the *fim* X gene. It is therefore possible that strain Taberman contains a third fimbrial gene either different or distinctly altered from the one characterised by Pedroni *et al.* (1988).

The hybridisation experiments using the 0.85kb *Sph* I fragment of pIL22 (the *fim* 2 probe) showed each cosmid insert which contained a fimbrial subunit gene also contained a copy of the insertion sequence IS481. It is not yet known whether this observation is of significance or whether it results by chance simply due to the number of insertion sequences within the genome.

The high number of insertion sequences within the *B. pertussis* genome also complicates interpretation of data involving the hybridisation of the *fim* 2 probe to digests of *B. pertussis* chromosomal DNA. In experiments involving the probing of the chromosomal DNA isolated from serotype variants no evidence was found to suggest that large scale DNA rearrangements had occurred (this observation is subjected to

the same limitations as those described earlier for the investigation into phase variation). McPheat *et al.* (1989) have also probed serotype variants with a probe containing a copy of IS481v2 and found no rearrangements around this loci.

The eight cosmid clones containing putative *fim* genes were then conjugated into *B. parapertussis* strain 10520. It was decided to use the cosmids containing the entire large insert rather than smaller sub-clones since the regions controlling expression of the fimbrial genes may be some distance from the actual subunit genes themselves. The cosmid-containing strains were then tested for the expression of various agglutinogens. At the time of the experiment the identity of AGG 1 had not been published and it seemed reasonable to check for expression of AGG 1 from a fimbrial subunit gene. Unfortunately, the polyclonal antibody raised against AGG 1 gave weak but consistent agglutination with the wild-type *B. parapertussis* strain which did not carry any cosmids. The cosmid-bearing strains did not show any difference in the degree of agglutination with the polyclonal AGG 1 antibody. A monoclonal antibody raised against AGG 1 was then used; this antibody did not react with any of the *B. parapertussis* strains, but did react with several *B. pertussis* strains (unpublished observations). These results allowed us to conclude that either the absorbed polyclonal antibody is not monospecific or that a molecule on the surface of *B. parapertussis* shares partial identity with the *B. pertussis* AGG 1, but can by differentiated using a monoclonal antibody raised against a single epitope. If, as current results suggest (Li *et al.*, 1988), AGG 1 is the

LOS A endotoxin it is unlikely that AGG 1 will be expressed from a gene homologous to a fimbrial subunit gene. However, since the production of liposaccharide endotoxins is a feature of Gram-negative bacteria it is not surprising that *B. parapertussis* expresses a similar molecule as part of the cell surface.

Two of the cosmid-carrying *B. parapertussis* strains reacted positively with agglutinin raised against AGG 3. These strains contained the cosmids pDA126 and pDA395. This region of chromosomal DNA obviously contains the strain Taberman *fim* 3 gene. The strength of the agglutination observed in these tests was similar to that observed for *B. pertussis* strains expressing AGG 3 from a chromosomal locus. This result demonstrates that the *B. parapertussis* virulence regulatory system is able to recognise and positively regulate genes from *B. pertussis*. It also further sugggests that the mechanism used by *B. parapertussis* to assemble and express fimbrial subunits functions equally well with fimbrial subunits derived from *B. pertussis fim* genes.

Lee *et al.* (1989) found that *B. parapertussis* containing a plasmid-borne PT operon could not export active PT outside the cell. The chromosomal PT operon is not expressed in *B. parapertussis* due to an accumulation of deleterious mutations in the promoter region of the operon, thus the maintenance of a PT export mechanism is wasteful and deleterious mutations have probably also occured in the genes concerned with PT export.

The six other cosmid-containing *B. parapertussis* strains did not react with agglutinins raised against either AGG 3 or AGG 2. Given the previous results indicating that active *B. pertussis* fimbrial subunit genes are expressed in *B. parapertussis* it was assumed that these clones contained silent copies of the *fim* 2 and *fim* X-like genes.

In order to remove the possibility that *trans*-acting factors may control the suppression of the various *fim* genes, the behaviour of the active *fim* 3 gene in different *B. pertussis* strains was tested. The strains used were all deficient in fimbrial serotype 3 expression; strain BP 347 has a transposon insertion within the *bvg* locus and does not express any of the *bvg*-regulated gene products, strain Tohama is a reference strain which expressess fimbrial serotype 2, but variants are known to express fimbrial serotype 3, strain ARG 40 is a derivative of strain Wellcome 28 which has lost the ability to express AGG 3 after passage through infant mice, and, strain 106V has lost the ability to express both AGG 2 and AGG 3 and is afimbriate. After conjugation of pDA126 into strains Tohama, ARG 40 and 106V the agglutination experiments revealed agglutination at levels comparable to the wild-type expression of AGG 3, no agglutination was observed with strain BP347 carrying pDA126.

From this experiment it was deduced that strains which have lost the ability to express AGG 3 do not contain *trans*-acting factors which repress expression of the *fim* 3 gene. There is no evidence to suggest that the specificity of the transport process is altered during serotype variation; synthesized

fimbrial subunits are assembled and exported as normal regardless of the status of the chromosomal *fim* gene.

