Genetic Studies with Bordetella pertussis

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DEDICATION

This thesis is dedicated to my Mother and Father who have supported me tirelessly in both undergraduate and postgraduate work.

It is also dedicated to my grandparents and to Kyle who have always been sources of support, happiness and love.

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"As a good Scot, I was in my youth, taught to be cautious".

Sir Alexander Fleming,

Discoverer of lysozyme and penicillin.

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SUMMARY

The primary aim of this research was to develop a reliable system for gene transfer in Bordetella pertussis to facilitate the study of the genetics of virulence in this species.

The starting point of the research was an attempt to repeat the procedures of Branefors (1964) and of Al-Sallami (1981) to introduce a streptomycin resistance (Str^R) chromosomal gene into a streptomycin sensitive (Str^S) B. pertussis recipient by DNA-mediated transformation. However, this system proved unsatisfactory and convincing evidence of transformation was not obtained.

Attention was next turned to developing a transfer system using conjugative R plasmids of the P incompatibility group. These were successfully employed to mobilise donor B. pertussis nutritional and antibiotic resistance genes. Several suitable plasmids, namely, R68.45, RP1 and derivatives containing transposon Tn501 and Tn7 were obtained in Escherichia coli hosts. The optimal conditions for plasmid transfer were determined for E. coli donors and recipients and, under these conditions, the plasmids were transferred by conjugation from E. coli to B. pertussis.

Having successfully transferred the plasmids into B. pertussis, the next step was to determine if B. pertussis chromosomal genes could be mobilised by the plasmids. The ability of each plasmid to mobilise antibiotic resistance genes between E. coli strains was first determined and all except one plasmid R68.45supdnaG315 mobilised genes at satisfactory frequencies. In B. pertussis, mobilisation of streptomycin and nalidixic acid resistance markers occurred but the relative mobilising abilities of the various plasmids differed. The presence of transposon Tn7 on a plasmid appeared to enhance mobilisation whereas transposon Tn501 had the opposite effect.

The isolation of auxotrophic mutants of B. pertussis which could be used as recipients for mobilised donor prototrophic genes was explored. This required the development of a minimal medium for isolation of auxotrophic mutants and for selection of prototrophic recombinants. These studies coincided with the report by Imaizumi et al. (1983) of the stimulatory effect of methylated β-cyclodextrin on the growth of B. pertussis on defined solid medium. In the present investigation, Cyclodextrin Solid Medium (CSM) was found to be suitable for the isolation and characterisation of the nutritional requirements of B. pertussis auxotrophic mutants. These were obtained by mutagenesis followed by enrichment by penicillin treatment.

The R plasmids were tested for their ability to mobilise prototrophic markers between strains of B. pertussis. In one case, linkage of the Gly^+ (glycine biosynthesis) and Nal^R (nalidixic acid resistance) markers was demonstrated.

Next, the expression of B. pertussis genes in B. parapertussis was studied. Using R plasmid-mediated chromosome mobilisation, it was possible to obtain expression of donor B. pertussis genes in a chloramphenicol resistant recipient.

A transposon insertion mutant of B. pertussis was used in preliminary experiments aimed at mobilising a virulent phase marker, haemolysin production (Hly^+), from a suitable donor. Although no mobilisation of Hly^+ was obtained, mobilisation of another donor gene, Str^R, occurred.

Plasmids which fail to replicate in B. pertussis were used to introduce transposons Tn5 and Tn501 into the organism to generate transposon insertion mutations. Although such mutations were generated, they were not characterised further.

A study was undertaken to attempt the isolation of bacteriophage from B. pertussis. No such bacteriophage were found, but the ability of B. pertussis to support lytic cycle growth of a mutant of bacteriophage Mu was demonstrated.

The susceptibility of B. pertussis and B. parapertussis chromosomal DNA to digestion by restriction endonucleases EcoRI and HindIII was investigated to determine if the DNA was modified at HindIII restriction sites. This was found to be the case.

Since the long term aim of this work was to further the study of the genetics of B. pertussis virulence, an opportunity was taken to examine the effect of plasmid presence on the interaction of B. pertussis with neutrophils. With B. pertussis BPT2 and E. coli JC3272 transconjugants, the presence of plasmids altered the levels of neutrophil stimulation, as measured by chemiluminescence, while little effect was apparent with B. pertussis 44122/7R transconjugants.

In conclusion, the way is now clear for detailed investigation of the genetics of B. pertussis virulence using R plasmid-mediated mobilisation of chromosomal genes. This would also allow further studies on linkage of antibiotic resistance and nutritional markers. The apparent ease with which B. pertussis genes can be recombined and expressed in B. parapertussis makes the latter a suitable recipient and lends support to the idea that B. parapertussis should be regarded as a variant of B. pertussis rather than as a separate species.

1. INTRODUCTION

1.1 WHOOPING COUGH - THE DISEASE

1.1.1 Historical background

The first clinical description of whooping cough was given by Guillaume de Baillou (1538-1616) (cited by Castiglioni, 1947), during an epidemic in France in 1578. He called it "quinta" or "quintana" but it later became known as "coqueluche". Baillou described "quinta" in Epidem. et ephemer Lib II p.237, published posthumously in 1640 (cited by Castiglioni, 1947). In the 18th century, there ensued a rapid spread of the disease, particularly in northern countries. For example, in Sweden alone, 40,000 children died of the disease between 1749 and 1764 (Castiglioni, 1947).

The aetiological agent of the disease was first cultured by Bordet and Gengou (1906). Initially, the organism was classified as a member of the genus Haemophilus due to its apparent requirement for growth on blood-containing media, but when the organism was later shown not to be dependent on blood nutritional factors, it was reclassified into a new genus. Thus, the organism responsible for whooping cough is now known as Bordetella pertussis (Moreno-Lopez, 1952).

1.1.2 Clinical symptoms

The onset of the disease is mild and is usually not recognised as being pertussis. Normally, the period of incubation is about 7 days and several distinct stages occur during the course of pertussis.

(i) Catarrhal stage. Upon inhalation of the organisms by a susceptible individual, the bacteria lodge between the cilia of the respiratory tract epithelial cells where attachment to the cells may be mediated by the fimbriae (Pittman, 1979). At this stage, the symptoms are those of a mild cold with some coughing episodes which increase in severity over the next 10 to 15 days.

(ii) Paroxysmal stage. The coughing attacks continue to increase in number and severity, with the characteristic "whoop" appearing due to inspiration of air through the narrowed glottis, into the lungs. At this stage, live organisms cannot usually be isolated from the patient. Patho-physiological changes at areas of the body distant from the infectious site occur. Systemic manifestations of the disease include lymphocytosis/leukocytosis, effects on blood glucose levels, possible neurotoxicity and sensitivity to histamine, caused by the action of pertussis toxin. (Sanyal, 1960; Tsukimoto and Lampkiss, 1976; Askelof and Bartfai, 1979; Askelof and Gillenius, 1982; Hewlett et al., 1983). Toxic damage may extend to lung tissues and blockage of bronchioles may lead to areas of lung collapse and emphysema. The most serious complications of the disease are secondary infections, seizures, encephalopathy and death. In treatment of pertussis, erythromycin is the antibiotic of choice as it is effective and safe for use in infants (Islur et al., 1975). Prophylactic erythromycin treatment for whooping cough contacts has been proposed but there is no evidence that this prevents the disease (Grob et al., 1981; Spencely and Lambert, 1981).

1.1.3 Vaccine

In the last one hundred years, the mortality rate from pertussis has declined rapidly due to improved health care and the introduction of pertussis vaccine. Field trials in the 1920's demonstrated that vaccine prophylaxis could control spread of the disease (Madsen, 1933). Vaccines used in these trials consisted of heat-killed cells of fresh isolates and similar results were obtained using a phenol-inactivated (Sauer 1933; 1937) and a thimerosal-inactivated vaccine (Kendrick and Eldering, 1936), but Doull et al. (1936) showed no significant protective effect between vaccinated and non-vaccinated groups. Such differences in protective efficacy led to the introduction of

the Intracerebral Mouse Protection Test which allowed comparison of vaccine efficacy and potency (Kendrick et al., 1947). Field trials in the U.K. with whole cell vaccines demonstrated that they were reasonably safe and effective in the control of pertussis (MRC, 1951; 1956). Present whole cell vaccines are suspensions of heat-killed B. pertussis cells which invariably contain agglutinogens 1, 2 and 3. Some problems exist in that the vaccines are reactogenic but public concern that the vaccine may constitute a greater risk than the disease (Miller et al., 1982) and be associated with brain damage in children (Stewart, 1979) appears to be unfounded. The U.K. National Childhood Encephalopathy Study findings indicate that no causal relationship exists between vaccine and serious neurological illness. (Joint Committee on Vaccination and Immunisation, 1981; Klein et al., 1982).