B. pertussis strain 106V is a derivative of a fimbrial serotype 2,3 strain which has lost expression of both AGG 2 and AGG 3. Electronmicrographs of this strain demonstrated that the loss of expression had rendered the strain afimbriate. The addition of pDA126 to this strain restored both the AGG 3 phenotype and the presence of fimbriae on the cell surface. This constitutes proof that the molecule referred to as AGG 3 and recognised by the Preston antiserum is a fimbrial structure. The presence of fimbriae on this strain was linked to the tetracycline resistance encoded by the cosmid. During the course of the experimental work the strain did not revert to expression of either AGG 2 or AGG 3 from chromosomal loci. An interesting experiment would compare the infectivity in animal models and adhesion in tissue culture models of strain 106V $\pm$ pDA126. There is still scant direct evidence to suggest that fimbriae are involved in the adhesion of *B. pertussis* to ciliated cells. The results from *in vivo* and *in vitro* studies of these isogenic strains may shed some light on the role fimbriae play in the disease process. These experiments may also allow investigation into the selective pressures responsible for serotype variation. *B. pertussis* cells containing cosmids encoding several virulence-related molecules were found to be retained during passage through mice even in the absence of antibiotic selection pressure (R. Brownlie and R. Parton, personal communication). Infection of mice immunised with various fimbrial subunits with sub-lethal doses of 106V $\pm$

pDA126 and other control strains may allow the recovery of a number of different serotype variants. Will the *fim* 3 gene carried on the cosmid be affected in the same manner as the chromosomal gene if the mouse is immunised with AGG 3 beforehand? If so, this would be a method of providing isogenic *fim* gene-containing cosmids which should differ only in the mutation(s) responsible for serotype variation of that gene. Nucleotide sequencing of these clones and subsequent comparison with the wild-type sequence will reveal the nature of the mutation(s). This method of selecting variants would not rely on passage of the strains through artificial media which may affect the mutation process.

In summary, the results of these experiments demonstrate that the *B. pertussis* chromosome contains three different unlinked genes with the potential to encode fimbrial subunit proteins. The products of the *fim* 2 and *fim* 3 genes are known, the product of the *fim* X gene, if there is one, is not known. The *B. pertussis* strain Taberman (fimbrial serotype 0,3) appears to contain a copy of the *fim* 3 gene which can be expressed in both *B. pertussis* and *B. parapertussis*. The chromosome of this strain also contains a silent *fim* 2 gene and a *fim* X-like gene. The behaviour of the *fim* 3 gene in a variety of different AGG 3$^-$ backgrounds was investigated. A hierachy of control levels was found. The *fim* genes require positive regulation by the *bvg* A gene product, this is the highest level of the hierachy, below this is the level of control responsible for serotype variation. The data generated by these experiments suggested that the control of serotype variation occurs at the

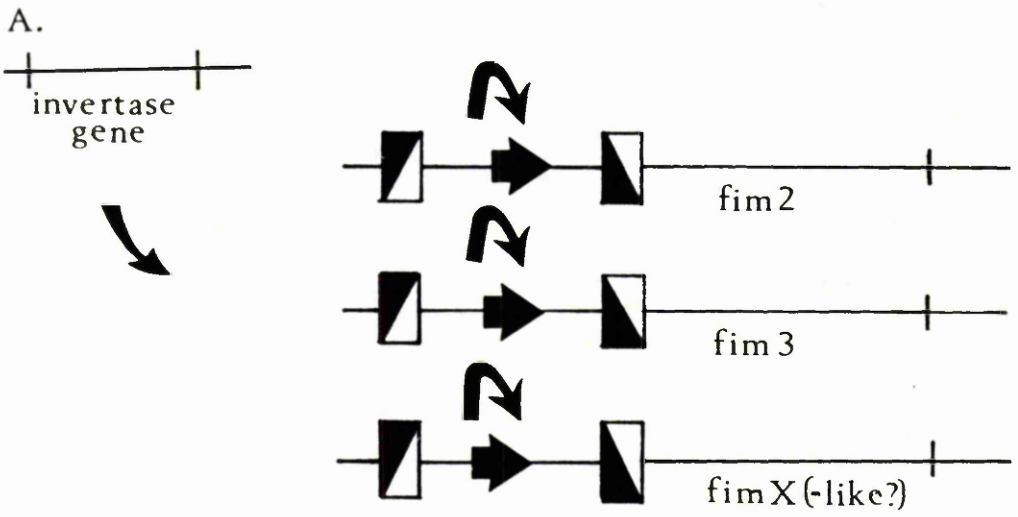
cis-acting level. If the three fimbrial subunits arose by gene duplication(s) it is reasonable to assume that the genes are all controlled by a similar mechanism. This assumption is supported by the similar rates of variation reported for the two fimbrial serotypes.

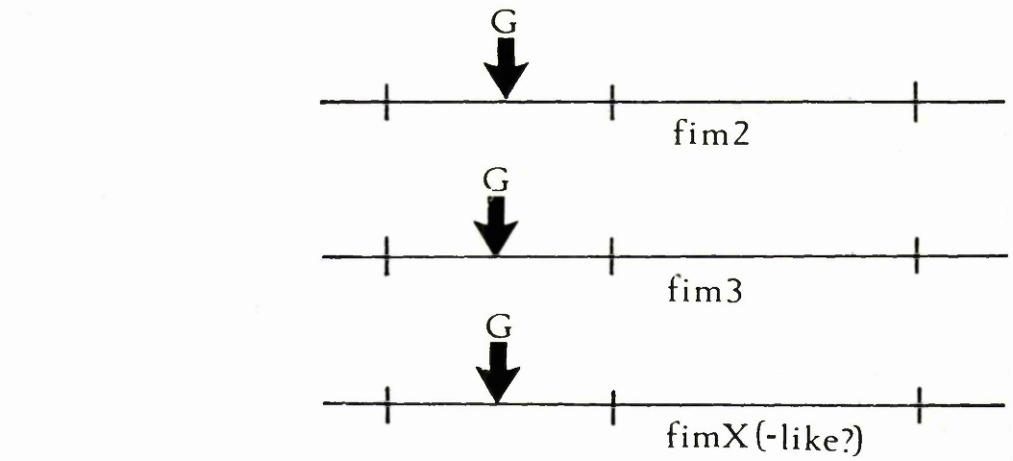
One of the complications in proposing a model for serotype variation is that the pattern of variation involves three different possible events. Unlike flagellar phase variation of *S. typhimurium* which involves the either/or expression of one of two flagella types, the change in expression being linked, or the variation in the Type 1 pili of *E. coli* which also occurs in an either/or manner between two distinct molecules, *B. pertussis* serotype variation involves the independent change in expression of two unlinked genes, with the possibility of a third gene being involved. A likely hypothesis for this situation involves each fimbrial subunit gene having its own seperately regulated *cis*-acting control region. These regions may be controlled in a global manner from a single gene, or each control region may be autonomously regulated. Various proposals for these systems are shown in Figure 22. Example A. illustrates how serotype variation could be controlled by a single invertase gene. Each fimbrial subunit gene possesses an upstream invertible control region which contains the BvgA-dependent promoter for the gene. The invertase gene produces an invertase molecule which acts independently upon each control region. The invertase protein must be expressed only in small amounts as any selective advantage conferred by serotype variation lies in the novelty of a new serotype. Deletion of

FIGURE 23. POSSIBLE MECHANISMS OF SEROTYPE VARIATION.

A. Invertase protein encoded by one gene independently causes inversion in control regions of *fim* genes. The invertible regions contain the *fim* gene promoters.

B. The control region of each *fim* gene contains a region susceptible to frame-shift mutations, analogous to the region within the *bvg* locus.


A.
invertase
gene
fim2
fim3
fimX (-like?)


B.
G
fim2
G
fim3
G
fimX (-like?)

the control region upstream of the *fim* X (-like) gene or a mutation in the recombination site which traps the gene in the 'OFF' position would result in the loss of any expression from that gene.

Example B. gives another method by which serotype variation could occur, this model is suggested by Mooi *et al.* (1990). The published sequence of the *fim* 3 gene contains a C-rich region upstream of the gene which appears also to be conserved upstream of the *fim* 2 gene. Mooi *et al.* (1990) suggest that this region may be involved in serotype variation in a manner analogous to the C-rich region in *bvgA* responsible for phase variation in several strains of *B. pertussis* (Stibitz *et al.*, 1989). This suggestion is circumstantially supported by the observation that the loss of fimbrial expression is more common than the gaining of expression. However, the rate of serotype variation is greater than that of phase variation, arguing against this hypothesis.

These possible mechanisms for serotype variation all assume that the event responsible for serotype variation occurs at the transcriptional level. No evidence has been published to support this assumption. A precedent does exist, however, in many other species of bacteria for variation of this kind to occur at the transcriptional level. Probing the mRNA from various serotype variants of *B. pertussis* for the presence or absence of fimbrial-specific mRNA molecules would establish whether the variation occurs at the transcriptional level or not. It would also be interesting to test a wide variety of

B. pertussis strains for the presence of *fim* X-specific mRNA, in an attempt to find the product of this gene.

The most obvious next step involving these cosmids would be the sequencing of the *fim* genes and their control regions. The data generated by these experiments could then be compared to the published sequences for differences that may be related to the mechanism of serotype variation. Sequencing may also shed light on the identity of the *fim* X-like gene present in the strain Taberman chromosome. Gene libraries made from the chromosomal DNA of isogenic serotype variants may prove useful in similar studies in case serotype variation occurs by a number of mechanisms, as may happen during phase variation. All the information at present indicates that serotype variation occurs only between established fimbrial molecules. No evidence exists to show that new fimbrial configurations are generated during variation. This is an observation of importance in the construction of a comprehensive, defined-component pertussis vaccine.

4.3. SEQUENCES WITHIN THE *B. pertussis* CHROMOSOME HOMOLOGOUS TO THE *hin* SEQUENCE OF *S. typhimurium*.

Foxall *et al.* (1990) used the plasmid pKK1001B as a probe to detect sequences within *B. pertussis* chromosomal DNA homologous to the entire invertible region of *S. typhimurium*. The major drawback of this experiment is that the probe contains adjacent sequences not directly related to the invertible region. These include the H2 flagellin gene and the *rh*1 gene. Their experiments suggested that hybridisation did not occur between *B. pertussis* DNA and these other genes.