Current research in the area of pertussis vaccine concentrates on the development of acellular vaccines, in particular, the isolation and purification of LPF (pertussis toxin) and filamentous haemagglutinin (F-HA) (Munoz et al., 1981; Irons and MacLennan, 1979a,b; Sato et al., 1983). Since October 1981, an acellular vaccine consisting mainly of F-HA, formalin-detoxified LPF and a trace of endotoxin has been in prophylactic use in Japan. This may be regarded as a first-generation acellular vaccine (Manclark and Cowell, 1984). Second generation vaccines may be more uniform in potency and in specified constituents and should have little or no endotoxin and little LPF toxicity. Third generation vaccines may include antigens involved in attachment and toxoids. Genetically-engineered antigens would allow production of cross-reactive peptides which are immunogenic but not toxic and could be produced, probably more economically, in an organism other than B. pertussis. Cloning and expression of B. pertussis outer membrane protein genes in E. coli has been demonstrated (Shareck and Cameron, 1984). Current studies on the genetic structure of B. pertussis will aid in the understanding of the control and expression of the virulence factors and protective antigens of the organism.

1.2 B. PERTUSSIS - THE ORGANISM

1.2.1 Growth

The initial isolation of B. pertussis in the early 1900's was on a medium which consisted of potato starch infusion, glycerol and defibrinated blood (Bordet and Gengou, 1906). In honour of the discoverers of the bacterium, the medium is now known as Bordet-Gengou Agar (BGA). B. pertussis is a Gram-negative coccobacillus which varies in size from 0.2 to 0.8μm. When grown on BGA at 35°C, the organism produces a glistening convex colony which may exhibit a hazy zone of haemolysis. Carbohydrates, lactate, pyruvate and intermediates of the Embden-Meyerhoff pathway are not utilised (Jebb and Tomlinson, 1957; Rowatt, 1955; Parker, 1976). Glutathione and ascorbic acid are required for growth in some media and there is an absolute requirement for nicotinamide or nicotinic acid. On BGA, cultures grown from small inocula may not produce colonies for 48-72h. In liquid culture, growth from small inocula can be obtained in medium containing nicotinamide, glutathione, ascorbic acid, salts, cysteine, glutamic acid and proline (Stainer and Scholte, 1971). This was the first defined medium described for the growth of B. pertussis, all previous liquid media having contained casamino acids or casein hydrolysate (Hornibrook, 1939; Cohen and Wheeler, 1946). Various blood-containing media were used for growth of B. pertussis until it was realised that unsaturated fatty acids present in media constituents, such as agar and peptone, and in cotton stoppers and on glassware, were toxic to the organism (Pollock, 1949; Proom, 1955; Rowatt, 1957a,b; Field and Parker, 1979). Additives to media which would adsorb fatty acids and permit growth of the organism included starch, charcoal, anion-exchange resins, blood, albumin and Heptakis 2,6-O dimethyl β-Cyclodextrin (MeβCD) (Sutherland and Wilkinson, 1961; Parker, 1976; Kloos et al. 1978; Imaizumi et al. 1983). The last named was capable, upon incorporation in a solid medium, of supporting growth from small inocula

as efficiently as BGA. No minimal medium suitable for genetic and nutritional studies on B. pertussis has yet been developed, although several attempts have been made (Parker, 1976; Kloos et al. 1978). Addition of MeβCD to a solid medium may be an important step in the development of such a medium.

1.2.2 Variation in B. pertussis

B. pertussis is known to undergo two types of variation.

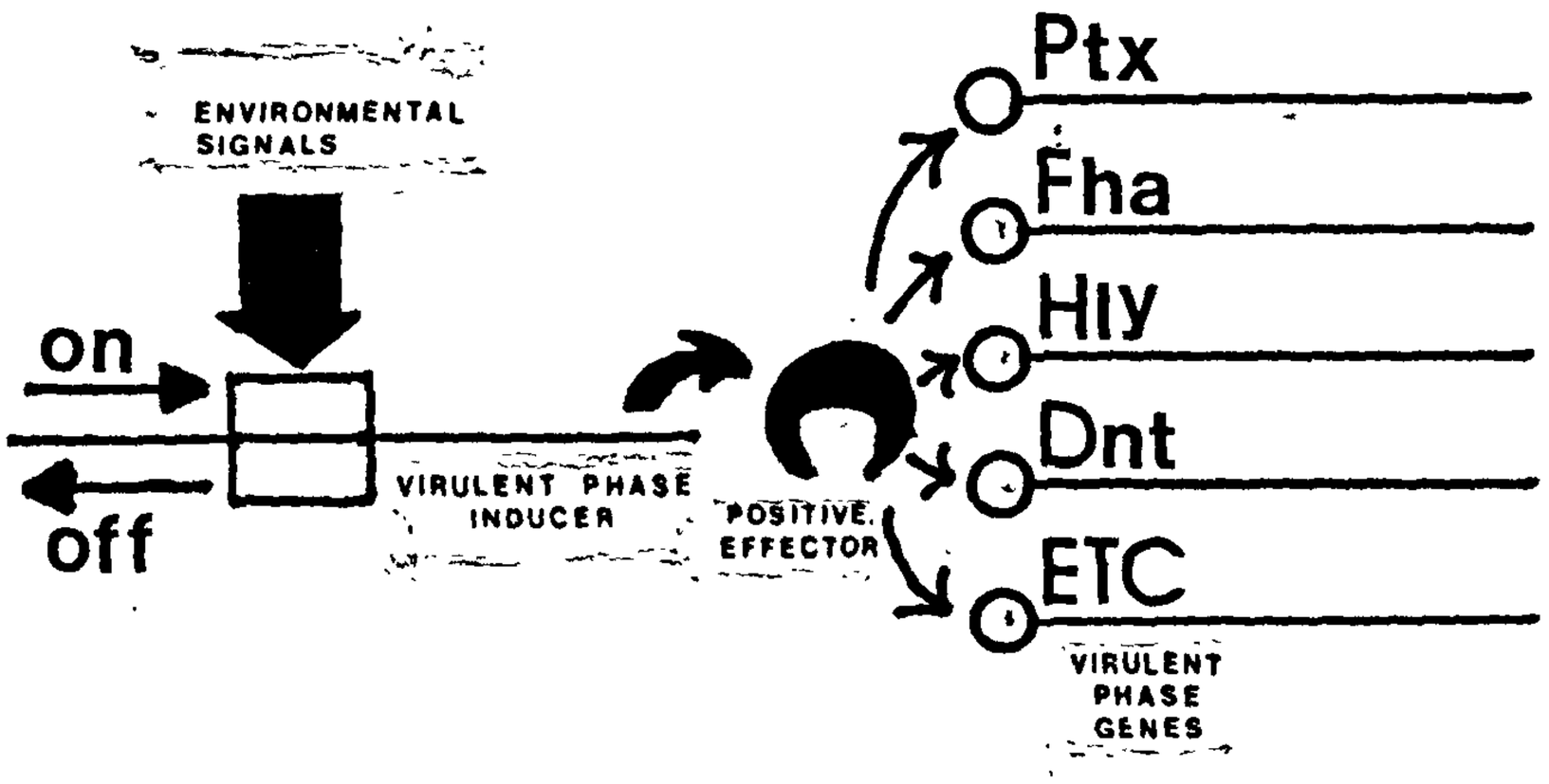
1.2.2.1 Phase variation

Upon repeated subculture, virulent strains of B. pertussis undergo a simultaneous loss of the ability to synthesise virulence factors such as pertussis toxin, adenylate cyclase, haemolysin, filamentous haemagglutinin and other components such as cytochrome d-629 and several outer-membrane proteins (Wardlaw and Parton, 1979; Wolff et al., 1980; Ezzell et al., 1981; Peppler, 1982; Cowell et al., 1982; Katada and Ui, 1982). The terminology used to describe phase variation has not been standardised, the process having been referred to as change from phase I to phase III (Kasuga et al., 1953), phase I to phase IV (Leslie and Gardner, 1931), fresh isolate to degraded state (Parker, 1979) or domed/haemolytic to flat/non-haemolytic (Peppler, 1982). However, the phase designations of Kasuga et al. are not equivalent to those of Leslie and Gardner : domed colony type and haemolysin production refer to only two of the many properties lost in phase shift and these have been mutated independently of other phenotypic characteristics of the virulent state (Weiss et al., 1983). A more descriptive designation - virulent phase to avirulent phase - has been used as a term for the potential of the bacteria to cause disease when they express all of the virulence-associated properties (Weiss et al., 1983). Regulation of virulence-associated genes may have a hereditary component since, under permissive conditions, the progeny of virulent or avirulent phase strains inherit the same phase. The change from virulent to avirulent phase was thought to occur by mutation and to be irreversible (Parker, 1979),

Figure 1 : A model for the regulation of virulence association genes by a positive effector in B. pertussis.

The lines represent the DNA coding regions for the regulatory gene and the virulence-associated genes. The circles represent the sites of action for the regulatory element. The trans-acting positive effector may be a protein encoded by the virulent-phase inducer gene, or this gene could specify an activity which synthesised a product required for the expression of virulence associated genes. Environmental signals such as temperature, Mg^{2+} ions or nicotinic acid could modulate expression of the positive effector. The molecular mechanism of the ON/OFF switch is unknown, but may be an invertible sequence.