The preliminary hybridisation experiments in this study repeated the work of Foxall *et al.* and produced similar results. However, they observed hybridisation between pKK1001B and a single high molecular weight *Eco* RI fragment of approximately 15kb. In this study hybridisation clearly occurs between pKK1001B and two high molecular weight *Eco*RI fragments, both approximately 15kb in length. This discrepancy may be explained by the differences in the stringency of the final washes given to the filters. The filters hybridised by Foxall *et al.* were given a final wash in 6 X SSC, 0.1%(w/v)SDS at 55°C for 1hr, whereas the filters used in this study were given their final wash in 2 X SSC, 0.1%(w/v)SDS at 55°C for 1hr. This increase in the final wash stringency may have been responsible for removing excess probe and revealing the hybridisation to the two similarly sized *Eco* RI fragments. The removal of excess probe also contributes to the higher background in the filters used in this study, due to a necessary increase in the exposure time.

One of the more interesting observations made from these results is the repeatable difference in the strength of hybridisation of the probe to Taberman and Tohama chromosomal DNA. In every experiment involving a *hin*-derived probe the chromosomal DNA from Taberman strains has bound the probe to a greater extent than DNA from Tohama strains. This effect does not appear to be due to the relative concentrations of chromosomal DNA loaded into each lane, as care was taken to load the same amount into each lane. The simplest explanation of this phenomenon is that there is greater homology between the sequence within the strain Taberman DNA and the probe than between the probe and strain Tohama DNA. Strain Taberman is a strain recently isolated from a patient whereas strain Tohama has a long history of laboratory use. The sequence in strain Tohama may have diverged due to inactivity during the laboratory history of the strain whilst the sequence in strain Taberman may have retained greater functional homology in the infecting population. A similar difference was also found between strains L84I and L84IV, the history of these strains is not as well known as that of the other strains and a similar explanation cannot be proposed for this difference.

The previously characterised *hin*-like invertible DNA systems consist of three component parts; the gene encoding the invertase, the conserved *dix* sites and a recombinational enhancer. The pKK1001B probe includes all of these components as well as several other genes such as the H2 flagellin gene. The advent of the random hexanucleotide primer radio-labelling technique by Feinberg and Vogelstein (1983) allowed the labelling of short linear DNA fragments using α-^{32}P-dATP to

high levels of activity. A short, highly specific DNA probe could thus be isolated from a larger piece of DNA with a known sequence. The sequence of the invertible region has been published (see Figure 3) and was scanned for restriction enzyme cleavage sites. The ensuing information allowed the selection of a probe which would be specific for a *hin*-like invertase gene. The 671bp fragment highlighted in Figure 3 appeared to be the optimum invertase specific probe which could be generated from the sequence.

This probe did not hybridise to *B. pertussis* DNA under high stringency, an unsurprising result, indicating that the invertase-like sequence differed from that of *S. typhimurium*. The major problem with the hybridisation protocol involved the large difference between the mol G+C% content of *S. typhimurium* and *B. pertussis* DNA, 50-53% and 66-70%, respectively. This difference implies that there will be a relatively large divergence between the two nucleotide sequences whilst the proteins retain a high degree of functional homology. Various studies have shown that a 1% mismatch reduces the T_M of the duplex by between 0.5 and 1.4°C, in this case the mismatch is expected to be spread throughout the length of the target sequence which further decreases the stability of the duplex (Anderson and Young, 1985). The ionic strength of the hybridisation and washing solutions can be used to help stabilise mis-matched duplexes, thus in these hybridisation experiments the probe was incubated in hybridisation buffer containing a higher salt concentration than normal (6.6 X SSC) and washed in higher salt buffers (2 X SSC).

The results indicate that a single copy of the *hin*

homologous sequence exists within the *B. pertussis* chromosome. This *hin* homologous sequence is located on a large *Eco*RI fragment and is closely bounded by *Cla* I (*Bsc* I) sites. The second high molecular weight *Eco* RI fragment that hybridises to the probe may contain a gene encoding a protein similar to the invertase such as a transposase. This may be due to the presence of numerous insertion sequences within the *B. pertussis* chromosome, one of which may contain a gene bearing sufficient homology to the probe to allow hybridisation under the lowest stringency conditions. Hybridisation to the larger of the two *Eco* RI fragments did not take place when the stringency of the hybridisation reaction was increased. Under the least stringent conditions the probe hybridised to a single 12kb *Bam*HI fragment. If the probe is picking up a closely related sequence it would appear that the two are linked on the same large *Bam*HI fragment.

Similar *hin* homologous sequences would appear to be present within the chromosomes of *B. bronchiseptica* and *B. avium*. Curiously these low stringency conditions did not demonstrate hybridisation between the probe and the chromosomal DNA of *B. parapertussis* 10520. This is unusual, since there is a close genetic relationship between *B. pertussis* and *B. parapertussis*. Homology between the plasmid pKK1001B and the chromosome of a *B. parapertussis* strain has been observed (C.J. Duggleby, personal communication) so it is possible that this sequence has simply been deleted at some point in the history of *B. parapertussis* strain 10520. The sequence present within the *B. bronchiseptica* genome is present on *Cla* I (*Bsc* I) and *Stu* I fragments of similar size to those in the *B. pertussis*

chromosome. The intensity of hybridisation appears to be less than that observed to the *B. pertussis* fragments, suggesting greater divergence from the probe.

The *B. avium* observations raise two interesting points. Firstly, *B. avium* DNA would appear to be refractory to digestion with *Cla* I (*Bsc* I). This would indicate that *B. avium* possesses a modification system which protects the *Cla* I (*Bsc* I) cleavage site. Secondly, the degree of hybridisation of the probe to the *B. avium* DNA samples is far greater than that observed for the *B. pertussis* samples. This may be due either to the mol G+C% content of *B. avium* being closer to that of the probe than *B. pertussis*, or the functional homology may actually be greater.

The major problem with the Southern blots using the low stringency conditions was the high degree of background interference. This continually made their interpretation difficult and also made their reproduction of little value. An attempt to increase the stringency of both the hybridisation and washing procedures to lower the background to acceptable levels was made. The concentration of formamide in the hybridisation buffer can be used to alter the T_M of the probe-target hybrid. A 1% increase in formamide concentration lowers the T_M of duplex DNA by 0.72°C. The optimum hybridisation signal is obtained using 33% (w/v) formamide, equivalent to a reduction in the T_M of 23.8°C. Standard protocols for the detection of perfectly matched probe-target hybrids recommend 50% (w/v) formamide concentration, which is equivalent to a reduction of 36°C in the T_M of a perfectly matched duplex.

Szekely and Simon (1981) detected homology between a *hin*-containing probe and Mu DNA with a final wash in 0.24M Na phosphate, 1 X Denhardt's solution and 0.2% (w/v)SDS at 55°C. When the wash temperature was reduced to 50°C the probe bound to P1 DNA. The salt concentration in the final wash used in this study is ≈0.2M NaCl at a temperature of 50°C, thus the homology between the *hin* probe and the *B. pertussis* DNA is approximately equal to that described by Szekely and Simon (1981). Under these conditions the hybridisation of the probe to *B. avium* DNA was still considerably greater than to that of the *B. pertussis* DNA. The exact degree of homology between the various Bordetella DNA samples and the probe could be investigated using a melting experiment similar to that described by Szkely and Simon (1981). Alternatively this information could be obtained by sequencing of the relevant loci and direct comparison.

The problems with high backgrounds did seem to be linked to the activity of the α-^{32}P-dATP because as the age of the isotope decreased the exposure time was increased and, consequently, the background increased. If the filters were washed stringently enough to totally reduce the background, the probe was also removed. A solution appeared to involve labelling the probe with biotin, which could be recognised with an enzyme conjugate. The activity of the label using this method did not decrease drastically over time and removed the need for autoradiography. However, a vital stage in the development of the signal using a biotin probe involves blocking the filter in a BSA solution at 65°C for one hour. The lack of signal in the *hin*-related experiments was probably due

to the fact that this blocking reaction was more stringent than the final wash and removed virtually all of the probe from the filter prior to development of the signal. If this is the case this marks a serious flaw in the technique if applied to cases where non-homologous hybridisation is needed. The stringency of the blocking reaction was lowered to well below the threshold which would have removed the probe but this simply increased the level of background until any possible signal was obscured.

The hybridisation conditions detailed above were also found to be membrane-specific. Hybridisation was observed between the probe and the target when the DNA was immobilised on Zeta-Probe and GeneScreen Plus membrane, but not when the DNA was immobilised on Hybond-N membranes. An investigation by Khandjian (1987) revealed large differences in the hybridisation signals obtained using different membranes under standard conditions. This study revealed that Hybond-N hybridised very poorly when the DNA was transferred using alkali, the method used thoughout this study.

These results demonstrate that the chromosome of *B. pertussis* contains a sequence homologous to the *hin* gene of *S. typhimurium*. From the hybridisation data it would appear that the sequence is as closely related to the *hin* sequence as the invertible sequences present in bacteriophages Mu and P1. A similar sequence also appears to be present within the chromosome of *B. bronchiseptica* and *B. avium*.

The final step in this preliminary investigation of the *hin*-like sequence in *B. pertussis* involved isolating the cosmid containing the relevant sequence from the Taberman gene library.

Restriction fragment analysis of pDI116 allowed a map to be constructed with respect to *Eco*RI, *Xho* I and *Cla* I (*Bsc* I) cleavage sites. The major restriction fragments are in the correct order, although ambiguity exists as to the exact order of the 3.0kb, 3.0kb and 0.6kb *Cla* I (*Bsc* I) fragments. This ambiguity will be removed after nucleotide sequencing of this locus since the 0.6kb fragment contains the *hin* homologous sequence. The order given in Figure 20 was chosen as this places the 0.6kb fragment next to the 11kb *Cla* I (*Bsc* I) fragment recognised by the recombination (target) site-specific oligonucleotide probe (Figure 21). The major use of this map is to suggest which fragment would be the most suitable to sub-clone for further investigation.