(After Weiss and Falkow, 1984).


ENVIRONMENTAL SIGNALS
on
off
VIRULENT PHASE INDUCER
POSITIVE EFFECTOR
Ptx
Fha
Hly
Dnt
ETC
VIRULENT PHASE GENES

but in one instance phase change has been shown to be reversible and may perhaps be due to regulation by a trans-acting gene product which induces virulence-associated genes and may itself be under the control of a DNA rearrangement similar to that which controls pilus expression in Salmonella. (Weiss and Falkow, 1984; Zieg et al., 1977).

1.2.2.2 Antigenic modulation

Lacey (1960) reported that B. pertussis underwent an antigenic transition when grown on BGA in which NaCl was replaced by $MgSO_4$. The change was reversible, growth on NaCl giving rise to the virulent phenotype while growth on $MgSO_4$ gave rise to the avirulent phenotype. These were referred to as X-mode and C-mode respectively due to their colony appearance (X-xanthic, C-cyanic), and the effect was termed 'antigenic modulation'. Other factors which induced modulation included growth in medium containing one of several salts, growth in high nicotinic acid concentration and growth at 28°C (Pusztai and Joo, 1967; Holt and Spasojevic, 1968; Livey et al., 1978; Wardlaw and Parton, 1979). Several virulent phase properties are lost during modulation : pertussis toxin (PT) activities such as HSF and LPF activity, heat-labile toxin (HLT), cell-envelope polypeptides, filamentous haemagglutinin (F-HA), agglutinogen 1 and adenylate cyclase (Holt and Spasojevic, 1968; Livey et al., 1978; Dobrogosz et al., 1979; Wardlaw and Parton, 1979). Some of these components such as adenylate cyclase, PT, HLT and F-HA may play a role in the pathogenesis of whooping cough (Pittman, 1979; Confer and Eaton, 1982), while the role of factors such as the membrane polypeptides are unclear.

Antigenic modulation may be under the control of the trans-acting gene product discussed previously. This gene product may be repressed when bacteria are in the C-mode condition and regulation of the gene may occur in response to environmental conditions such as temperature, magnesium ion or nicotinic acid concentrations (see Diagram, Figure 1) (Weiss and Falkow, 1984).

1.2.2.3 Variation as a survival strategy.

Avirulent phase organisms have been isolated from patients during the course of infection (Kasuga et al., 1954); the proportion of avirulent phase organisms increasing as the infection proceeded. Expression of virulence factors occurs only during the virulent or Phase I stage, although loss of one virulence associated gene product does not lead to the loss of others (Weiss et al., 1983). Thus mechanisms which allow switching on and off of virulence factor synthesis may be essential for the existence of the organism in vivo. Phase change may be a mechanism which, by regulation of virulence factor expression via the trans-acting effector protein, allows escape from immune detection. Accompanying phase change there is an increased tolerance to erythromycin and other antibiotics (Weiss and Falkow, 1984; Peppler and Schrumpf, 1984). This may permit survival of avirulent phase organisms in the presence of antibiotics used in treatment of whooping cough. Environmental signals such as temperature, Mg^{2+} ions, nicotinic acid or the immune status of the host may modulate expression of the positive effector. The phase switch could therefore allow survival of the organism in human or non-human environments in an asymptomatic state, until a switch back to the virulent phase occurs in a susceptible individual.

1.2.3 Virulence factors of B. pertussis.

The organism produces a range of biologically active components which may contribute to the ability of the organism to cause disease.

1.2.3.1 Filamentous haemagglutinin (F-HA)

This component is a protein of molecular weight 107,000 to 130,000 Daltons (Sato et al., 1983) and appears, in electron microscopy, as filaments 40-100nm in length (Arai and Sato, 1976; Sato et al., 1982). The protein is most efficiently isolated from supernates of cultures grown statically in shallow

culture (Arai and Munoz, 1979; Ashworth et al., 1982) or in shake flasks with Cyclodextrin-containing media (Suzuki et al., 1984). It can be purified by column chromatography on hydroxylapatite with removal of contaminating LPF by passage through haptoglobin - or fetuin - Sepharose (Cowell et al., 1982; Sato et al., 1983). The protein is a haemagglutinin and is subject to inhibition by cholesterol (Irons and MacLennan, 1979a) and may be fimbrial in origin (Sato et al., 1979), although recent findings have cast doubt on this hypothesis (Ashworth et al., 1982).

1.2.3.2 Pertussis toxin

Also known as Lymphocytosis-Promoting Factor (LPF), Histamine-Sensitising Factor (HSF), Islet-Activating Protein (IAP) and Pertussigen, the array of names used to describe this protein reflects its wide range of biological activities. It is produced by Phase I (X-mode) cells and is believed to be a major virulence factor and protective antigen, since highly purified crystalline toxin is protective and remains so after toxoiding (Munoz et al., 1981). The concept that a single component may be responsible for mouse-protective activity, histamine sensitisation and heat-labile adjuvancy was first proposed in a 'Unitarian Hypothesis' (Levine and Pieroni, 1966). HSF was later shown to be a haemagglutinin distinct from F-HA (Arai and Sato, 1976; Morse and Morse, 1976) and to be responsible for production of leucocytosis (Lehrer et al., 1974) implying that a single component was responsible for these activities.

Pertussis toxin is a globular protein which can be purified from culture supernates by chromatography and gel filtration on haptoglobin or fetuin. These sialic acid-containing proteins are known to inhibit haemagglutination by pertussis toxin (Irons and MacLennan, 1979a,b). Molecular weight estimations vary from about 80,000 (Yajima et al., 1982) to 117,000 (Tamura et al., 1982). The protein appears to be composed of five subunits (Tamura et al.,

1982) termed S-1 to S-5, with molecular weights of 28,000, 23,000, 22,000, 11,700 and 9,300 respectively. S-2 to S-5 form the binding or B-oligomer which can block action of the whole toxin by competing for receptor occupancy and may mediate toxin binding to host cell membranes. This oligomer may be responsible for several of the biological activities (Nogimori et al., 1984). The S-1 subunit (active or A-protomer) interacts with the B-oligomer to form intact toxin and has been shown to be an ADP-ribosyl transferase which catalyses ADP ribose transfer from NAD^+ to a 41,000 Dalton host cell membrane protein (Tamura et al., 1982; Hildebrandt et al., 1983).

1.2.3.3 Agglutinogens

The genus Bordetella can be differentiated by species-specific agglutinogens (Anderson, 1953; Eldering et al., 1957). Agglutinogen 7 is common to all members of the genus, and agglutinogen 1 is present in all B. pertussis strains while 2, 3, 4, 5 and 6 may vary between strains. Although the nature and location of these antigens is uncertain, Ashworth et al. (1982) have suggested that agglutinogen 2 is located on fimbriae. It may be that agglutinogens are important protective antigens and should be included in acellular vaccines (Preston 1963; Eldering et al., 1967) since disease does not occur in the presence of agglutinins of high titre (Miller et al., 1943).

1.2.3.4 Adenylate cyclase

B. pertussis produces a heat-stable, calmodulin-activated, adenylate cyclase which is extracytoplasmic and can be released into the supernate or remain cell-associated. The enzyme, in supernates, has a molecular weight of about 70,000 (Hewlett and Wolff, 1976). It may contribute to virulence by interfering with the function of phagocytic cells (Confer and Eaton, 1982), allowing survival in a hostile environment, and it also exhibits effects such

as a reduction in host inflammatory response and enhancement of primary infection. Further evidence for a role in virulence now exists, since strains which do not produce the enzyme are severely impaired in their ability to infect mice (Weiss et al., 1984).

1.2.3.5 Heat-labile toxin (HLT)

HLT (Dermonecrotic Toxin) can be isolated from the cytoplasmic fraction of cell lysates of B. pertussis and is sensitive to heating at 56°C for 10 minutes (Munoz et al., 1959; Cowell et al., 1979). When toxoided, active HLT becomes antigenic (Munoz, 1971). Purification of HLT from B. pertussis and B. bronchiseptica, which is free from pertussis toxin has been carried out. The toxin preparation induced vasoconstriction, in peripheral blood vessels in guinea-pigs or mice, leading to formation of local lesions, partial haemorrhage and oedema (Nakase and Endoh, 1984). Anti-inflammatory agents, such as prednisolone and meclofenamate were found to inhibit the skin reactions in young mice (Parton, 1986).

1.2.3.6 Lipopolysaccharide endotoxin (LPS)

Two distinct LPS endotoxins exist in B. pertussis, designated LPS-I and LPS-II (Le Dur et al., 1978). Like other bacterial LPS, it exhibits toxicity and pyrogenicity in animals, Shwartzmann reaction, adjuvanticity and non-specific resistance to infection (Farthing, 1961). Although the LPS role in pathogenesis is unclear, antibodies are produced in humans following vaccination with whole-cell vaccine (Ashworth et al., 1983).

1.3 GENETIC TRANSFER IN PROKARYOTES

Three mechanisms exist which allow transfer of genetic information between bacteria.