Cloning of the 10kb *Eco*RI/*Xho* I fragment which contains the 0.6kb *Cla* I (*Bsc* I) fragment would yield a DNA fragment which could be sequentially deleted in both directions, placed under the control of a strong promoter and tested for invertase activity. The 0.6kb *Cla* I (*Bsc* I) fragment could also be placed under the control of a strong promoter but the fragment may well be too small to produce an active protein. This 10kb fragment, or the entire 15kb *Eco*RI fragment, would also be candidates for nucleotide sequencing. These fragments contain not only the *hin* homologous fragment but enough surrounding DNA to give a clue as to the possible function, if any, of the invertase gene.

The 0.85kb *Sph* I fragment of pIL22, which contains the *fim* 2 gene and a portion of IS481v1, hybridised to a single 2.65kb *Sal* I fragment of pDI116. This fragment is different from any of the *Sal* I fragments the probe hybridised to in the

cosmids containing the *fim* genes. This result implies that the *hin*-like sequence carried by pDI116 is not linked with any *fim* genes but is linked with another copy of IS481 (McPheat *et al.*, 1989). As mentioned before it is not known whether this juxtaposition of insertion sequences and possibly virulence-associated genes is meaningful or simply the result of the number of insertion sequences within the genome.

A number of assays exist which can test for invertase activity encoded by plasmid borne invertase genes. The major assay used in this study involved the plasmid pSH1313 (Huber *et al.*, 1985). In its 'ground' state this plasmid encodes resistance to ampicillin and chloramphenicol, in the presence of an exogenous invertase the cartridge containing the resistance genes (see Figure 9) inverts and the plasmid then confers resistance to ampicillin and kanamycin. The experiments involving this plasmid and pDI116 did not reveal any invertase action encoded by pDI116. This may have been due to several reasons:- *a*) the gene simply does not encode a functional invertase in *E. coli* or even in its natural state in *B. pertussis*, *b*) the promoter of the *hin* homologous gene may require positive regulation by Bvg A, *c*) the recombination sites of pSH1313 are derived from the *cin* system of bacteriophage P1 and may not be complementary with the invertase encode by the *hin* homologous gene. The experiments involving *E. coli* strain RZ211 lead to the same conclusions.

The invertase gene, plus attendant recombinational enhancer sequence, form only one half of a functioning invertible DNA sequence. Inversion also requires a pair of inverted repeat target sequences to act as the recombination sites between

which inversion occurs. In the *hin*-like invertible sysytems so far characterised the recombination sequences are fairly highly conserved. The newly discovered invertible DNA sequence responsible for pilin variation in *M. bovis* has recombination sites which share homology with the *hin*-like recombination sites. This high degree of homology between the various recombination sites was exploited in the creation of oligonucleotide probes which should have had the ability to detect related sequences. At the time of synthesis the sequence of the *M. bovis* recombination sites was not known and the information was not incorporated into the design of the oligonucleotide probes.

The final probe synthesised was able to hybridise, albeit weakly, to *B. pertussis* chromosomal DNA. The low hybridisation signal suggested that either only one of the 32 possible sequences was able to hybridise under the conditions used or that several hybridised very weakly. The major finding of these experiments was that sequences related to the *hin*-like recombination sites do occur within the *B. pertussis* genome. The probe hybridised to different sized bands in the chromosome of strain Taberman and strain Tohama, this may be indicative of an inversion event which has occurred at some point in the history of one of the strains. The *bvg* and *fim* 2 probes used in this study found no differences in the banding patterns between these two strains.

Weiss and Falkow (1984) had suggested that an invertible DNA sequence may be responsible for phase variation in *B. pertussis*. The oligonucleotide probe did not hybridise to any of the Eco R1 fragments implicated in the *bvg* locus. The

nucleotide sequence of the *bvg* locus from a number of sources (Stibitz *et al.* 1988; Relman *et al.*, 1989) does not share any homology with the *hin*-like invertible regions. It therefore seems a fair conclusion that the sequences within the *B. pertussis* genome homologous to invertible DNA sequences do not play any role in the phase variation process. The sequence data of the *fim* genes (Livey *et al.*, 1987; Pedroni *et al.*, 1988; Mooi *et al.*, 1990) also reveals no homology to invertible sequences around these loci. The *fim* containing cosmids could be probed with a range of mixed-sequence oligonucleotide probes homologous to variants of the consensus sequence to ascertain if they contain sequences related to invertase recombination sites. However, if the suggestion by Mooi *et al.* (1990) that serotype variation occurs via a frame-shift mutation is correct it would appear that the *hin*-homologous sequences play no part in serotype variation. This raises the question of what function these sequences may play.

The *pin* invertible sequence in several *E. coli* strains has been cloned (Kutsukake *et al.*, 1985) and sequenced (Plasterk *et al.*, 1983), yet no group has so far been able to ascribe a function to this sequence. The invertase gene and the recombination sites are usually linked as are the invertible region and the area under its control. Thus, probing the gene bank with an oligonucleotide probe with a greater degree of specificity to the *B. pertussis* sequences should allow the cosmid containing the genes under the control of the invertible region to be isolated. This cosmid may prove to be one which codes for a known virulence-associated factor, in which case the function of the invertible sequence should be fairly

straightforward to deduce, or may contain DNA with no apparent function, in which case the role of the invertible sequence may be almost impossible to define.