1.3.1 Transformation

Genetic transformation is a process by which a bacterial cell takes up DNA from the surrounding medium and incorporates it into the genome to acquire an altered genotype that is heritable. In genera such as Bacillus, Streptococcus and Haemophilus transformation is comparable in importance to conjugation and transduction as a means of gene transfer (Graham and Istock, 1978; Smith et al., 1981). These organisms have evolved highly efficient mechanisms for release, uptake and incorporation of DNA. The transformation process was first observed in the pneumococcus (Griffith, 1928) and can be divided into several discrete steps :-

(i) Development of the ability to take up DNA (competence)

(ii) Binding and uptake of DNA.

(iii) Preintegration and integration.

Both Gram-positive and Gram-negative bacteria follow these basic steps, although they employ different mechanisms to perform each step, possibly reflecting differences in cell envelope structure. In Haemophilus and Neisseria sp. recognition of the species' own DNA occurs at the cell surface and in Haemophilus, an 11 base pair sequence in the donor DNA identifies the DNA to the cell (Zoon et al, 1976).

1.3.1.1 Transformation in Gram-negative bacteria

The initial stage in transformation, in H. influenzae is competence development. This occurs in conditions which allow protein synthesis to continue

but do not permit cell division (Herriott et al., 1970). Accompanying this are a number of changes in the cell surface, such as induction of specific inner and outer membrane proteins which parallel competence development (Zoon and Scocca, 1975; Zoon et al., 1976) and the transfer of cytoplasmic proteins to the cell membrane. Under conditions conducive to transformation, some cells in the population release DNA by autolysis or secretion, following which, binding to the recipient and uptake occurs. Competent cells usually bind and absorb only double stranded DNA from the same or closely related species, due to the specificity of the DNA uptake system (Scocca et al., 1974; Dougherty et al., 1979). Foreign DNA may bind to the cell surface but does not compete well with homologous DNA for uptake. Donor DNA enters the recipient cell as double-stranded molecules via membraneous extensions - transformasomes - which protect the DNA from exonuclease or restriction enzyme action (Kahn et al., 1984). One strand of the DNA is incorporated into the host chromosome (Natani and Goodgal, 1966) while the other strand and the displaced host strand are degraded (Steinhart and Herriott, 1968).

1.3.1.2 Transformation in Gram-positive bacteria

In Gram-positive bacteria, the onset of competence is due to secretion of a small protein called competence factor (Tomasz and Hotchkiss, 1964), although the mechanism of action remains obscure. Binding of double-stranded DNA is followed by uptake into a cell compartment such as the periplasmic space or the cytoplasm. Heterologous DNA is taken up, so no system of specific sequence recognition is present (Ciferri et al., 1970; Ranhand, 1980). Uptake is accompanied by degradation of one of the strands (Lacks, 1962) while the other forms a temporary eclipse complex before integration. The eclipse complex consists of the single-stranded donor associated with a polypeptide and in this state, the transforming activity is temporarily lost. Integration occurs

by displacement of a homologous strand of the recipient chromosome.

1.3.2 Generalised transduction.

Generalised transduction occurs when bacterial DNA is encapsulated within a phage head during the lytic cycle, in a donor cell, and is carried by the resultant hybrid particle to a recipient cell. The bacteriophage promotes entry of the transducing DNA into the recipient cell by the mechanism used for infection of normal phage genomic DNA. Within the recipient cell, the transduced DNA may undergo recombination with the host genome to yield a stable transductant or, more likely, it will fail to do so and will be lost from the population. Generalised transduction was discovered in 1952 by Zinder and Lederberg during a search for conjugation-mediated recombination between auxotrophs of Salmonella typhimurium. The transducing agent was found to be bacteriophage P22 and later, phage P1 of E. coli was found to be capable of mediating generalised transduction (Lennox, 1955). Compared with specialised transduction, generalised transduction is a more significant means of gene transfer since any chromosomal gene may be carried from donor to recipient, while in specialised transduction, mediated by phage such as lambda (λ), the only genes which can be transduced are those which are adjacent to the site of lysogeny.

Transducing phage such as P1 and P22 are packaged from concatamers of several genome equivalents and the formation of transducing phage occurs when the packaging mechanism seizes upon host DNA instead of the phage concatamers (Ikedu and Tomizawa, 1965). Upon injection into the recipient cell, one of three possible fates awaits the transduced gene :-

(i) Recombination with a homologous area of the chromosome to yield complete transductants.

(ii) Persistence in the cytoplasm in a non-replicating, but stable state - this is called abortive transduction.

(iii) Total degradation by nuclease action.

The processes which lead to recombination of transduced DNA with homologous DNA are unknown (Masters, 1985). Most of the transducing DNA is abortive and does not undergo recombination. There is little understanding of the processes which regulate establishment of complete transductants. In abortive transduction, the DNA fragment, although capable of complementing a mutation, cannot replicate and so, upon cell division, the gene segregates to only one of the daughter cells. However, the protein gene product is divided between daughter cells, a process which continues until a daughter is produced with insufficient product to allow growth. This gives rise to a small colony which is incapable of exponential growth. Thus, abortive transductants are recognised as very small colonies among the normal sized colonies of complete transductants (Ozeki and Ikeda, 1968).

1.3.3 Plasmids and conjugation

Plasmids are extra-chromosomal circular pieces of DNA which can be stably inherited without being linked to the bacterial chromosome. They are not essential for the normal growth of their host but can confer upon the host, selective benefits. Plasmids are classified into incompatibility groups, a group comprising plasmids which cannot co-exist together in the same host cell (Datta, 1975). Plasmids can be either conjugative or non-conjugative. Those which are conjugative are, as their name implies, capable of promoting conjugation, a process whereby DNA is transferred between bacterial cells by a mechanism requiring cell to cell contact (Davis, 1950). For several years, the sex factor F (Hayes, 1952) was considered to be the only conjugative plasmid and conjugation was thought to be an unusual phenomenon (Clark and Warren, 1979).

However, conjugation and conjugative plasmids are now known to be common to many bacterial genera and over 20 incompatibility groups have been established, with plasmids of the same group possessing similar conjugation systems (Datta, 1975).

Of the many plasmid conjugation systems, only that of F has been studied in detail (Willetts and Skurray, 1980). Conjugation can be divided into two major components : formation of mating pairs and DNA transfer. During mating pair (or aggregate) formation, the cells are brought together by the plasmid encoded F-pilus (Bradley, 1980) which recognises the recipient cell. Following this, surface exclusion proteins in the membrane prevent the cell being used as a recipient by another donor cell which carries the same plasmid or one of the same incompatibility group. Upon contact between pilus and recipient, the pilus may act as a DNA transfer bridge (Ou and Anderson, 1970) or the pilus retracts bringing donor and recipient into contact, allowing DNA transfer to take place (Novotny and Fives-Taylor, 1978; Achtman *et al.*, 1978). Next, a plasmid strand is nicked and transferred to the recipient cell, led by the 5' terminus (Rupp and Ihler, 1968; Ohki and Tomizawa, 1968) beginning at the transfer origin (*ori* T). The transferred strand is replicated in the recipient cell by host specific enzymes, and only the initiation of transfer requires expression of the plasmid *tra* genes (Kingsman and Willetts, 1978). Meanwhile, the strand remaining in the donor is also replicated. *Tra* genes occupy about one third of the F plasmid while the *ori* T region is located at one end of the transfer genes.

1.3.3.1 Chromosome transfer by the F factor

The F plasmid is capable of promoting transfer of donor chromosomal markers by two discrete systems :-

i. Formation of F-primes (F')

An F-prime is formed by pickup of chromosomal DNA by recombination into, and aberrant excision from, the bacterial chromosome by the F plasmid (Jacob and Wollman, 1961; Campbell, 1969). Single genes may be transferred at low frequencies and the system does not allow the determination of the order of donor chromosomal genes.

ii. High frequency of recombination (Hfr)

Hfr strains are capable of promoting transfer of donor chromosomal genes at high frequencies and in one direction from the origin of transfer (Jacob and Wollman, 1958). This arises by physical integration of the F plasmid into the chromosome by recombination between homologous regions and allows ordered transfer of chromosomal genes (Davidson et al., 1975).

1.3.3.2 R plasmids

R plasmids, a group of conjugative plasmids much more widely distributed than F, were discovered in 1959 in Japan as the cause of multiple drug resistance in Shigella. R plasmids have now been found in many bacterial species which are pathogenic for man and animals and are particularly common in the Enterobacteriaceae such as E. coli, Salmonella sp. Shigella sp. and in Pseudomonas sp. Those plasmids which belong to the P incompatibility group may be especially important in disseminating drug resistance to a wide range of organisms. Use of antibiotics has not induced, but has selected for, resistance plasmids since strains of E. coli which were freeze-dried before the widespread use of antibiotics have been found to carry R plasmids (Smith, 1967).

Typical R plasmids are large broad host-range, low copy number, self-transmissible plasmids with two functionally distinct parts : the resistance

transfer factor which contains genes for replication and conjugation (tra genes), and the resistance determinant, many of which are located on transposable elements (Datta et al., 1971). The medical significance of such plasmids cannot be underestimated since selection for R plasmids creates a growing threat to the successful use of antibiotics in treatment of bacterial disease (Falkow, 1975).

The occurrance of two epidemics in Central America within a short space of time caused by Shigella dysenteriae and Salmonella typhi strains which showed identical, transferable antibiotic resistance patterns (Farrar and Eidson, 1971; Gangarosa et al., 1972), but which produced a more severe and protracted typhoid epidemic than is typical of the disease, led to speculation that the R plasmid may have increased the virulence of these pathogens (Gangarosa et al., 1972). This may be the case since R plasmid-encoded functions have subsequently been shown to increase resistance to the bactericidal action of serum (Fietta et al., 1977) and to enhance resistance to phagocytosis (Agvero et al., 1984) by modification of the cell envelope to favour invasiveness.

1.3.4 Bacterial transposable elements

Transpositional recombination is a chromosome restructuring mechanism in which the vectors of recombination, called transposable elements, are specific DNA sequences that can move from one genetic location to another in the absence of the normal homology-dependent recombination systems. Although studied mainly in bacteria, transposable elements were initially discovered in maize (McClintock, 1941). Three classes of transposable elements exist.

1.3.4.1 IS (insertion sequence) elements.

IS elements are short DNA segments (760-1600bp) which encode only one or two genes involved in transpositional recombination. They are found singly as natural constituents of chromosomes, or as pairs, acting as the driving force

for transposition in composite transposons (DNA segments which may carry on antibiotic resistance gene, flanked by two copies of the IS). IS elements were first identified as DNA insertions which induced polar mutations in the control region of the gal operon of E. coli (Jordan et al., 1968; Hirsch et al., 1972). IS elements encode a protein, a transposase, which is required for recombination and transposition. This may be either a replicative process, in which a copy of the IS element is retained at the donor site, or a conservative process in which the element is excised from the donor without replication.

1.3.4.2 Drug resistance transposons.

Drug resistance transposons can be divided into two categories - class I and class II - by their physical organisation. Class I transposons are composed of a drug resistance determinant flanked by copies of an IS element which provide the transposition functions and act in unison to transpose the intervening drug resistance gene. A typical class I transposon is Tn5 which encodes kanamycin resistance. The properties of class I transposons are similar to those of IS elements. Tn5 is also known as IS50 and because it has no preferential site of insertion in the chromosome of many bacteria, it has been widely used in creation of transposon insertion mutants.

Class II transposons contain a sequence which encodes transposition functions and drug resistance and is flanked by inverted repeats 30 to 40bp in length. The prototype of the group is TnA, now known as Tn1, which is identical to the best studied member of the group, Tn3. The Tn3 family form a distinct class of transposons which share a common two-step transposition mechanism (Heffron et al., 1977; Gill et al., 1979; Grindley and Reed, 1985). Transposon encoded antibiotic resistance was first observed as a transfer of penicillinase activity between plasmids and between plasmids and chromosomes (Datta et al., 1971; Richmond and Sykes, 1972). Tn3-like transposons produce two gene products

which are essential in transposition : a transposase (MW = 120,000) and a resolvase (MW = 21,000). Upon transposition, a 5bp target site is duplicated. The Tn3 family can be divided into two subgroups based on the genetic organisation and ability of recombination functions to complement one another. These two groups can be represented by Tn3 and Tn501. Tn3 transposition involves fusion of donor and target replicons by the transposase. This forms a cointegrate, a structure which, under normal conditions, is rapidly broken down by the resolvase to yield an insertion of the transposon in the target replicon and regeneration of the donor replicon (Shapiro, 1979; Gill et al., 1979). Tn501, initially discovered on a Pseudomonas plasmid (Stanisich et al., 1977), has similar transposase activity to Tn3, but efficient cointegrate resolution only occurs in the presence of mercury salts. Although transposition can occur in the absence of mercury salts, little or no cointegrate resolution occurs (Sherratt et al., 1980).

Transposon Tn7 is a class II transposon which differs from the Tn3 family in several respects. It encodes resistance to trimethoprism and streptomycin in E. coli (Barth et al., 1976). It differs from Tn3-like transposons in that it is larger and encodes a third transposition function which is required only for transposition from chromosome to plasmid (Hauer and Shapiro, 1984). The inverted repeats which flank the transposition and resistance genes are shorter than those in Tn3, being about 19bp long. Tn7 has been shown to insert preferentially into chromosomal hot-spots in several species (Barth et al., 1976; Lichtenstein and Brenner, 1981; Ely, 1982; Thomson et al., 1981).

1.3.4.3 Bacteriophage Mu.

Mu represents the transposing bacteriophages and was the first prokaryote transposable element to be described (Taylor, 1963). It shares with IS elements and transposons, all the properties characteristic of transposable elements

and transposes at random, inactivating the gene in which it inserts. Transposition occurs in both lytic and lysogenic cycles and by inducing rearrangements of its host genome while it transposes, Mu generates mutations in about 3% of the lysogens. During the lytic cycle, Mu generates DNA rearrangements by transposing to new locations on the chromosome which results in formation of strong polar mutations (Jordan et al., 1968). The functions required for integration appear to be expressed early in the lytic cycle and Mu integration may be related to the formation of covalently closed circles which appear after prophage induction (Waggoner et al., 1974; Razzaki and Bukhari, 1975). Many properties of Mu are shared with the IS elements and transposons, so that Mu is now thought to be a large transposon of 39kb, which can exist as a virus.

1.3.4.4 Transposon mutagenesis

Transposons are convenient tools for use in genetic analysis of bacteria, since they carry antibiotic resistance which are selectable properties. They are powerful mutagens since they can insert into DNA replicons in the absence of the recA gene function. Where insertion into a gene occurs, non-leaky polar mutations arise and insertion can be mapped by the presence of the antibiotic resistance marker. The insertion mutations are generated by introducing the transposon into the bacterial cell on a conjugative plasmid which fails to replicate in the new recipient. Several bacterial properties have been studied using such a system : the nitrogenase activity of Rhizobium japonicum (Horn et al., 1984), motility in Caulobacter crescentus (Ely and Croft, 1982) and bioluminescence in marine Vibrio sp. (Belas et al., 1984). Tn5 mutagenesis in Erwinia carotovora has yielded auxotrophic mutants and mutants defective in carbohydrate metabolism (Zink et al., 1984) while bacteria of medical and agricultural importance such as Vibrio cholerae, Agrobacterium tumefaciens and B. pertussis have been studied using these techniques (Newland et al., 1984; Pischl and Farrand, 1984; Weiss and Falkow, 1983).

1.3.4.5 Role of IS elements and transposons in chromosome mobilisation.

As discussed above, the sex plasmid F can promote mobilisation at high frequencies, by formation of Hfr donors. Integration of the plasmid into the chromosome of E. coli K12 is now known to occur by homologous recombination via insertion sequences carried on the F plasmid and on the bacterial chromosome (Ohtsubo et al., 1974; Deonier and Davidson, 1976; Cullum and Broda, 1979).

R plasmids such as RP1, RP4 and R68 were found to be capable of promoting mobilisation of the bacterial chromosome in strains of Ps. aeruginosa (Stanisich and Holloway, 1971). Examination of crosses in which the donor strain carried plasmid R68 showed that the plasmids present in recombinant clones had acquired increased chromosome mobilising ability (Cma) (Haas and Holloway, 1976; 1978). One such plasmid was designated R68.44, which tended to lose Cma and kanamycin resistance concurrently. A mutant of this plasmid, R68.45, was a thousand times more efficient than R68 at promoting mobilisation and the stability of Cma could be increased by maintenance on kanamycin (Haas and Holloway, 1976). Cma in R68.45 is associated with a DNA insertion of about 2.05kb (Jacob et al., 1977) which is a repeat of a sequence already present on R68 (Riess et al., 1980) and is close to the kanamycin resistance determinant (Currier and Morgan, 1982). The duplicated regions may be insertion sequences - IS elements - and duplication may be the result of intra-molecular transposition. Such an IS element, IS21, exists on R68.45 and the Cma of this plasmid may be due to formation of an R68.45-chromosome cointegrate during transposition of IS21 (Willetts et al., 1981), although the exact mechanism remains unclear.

The ability of plasmid R68.45 to promote chromosome mobilisation can be improved by insertion into the plasmid and into the chromosome of homologous nucleotide sequences such as transposons, which can promote formation of Hfr-like donors by recombination of the plasmid into the chromosome. This has been demonstrated using R68.45::Tn5 in Agrobacterium tumifaciens (Pischl and Farrand, 1983) and

with RP1::Tn501 in Rhodopseudomonas sphaeroides (Pemberton and Bowen, 1981). Recent advances have used the oriT region of RP4 cloned into Tn5 which, in the presence of a helper plasmid to supply transfer functions in trans mobilises the chromosome at high frequencies (Simon, 1984; Zokobson and Guiney, 1984).