The results generated by this study with regard to the *hin*-like sequences found within the *B. pertussis* genome can be regarded only as preliminary studies. Nucleotide sequencing of the invertase gene and surrounding DNA will shed light not only on the relationship of the sequence to other invertible DNA systems but may reveal the role of this sequence within the *B. pertussis* genome.

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APPENDIX 1.

All media and sterile equipment used in this study was sterilised for 15 mins at 121°C before use.

A.1.1. Stainer and Scholte medium (+ cyclodextrin).

For one litre medium:-

Sodium L-glutamate	10.70g
L-proline	0.24g
NaCl	2.50g
KH_2PO_4	0.50g
KCl	0.20g
$MgCl_2 \cdot 6H_2O$	0.10g
$CaCl_2$	0.02g
Tris	1.50g
Heptakis(2,6-O-dimethyl)β-cyclodextrin	1.00g)

Distilled water added to a volume of 1 litre and the pH adjusted to 7.4 with concentrated HCl. The medium was then autoclaved and stored at 4°C.

The following growth supplements were prepared:-

L-cysteine	0.04g
$FeSO_4 \cdot 7H_2O$	0.01g
Niacin	0.004g
Glutathione (reduced)	0.10g
Ascorbic acid	0.02g

The supplement was made up in 100ml of distilled H_2O and filter sterilized (Millipore filters, pore size 0.22μm). The

supplement was stored at 4°C for no longer than 2 weeks. The supplement was added as 1ml per 100ml basal medium

For Stainer and Scholte medium plus α-cyclodextrin 1g/litre of cyclodextrin and 1%(v/v) casamino acids were added to the basal medium described above. Casamino acids (Difco) were made up as a 25%(w/v) solution in distilled H_2O, autoclaved and stored at 4°C. Cyclodextrin was supplied by Dr. J. Shimizu, Medical and Pharmaceutical Division, Teijin Ltd, Tokyo, Japan.

A.1.2. Bordet Gengou agar.

40g of BG agar (Gibco-BRL) and 10g of glycerol were made up to 900ml with distilled H_2O and autoclaved. The agar was allowed to cool to ≈50°C before the addition of 100ml of defibrinated horse blood (InterMed).

For sandwich plates the medium was split into 600ml and 400ml lots. The first was poured without the addition of blood and allowed to set. The blood was added to the second lot and this was poured thinly over the set plates.

A.1.3. CSM Media.

For one litre medium:-

Sodium L-glutamate	10.70g
L-proline	0.24g
NaCl	2.50g
KH_2PO_4	0.50g
KCl	0.20g
$MgCl_2 \cdot 6H_2O$	0.10g

$CaCl_2$	0.02g
Tris	6.10g
Casein hydrolysate peptone 5 (Gibco)	0.50g
Heptakis (2,6-O-dimethyl)β-cyclodextrin	1.00g
Bacto-Agar (Difco)	18.00g

The pH was adjusted to 7.4 prior to autoclaving.

The following growth supplements were prepared:-

L-cysteine	0.04g
$FeSO_4 \cdot 7H_2O$	0.10g
Niacin	0.004g
Glutathione (reduced)	0.15g
Ascorbic acid	0.02g

The supplement was made up in 10ml of distilled H_2O and filter sterilised (Millipore filters, pore size 0.22μm). The supplement was stored at 4°C for no longer than 2 weeks. The supplement was added to 1l basal medium immediately prior to use.

A.1.4. Nutrient agar.

Nutrient agar (Oxoid) was made up with 2.8g per 100ml distilled H_2O.

A.1.5. Nutrient broth.

Nutrient broth number 2 (Oxoid) was made up as 1.4g per 100ml distilled H_2O.

A.1.6. Minimal medium

For 1 litre of minimal medium:-

NaCl	5.0g
NH_4NO_3	1.0g
Na_2SO_4	2.0g
K_2HPO_4	3.0g
KH_2PO_4	1.0g
$MgSO_4.7H_2O$	0.1g
(Noble Agar	15.0g if needed).

A.1.7. 4-chloro-1-naphthol solution.

Mix 1 part solution A with 5 parts solution B immediately prior to use.

A) 3mg/ml 4-chloro-1-naphthol in methanol.

B) 0.018%(v/v) H_2O_2.

APPENDIX 2.

Solutions and buffers used in DNA manipulation techniques throughout this study. Unless otherwise stated all solutions were made up in sterile distilled water.

A.2.1. Tris-EDTA buffer (TE).

10mM Tris.HCl (pH8.0)

1mM EDTA

A.2.2. Chromosomal lysis buffer.

25% (w/v) sucrose

50mM Tris.HCL (pH 8.0)

1mM EDTA.

A.2.3. TE-saturated phenol.

Hydroxyquinoline was dissolved in liquid phenol to a final concentration of 0.005g/ml. The phenol was twice mixed with an equal volume of 1M Tris.HCl (pH 8.0) and the excess buffer removed. The phenol was then mixed with TE + 0.1% (v/v) β-mercaptoethanol and excess buffer removed. Phenol was then stored at 4°C under the final buffer.

A.2.4. Plasmid lysis buffer.

50mM glucose

25mM Tris.HCl (pH8.0)

10mM EDTA

4mg/ml lysozyme (Sigma) added immediately prior to use.

A.2.5. Acidified potassium acetate solution.