Many studies on gene transfer in Gram-negative bacteria have used a conjugation-mediated system with R plasmids of the P incompatibility group. The plasmids have a broad host range (Datta and Hedges, 1972; Olsen and Shipley, 1973) and are capable of promoting recombinational exchange of chromosomal genes in many Gram-negative bacteria (Holloway, 1979) such as Rhodopseudomonas sphaeroides (Tucker and Pemberton, 1979), methylotrophic Pseudomonas (Tatra and Goodwin, 1983) and Legionella pneumophila (Dreyfus and Iglewski, 1985). Such studies, combined with the use of transposons, as mutagens permits genetic analysis of poorly characterised organisms.

1.4 GENETICS OF B. PERTUSSIS.

1.4.1 Transformation

Branefors (1964) transformed streptomycin resistance to a strain of B. pertussis with DNA from a Str^R donor. In addition, starch independence was believed to have been co-transferred. Further transformation studies used auxotrophic mutants of B. pertussis as recipients which were reported to have been transformed to prototrophy by DNA from prototrophic donor strains; the first study of genes associated with biosynthesis in B. pertussis (Kloos et al., 1978). Al-Sallami (1981), in repeating the work of Branefors, associated transformation of Str^R with increased virulence in mice, and characterised the Branefors strains with respect to their biological properties. No subsequent reports of transformation by chromosomal genes have appeared, but transformation by plasmid DNA was demonstrated (Weiss and Falkow, 1982) by using cold shock and freeze-thaw procedures. However, plasmid DNA from E. coli could not be trans-

formed if the DNA contained HindIII restriction sites. This supported the findings of Greenaway (1981) who reported the presence of a HindIII-like restriction enzyme in B. pertussis.

1.4.2 Theories of phage conversion of B. pertussis.

The first suggestion of the existence of bacteriophage specific for B. pertussis was a report of lytic activity against the organism observed in respiratory secretions, homogenised human lung and soil (Grant, 1979). Mebel et al. (1981) demonstrated mitomycin C induction of bacteriophage which gave rise to plaques on B. parapertussis indicator bacteria. In addition, spontaneous release of phage from B. pertussis vaccine strains was observed. Further study showed that lysogenisation of B. parapertussis by phage from B. pertussis caused expression and production of toxic properties and agglutinogens which were characteristic of the donor (Lapaeva et al., 1982), but the lysogens did not possess protective activity. These findings led to theories of conversion of B. parapertussis to B. pertussis by uptake of a lysogenic phage (Mebel and Lapaeva, 1982; Granstrom and Askelof, 1982) which may explain some anomolous observations from Linnemann (1977). He isolated B. pertussis from family members and relatives of children infected with B. parapertussis and demonstrated that infection with this organism produced lymphocytosis. These observations could be explained, however, by the Weiss and Falkow model of phase variation and virulence factor expression, if it is accepted that avirulent phase variants of B. pertussis were misidentified as being B. parapertussis.

1.4.3 Plasmids in B. pertussis.

The first report of plasmid DNA in B. pertussis was of a small cryptic plasmid in several strains of the organism, and in B. parapertussis (Kloos et al., 1978). Strains of B. bronchiseptica have been shown to harbour large R plasmids

(Terakado et al., 1973; Hedges and Jacob, 1974), although to date, no such plasmids have been found in B. pertussis. It has been possible however, to transfer plasmids into the organism by conjugation with E. coli donors. The broad host-range plasmids R388 and RP4 have been introduced into the organism in this way (Weiss and Falkow, 1982). Although less promiscous plasmids of the FI and FII groups did not transfer to B. pertussis, a chimeric plasmid pUW942 was used to introduce Tn501 into B. pertussis and by promoting integration into the chromosome, create genetic donors (Sato et al., 1981; Weiss and Falkow, 1983), which could mobilise chromosomal markers. A second chimeric plasmid, pUW964 was subsequently used to begin a new approach to the determination of the role of each virulence factor in the establishment of whooping cough. This plasmid was used to deliver Tn5 transposition induced mutations in virulence-associated genes and allowed study of virulence factor expression and phase variation, leading to a model for virulence factor expression (Weiss and Falkow, 1983; 1984; Weiss et al., 1983; 1984).

1.4.4 Development of a minimal medium for B. pertussis.

The development of a gene transfer system in B. pertussis has been hampered by the lack of suitable markers which could act as selectable properties. The development of a minimal medium was considered by Parker (1976) who, in attempts to isolate mutants resistant to inhibitors or deficient in amino acid metabolsim used a medium based on Stainer and Scholte liquid medium (Stainer and Scholte, 1971). The medium included sodium acetate, which is stimulatory for B. pertussis (Bundeally and Rao, 1965), Dowex-1 chloride to remove inhibitors (Sutherland and Wilkinson, 1961) and agarose. Agar was unsuitable as a solidifying agent since it is inhibitory to B. pertussis (Verwey et al., 1949). The Parker medium supported growth of B. pertussis vaccine strains and fresh isolates from small inocula. No attempt was made to isolate auxotrophic mutants.

Kloos et al. (1978) extended the transformation studies of Branefors (1964) to include genes associated with biosynthesis of amino acids. For these studies, a medium based on that of Stainer and Scholte (1977) was used which contained casein hydrolysate or, for screening and selection of auxotrophs and prototrophic recombinants, a combination of up to 11 amino acids. For growth of Phase I strains, activated charcoal was added but this was omitted for Phase IV strains, SSA containing casamino acids being selective for Phase IV strains (Dobrogosz et al., 1979). This selective medium, with 1% Noble Agar as a solidifying agent, was used to isolate colonial variants of B. pertussis which had lost some Phase I characteristics (Peppler, 1982). The medium used in his study also contained BSA addition of which allowed growth of Phase I organisms and may have overcome the inhibitory effect of agar. In their studies on Tn501-induced donor formation, Weiss and Falkow (1983) used Stainer and Scholte agarose (SSA) plates containing BSA to select recombinant prototrophic colonies which carried a mobilised donor nutritional marker.

The most significant development was the discovery that cyclodextrins, in particular Heptakis (2,6-O-Dimethyl)β-Cyclodextrin, (MeβCD) when incorporated in SSA, allowed growth of B. pertussis from small inocula (Imaizumi et al., 1983). This medium, Cyclodextrin Solid Medium (CSM) supported growth at a level comparable to that of BGA, and may be suitable for use as a B. pertussis minimal medium.

OBJECT OF RESEARCH

The primary objective of this research was to develop a system of genetic transfer in Bordetella pertussis which would enable virulence determinants to be expressed individually in suitable recipient strains. Procedures such as transformation and plasmid-mediated chromosome mobilisation were explored for this purpose, together with attempts to identify bacteriophage in B. pertussis.

In parallel with these studies, markers suitable for following genetic exchange were created by the isolation of drug resistant and auxotrophic strains of B. pertussis.

2. MATERIALS AND METHODS

2.1 BACTERIOLOGICAL

2.1.1 Bacteria and plasmids

B. pertussis strains 44122/7R and 44122/7S as used in studies on transformation by Branefors (1964) and Al-Sallami (1981) were employed. The latter author also characterised the strains with respect to intranasal and intracerebral virulence, and HSF activity. 44122/7R is resistant to streptomycin (>500μgml^{-1}) while 44122/7S is sensitive. 44122/7S has been used for vaccine production in Sweden. Both strains were obtained from P. Branefors, Karolinska Institute, Stockholm, Sweden.

Taberman. This strain was isolated pernasally before the death of an infant, and was obtained from Professor G.T. Stewart, Ruchill Hospital, Glasgow. Since the wild-type is sensitive to most antibiotics which are commonly used for selection in genetic transfer systems, several antibiotic resistant mutants were isolated. R1, R2, R3 are rifampicin resistant mutants, while P1, P2 and P3 are phosphonomycin resistant mutants which were obtained by N-Methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis.

BPT1 to BPT6 are nalidixic acid resistant mutants which were also obtained by NTG mutagenesis. BPT2 was used in subsequent genetic manipulations.

Tohama. This strain was obtained from Dr. A. Robinson, PHLS, Porton Down, Wiltshire and was used in gene transfer studies by Weiss and Falkow (1983). A nalidixic acid resistant mutant (BPN6) was obtained which was subsequently used for isolation of threonine requiring auxotrophic mutants of B. pertussis (BPN6 Thr^-) (see Results).

D3O042 Phase I. was obtained from Dr. J. Dolby, Clinical Research Centre, Harrow, Middlesex. A streptomycin resistant mutant was isolated (CSP1) and was used for subsequent isolation of two auxotrophic mutant strains (CSP1

Trp^- and CSP 1 Gly^-), which are tryptophan and glycine requiring respectively.

L84 Phase I was obtained from the Microbiology Department culture collection.

18334 was originally obtained from Connaught Laboratories, Toronto, Canada and is used in vaccine production.

18323 was obtained from Dr. F. Sheffield, National Institute for Biological Standards and Control, Hampstead, London, and is virulent for mice by the intracerebral route being the standard intracerebral challenge strain.

134. This strain was obtained from the departmental culture collection. It was the strain used by Pillemer (1950) in preparation of his erythrocyte adsorbed protective antigens.