The acidified potassium acetate solution is prepared as follows:-

60ml 5M potassium acetate

11.5ml glacial acetic acid

28.5ml distilled H_2O

A.2.6. Å solution.

0.1mM dCTP

0.1mM dGTP

0.1mM dTTP

A.2.7. Loading buffer.

10% (w/v) Ficoll

0.05% (w/v) bromophenol blue

0.005% (w/v) xylene cyanol FF

0.05% (w/v) orange G

5µl loading buffer used per 20µl of sample.

A.2.8. TBE buffer.

0.089M Tris-borate

0.089M boric acid

0.002M EDTA

pH 8.2

Made up as 10 X concentrate.

A.2.9. TAE buffer.

0.04M Tris-acetate

0.002M EDTA

pH 8.2

Made up as 50 X concentrate.

A.2.10. 30% (w/v) acrylamide solution.

acrylamide	29g
N,N'-methylene bisacrylamide	1g

H_2O to 100ml.

A.2.10. Denaturing buffer.

1.5M NaCl

0.5M NaOH

A.2.11. Neutralising buffer.

1.5M NaCl

1M Tris-HCl (pH8.0)

A.2.12. SSC solution.

1 X SSC = 0.15M NaCl

0.015M sodium citrate

Made up as 20 X SSC.

A.2.13. High stringency hybridisation buffer.

6 X SSC

1% (w/v) SDS

5 X Denhardt's solution

50% formamide

sssDNA 100μg/ml

Denhardt's solution = 0.02% (w/v) bovine serum albumin

0.02% (w/v) Ficoll

0.02% (w/v) polyvinyl pyrrolidine

6 X SSC

sssDNA = single-stranded salmon sperm DNA made up at 10mg/ml, denatured by repeated passage through a G25 hypodermic needle. Before use the sssDNA is boiled for 5 minutes and added to the hybridisation buffer immediately.

A.2.14. Low stringency hybridisation buffer.

7 X SSC

1% (w/v) SDS

5 X Denhardt's solution

33% deionised formamide

sssDNA 100μg/ml

A.2.15. OLB buffer.

OLB buffer made up as follows:-

Solution O = 1.25M Tris.HCl (pH8.0)

0.125M $MgCl_2$

Solution A = 1ml solution O

18μl β-mercaptoethanol

5μl dCTP (100mM in TE)

5μl dGTP (100mM in TE)

5μl dTTP (100mM in TE)

Solution B = 2M Hepes pH 6:6

Solution C = hexadeoxynucleotides (Pharmacia) suspended in TE to 90 OD units per ml.

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

A.2.16. STOP buffer.

20mM NaCl

20mM Tris.HCl (pH7.5)

2mM EDTA

0.25% (w/v) SDS

1μM dATP

Stored at -20°C.

A.2.17. T4 polynucleotide kinase buffer.

0.05M Tris.HCl (pH8.0)

0.01M $MgCl_2$

50mM dithiothreitol (Sigma)

0.1mM spermidine (Sigma)

0.1mM EDTA

Made up as a 10 X concentrate, stored at -20°C.

A.2.18. Oligonucleotide pre-hybridisation buffer.

6 X SSC

1 X Denhardt's solution

1% (w/v) SDS

sssDNA 100μg/ml

0.05% (w/v) sodium pyrophosphate

A.2.19. Oligonucleotide hybridisation buffer.

6 X SSC

1 X Denhardt's solution

0.05% (w/v) sodium pyrophosphate

20μg/ml tRNA

A.2.20. S1 nuclease buffer.

300mM NaCl

30mM sodium acetate (pH4.6)

4.5mM $ZnCl_2$

100μg bovine serum albumin/ml

APPENDIX 3.

A.3.1. REact™ BUFFERS.

REact™ buffers are supplied with enzymes ordered from BRL at 10 X concentration. The formulas given below are the working concentrations.

REact™1: 50mM Tris-HCl (pH8.0)
10mM $MgCl_2$

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™4: 20mM Tris-HCl (pH8.0)
5mM $MgCl_2$
50mM KCl

REact™6: 50mM Tris-HCl (pH8.0)
6mM $MgCl_2$
50mM KCl
50mM NaCl

REact™8: 20mM Tris-HCl (pH8.0)
10mM $MgCl_2$

A.3.2. Other buffers.

Bsc I buffer: supplied by NBL at 10 X concentration:
50mM Tris-HCl (pH7.5)
100mM NaCl
10mM $MgCl_2$
1mM dithioerythritol

M buffer: 6mM Tris-HCl (pH8.0)

50mM NaCl

6mM $MgCl_2$

6mM β-mercaptoethanol

APPENDIX 4: SINGLE LETTER CODES FOR AMINO ACIDS.

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Asparagine or Aspartic acid	B
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glutamine or Glutamic acid	Z
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

APPENDIX 5: LENGTHS OF NUCLEIC ACID SIZE MARKERS.

λ*Hin* dIII FRAGMENTS	ΦX174*Hae* III FRAGMENTS
23.1kb	1353bp
9.4kb	1078bp
6.5kb	872bp
4.3kb	605bp
2.3kb	310bp
2.0kb	281bp
0.56kb	271bp
0.125kb	234bp
	194bp
	118bp
	72bp