BP 347. This strain was obtained from A.A. Weiss, Stanford University School of Medicine, Stanford, California, U.S.A. and is a transposon Tn5-induced mutant defective in production of haemolysin, fimbrial haemagglutinin, pertussis toxin and dermonecrotic toxin. (Weiss et al., 1983).

B. parapertussis NCTC 10520, a strain which carried intrinsic resistance to chloramphenicol and streptomycin, was used as a recipient in genetic transfer studies.

Several strains of E. coli were also used :-

E. coli K12. A prototrophic strain commonly used in genetic studies.

J53-1. This strain has a chromosomal mutation which confers resistance to nalidixic acid (Nal^R), in addition to mutations which make it auxotrophic for proline (pro) and methionine (met). J53-1 carries plasmid R68-45.

JC3272. This strain carries chromosomal mutations which render it auxotrophic for histidine, (his), tryptophan (trp) and lysine (lys), and resistant to streptomycin (Achtman et al., 1971). J53-1 and JC3272 were

supplied by Dr. B.E.B. Moseley, Department of Microbiology, University of Edinburgh.

JMP543. This strain is auxotrophic for threonine (thr), leucine (leu), serine (ser) and vitamin B1 (thi) and carries plasmid RP1 containing the mercury resistance transposon Tn501. JMP543 is also resistant to streptomycin (Str^R) and rifampicin (Rif^R).

JMP502. This strain differs from JMP543 only in that Tn501 is not present on the plasmid RP1. Both strains were obtained from J.M. Pemberton, University of Queensland, Australia.

UW937/pUW942. This strain is auxotrophic for threonine (thr), leucine (leu), thiamine (thi) and also carries lac Y1, sup E44, ton A21 and gyr A mutations. Plasmid pUW942 is present in this strain.

HB101/pUW964. This strain is deficient in arabinose fermentation (ara) and is auxotrophic for proline (pro). It also carries the lac Y1 mutation and is Str^R. This strain carries plasmid pUW964.

J53-1/RP4::Tn7. This is the same J53-1 host strain as described above, but contains plasmid RP4 carrying the trimethoprim and streptomycin resistance transposon Tn7.

Strains UW937/pUW942 and HB101/pUW964 were obtained from A.A. Weiss, University of Virginia Medical School, U.S.A. and J53-1/RP4::Tn7 was obtained from D. Platt, Glasgow Royal Infirmary.

Plasmids.

R68.45, RP1 and RP4. These plasmids were present in E. coli hosts as described above. The plasmids are members of the P-1 incompatibility group and encode resistance to kanamycin, carbenicillin, tetracycline and neomycin. Derivatives were also used which contained transposons Tn501 (plasmid:RP1::Tn501) and Tn7 (plasmid:RP4::Tn7).

R68.45supdnaG315 This plasmid is a dnaG suppressing mutant of R68.45 which enhances chromosome mobilisation in Rhizobium sp. (Ludwig and Johansen, 1980). It was obtained from Dr. E. Johansen, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.

pUW942. This is a derivative of the chimeric plasmid pAS8 Rep-1 (Sato et al., 1981). It carries RP4 conjugation genes and a Col E1 replication origin and will only replicate in a limited number of species. In addition, it contains transposon Tn501 (Weiss and Falkow, 1983).

pUW964. This is also a chimeric plasmid of the type described above and was constructed from a derivative of pRK2013, called pRKTV5 (Figurski and Helinski, 1979), in which a kanamycin resistance determinant had been inactivated by a Tn7 insertion thus allowing transposition of Tn5, which encodes resistance to kanamycin, onto the plasmid. This plasmid is also limited in its host range (Weiss et al., 1983).

RP4::Mucts62. This plasmid is the P group plasmid RP4 carrying the thermosensitive transposable bacteriophage Mucts62 (Howe, 1973; Boucher et al., 1977). At 42°C, the lytic cycle of infection is induced giving rise to viable phage particles.

2.1.2 Media

For growth of E. coli, B. pertussis and B. parapertussis,and selection of transconjugants, four types of media were used.

Bordet-Gengou Agar (BGA) (Gibco Europe Ltd., Paisley, Scotland) supplemented with 20% sterile defibrinated horse blood (Gibco) (Appendix 1) was used for routine growth of B. pertussis.

Cyclodextrin Solid Medium (CSM) was prepared according to the recipe of Imaizumi et al. (1983) (Appendix 1) when used for routine growth of B. pertussis the medium contained Casamino Acids (Casein Hydrolysate Acid, Peptone No. 5,Gibco).

For isolation of auxotrophic mutants and selection of prototrophic recombinants in gene transfer experiments, casein hydrolysate was omitted but amino acid 'pools' were incorporated in the medium (Appendix 2). Methylated β-cyclodextrin (MeβCD) was obtained from Dr. Imaizumi, Teijin Institute for Biomedical Research, Tokyo, Japan. MacConkey Agar (Difco) was used for growth of B. parapertussis as was BGA.

E. coli strains were grown on nutrient agar (Gibco).

E. coli K12 recombinants were selected on E. coli Minimal Agar (ECMA) (Clowes and Hayes, 1968) (Appendix 1).

In liquid culture, B. pertussis and B. parapertussis were grown in Cohen-Wheeler Medium (Cohen and Wheeler, 1946) (Appendix 1) or Stainer-Scholte defined liquid medium (Stainer and Scholte, 1971) (Appendix 1).

E. coli liquid cultures were grown in nutrient broth (Gibco).

2.1.3 Growth of bacteria

Freeze-dried cultures of B.pertussis were reconstituted in 1% (w/v) Casamino Acids (CAA) (Appendix 1) and plated on BGA. Plates were incubated at 37°C for 2-4 days in a closed plastic box containing a beaker of water to saturate the atmosphere.

Cultures were grown from frozen stocks by thawing and spreading 100µl of bacterial suspension on BGA, and incubating as above.

B. pertussis was grown in 25ml amounts of Cohen-Wheeler Medium in dimpled 250ml flasks at 37°C for 48-72h with shaking on an orbital incubator at 70 r.p.m. Where B. pertussis transconjugants were used subsequently as genetic donors, or for plasmid DNA preparations, tetracycline (10µg/ml) was added to the medium.

B. parapertussis was grown from frozen stocks, on nutrient agar or on BGA.

Nutrient agar was, on occasion, supplemented with tyrosine (0.2% w/v).

B. parapertussis was also grown on MacConkey Agar. All plates were incubated at 37°C for 2-3 days, in a moist atmosphere.

In liquid culture, B. parapertussis was grown in 25ml amounts of Cohen and Wheeler medium or nutrient broth at 37°C for 48h with shaking at 70 r.p.m.

Freeze-dried E. coli strains were reconstituted in nutrient broth and 100µl of the suspension was spread on nutrient agar. Plates were incubated at 37°C overnight.

Liquid cultures of E. coli were grown in 25ml amounts of nutrient broth overnight at 37°C with shaking. Where E. coli strains were used as genetic donors, or for preparation of plasmid DNA, the medium was supplemented with tetracycline (10µg ml^{-1}).

2.1.4 Culture purity checks

All strains were checked regularly for purity by Gram stain. B. pertussis Phase I cultures were also checked by their inability to grow on nutrient agar, and their ability to agglutinate in specific antiserum (concentrated rabbit anti-B. pertussis sonicate, prepared by J. Hertz, 1978). Two drops of a turbid cell suspension were placed on a clean microscope slide about 4cm apart. To one, a drop of antiserum was added while saline was added to the other. The slide was agitated gently for several minutes to allow agglutination to occur.

2.1.5 Determination of antibiotic sensitivity.

a) Faintly turbid cell suspensions of B. pertussis cells were made in 1% Casamino Acids solution (CAA). From the suspension, 100µl was spread on BGA and allowed to dry. An Oxoid S3 Multodisk (Appendix 2) was placed, using

sterile forceps, on the surface of the plate, which was incubated as described previously. After 48h, test strains were determined to be resistant (R) or sensitive (S) to each antibiotic.

b) Varying concentrations of the test antibiotic were incorporated in BGA by adding the sterile antibiotic solution before pouring. Plates were streaked with the test organism and incubated as described previously. The Minimum Inhibitory Concentration (MIC) of the antibiotic was recorded as being the lowest concentration of antibiotic which gave complete inhibition of bacterial growth.

2.1.6 Preparation of antibiotic solutions.

Stock solutions of antibiotics were made at the following concentrations (% w/v) :-

streptomycin (Sm) - 4%, kanamycin (Km) - 2%, carbenicillin (Cb) - 2%, tetracycline (Tc) - 0.2%, nalidixic acid (Nal) - 0.4%, chloramphenicol (Cm) - 1%, trimethoprim (Tp) - 4%, erythromycin (Em) - 0.25%, phosphonomycin (Pm) - 0.5%, rifampicin (Rif) - 0.4%, penicillin G (PenG) - 1000 units ml^{-1}.

Streptomycin, kanamycin, chloramphenicol, carbenicillin, phosphonomycin and penicillin G were dissolved in distilled water and filter sterilised by passing the solutions through a Sartorius Minisart NML filter (pore diameter 0.45µm). Nalidixic acid and trimethoprim were dissolved in methanol, in a hot water stream to ensure solubility, while tetracycline, erythromycin and rifampicin dissolved readily in methanol at room temperature. All antibiotic solutions were stored at -20°C except nalidixic acid and trimethoprim which, due to their tendency to come out of solution, were prepared fresh immediately before use. All antibiotics were obtained from the Sigma Chemical Co., Poole, Dorset.

2.1.7 Mercuric chloride solution.

2% (w/v) stock solutions were prepared by dissolving mercuric chloride (Koch-Light Laboratories, Buckinghamshire) in distilled water and filter sterilising as described above. The stock solutions were stored at -20°C.

2.2 GENETIC

2.2.1 Preparation of chromosomal DNA.

Chromosomal DNA from several strains of B. pertussis was prepared using a combination of lysis, by the procedure of Schwinghamer (1980), and extraction by the method of Marmur and Lane (1961). To avoid DNA shearing during mechanical cell disruption lysozyme was used to produce sphaeroplasts for gentle lysis of cells. A mild osmotic shock in the presence of EDTA allows lysozyme to penetrate the outer membrane and to degrade the murein layer. The procedure was as follows :

500ml of a 48h shaken culture of a shaken culture of B. pertussis was centrifuged at 12000xg for 20 min at 4°C, and the cell pellet was resuspended in 50ml cold 0.05M Tris-HCl buffer containing 0.05M NaCl pH 8.0. 2ml of sodium lauryl sarcosinate 2.5% w/v was added and the mixture vortexed for 30 sec, followed by centrifugation at 12000xg at 4°C for 20 min. The supernate was discarded and the pellet drained well. Cells were resuspended in 0.8ml 0.05M Tris-HCl buffer, pH 8.0 containing 0.05M NaCl. 0.7ml of 1.6M sucrose, 0.55M Tris, 0.1M EDTA was added. The mixture was then kept on ice for 20 min, after which 0.3ml of lysozyme (Sigma) (5mg ml^{-1} in 0.05M Tris, pH 8.0) was added, followed by 7.2ml of cold 0.01M EDTA. The mixture was kept on ice for a further 20 min. Next, 5.0ml of sodium lauryl sarcosinate 2.5% w/v was added and mixed slowly to give a clear lysate. The lysate was extracted twice with phenol, followed by extraction twice with ether. One

tenth volume of 3M sodium acetate was added, followed by addition of two volumes of cold absolute alcohol. After mixing, the preparation was stored at -20°C overnight. Following centrifugation at 12000xg the supernate was discarded and the pellet was dissolved in 2.0ml SSC (0.15M NaCl, 0.015M trisodium citrate, pH 7.0). 0.1ml of 0.1% RNase A (Sigma) in 0.15M NaCl pH 5.0 was added. To destroy DNase activity the RNase was heated at 80°C for 10 min before addition. The preparation was incubated at 37°C for a further 30 min. The mixture was extracted several times with phenol, until the interphase between the two layers was clear. This was followed by extraction twice with ether, followed by addition of one tenth volume of 3M sodium acetate and two volumes of cold absolute alcohol to precipitate DNA. The DNA was spooled off on a glass rod and dissolved in 1.0ml of TE buffer (0.01M Tris-HCl, 0.001M EDTA pH 8.0). Half the volume of 7.5M ammonium acetate was added, followed by 2 volumes of cold absolute alcohol. Following mixing, the preparation was stored at -20°C overnight then centrifuged at 2900xg. The pellet was taken up in 0.5ml TE buffer. The DNA was stored in test tubes in 5 x 0.1ml aliquots at -20°C.

2.2.2 Preparation of plasmid DNA.

Plasmid DNA was isolated from E. coli and B. pertussis using two rapid extraction methods.

i) Single colony final sample buffer (SCFSB).

This procedure is commonly used in the laboratory of D.J. Sherratt, Institute of Genetics, University of Glasgow. From growth on solid media, bacteria were removed using a sterile toothpick and were resuspended in 200µl SCFSB. This was prepared as a 5x normal strength solution containing Ficoll 10% w/v (Sigma), sodium dodecyl sulphate 5% w/v and bromophenol blue 0.05% w/v and was diluted to normal strength, in distilled water, before use.

Following suspension of bacteria, the preparation stood at room temperature for 5-10 min, and was then centrifuged at 12000xg for 15 min at room temperature (MSE Micro Centaur), giving a gelatinous pellet. 20µl of the supernate was examined by agarose gel electrophoresis.

ii) Boiling procedure.

Plasmid DNA was also prepared using the method of Holmes and Quigley (1981). Cells from 1.5ml of an overnight E. coli or 48h B. pertussis liquid culture were harvested by centrifugation at 20,000xg in a Sarstedt Microfuge. The pellet was drained well on tissue paper and resuspended in 350µl of STET Buffer. (sucrose 8% w/v, EDTA 50mM, Tris-HCl 50mM, Triton X-100 5% v/v,pH 8.0). 25µl of lysozyme (10mg ml^{-1} freshly prepared in STET) was added and mixed well. Tubes were then placed in a boiling water bath for 40 sec, followed immediately by centrifugation at $4^{o}C$ for 15 min at 20,000xg,to remove cell debris. The pellet was removed with a toothpick. The condition of the pellet gave an indication of how well the lysis procedure worked, a white 'fluffy' pellet indicating good lysis. 40µl of 3M sodium acetate was added to the supernate, followed by 400µl of ice cold isopropanol. The two phases were mixed by inversion of the tube and were centrifuged at 20,000xg for 7 min at $4^{o}C$. The supernate was discarded and the pellet was drained well on tissue paper before addition of 1ml ethanol, 70% v/v. This was discarded after a few seconds, without resuspending the pellet which was white at this stage. The pellet was dried in a vacuum dessicator followed by incubation at $37^{o}C$ for 10-15 min, to drive off the remaining ethanol. The pellet was now gelatinous and transparent and was dissolved in 50µl of TE buffer. Plasmids were visualised by electrophoresis in 0.6% agarose gels (Section 2.2.9).

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2.2.3 Isolation of antibiotic resistant B. pertussis mutants.

To increase the range of mutants which would be suitable for use in gene transfer experiments, the following procedure was used. A filter paper disk (Whatman No. 1), 0.5cm in diameter, was soaked in a solution of N-methyl, N'-nitro, N-nitrosoguanidine (NTG) (Aldrich Chemicals, Wembley, Middx.) (200µg ml^{-1}). A heavy suspension of the strain in use was adjusted to contain about 10^9 bacteria per ml by comparison with the Fifth International Reference of Opacity (World Health Organisation International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Holly Hill, London NW3 6RB). 100µl of the suspension was spread on BGA containing the selective antibiotic and allowed to dry. The NTG soaked disk was placed in the centre of the plate, which was then incubated at 37°C as previously described. After 4-5 days incubation, antibiotic-resistant mutants had grown to single colonies at a distance of 1-2cm from the centre of the disk. These colonies were subcultured on BGA containing the antibiotic.

2.2.4 Isolation of B. pertussis auxotrophic mutants.

B. pertussis was grown in 25ml Stainer and Scholte medium for 48h at 37°C, with shaking. NTG was added to a final concentration of 200µg ml^{-1} and incubation continued for a further 5h at 37°C. 4ml of this culture was transferred to fresh Stainer and Scholte medium and incubated for a further 48h at 37°C. From this culture, 0.4ml was transferred into 1.6ml of sterile saline (0.85% w/v) and the total 2ml volume was used to inoculate 25ml of Stainer and Scholte medium. Penicillin G was then added to a final concentration of 6 Units ml^{-1} and the culture was incubated at 37°C for 24h. During this time any prototrophs would be subject to the action of penicillin and would be killed, whereas non-growing auxotrophs would remain viable. Following incubation, six platings each of 100µl were made from each dilution in a series of 10-fold dilutions

of the cultures. Platings were on BGA and following incubation for 4-5 days at 37°C, single colonies were restreaked on CSM without CAA, as well as on BGA. Plates were incubated for 48h, following which presumptive auxotrophs were identified as those colonies which failed to grow on CSM but grew on BGA. The nutritional requirement of each presumptive auxotroph was characterized by restreaking on a series of CSM plates, each of which contained a combination of amino acids, purines and pyrimidines (see Appendix 2). Amino acids, purines and pyrimidines were each present in the pools at concentrations of 0.2% and 100µl amounts were spread on a CSM plate, giving a 'pool plate' which contained approximately 10µg ml^{-1} of each amino acid in the pool.

2.2.5 Transformation procedures

100ml of Cohen and Wheeler medium was inoculated from a BGA plate on which a recipient strain had been grown from a freeze-dried culture. The Cohen and Wheeler medium contained casein hydrolysate, 1% w/v and was incubated at 37°C for 18-30h. This gave the pre-culture from which 10ml aliquots were transferred to several flasks each containing 100ml of Cohen and Wheeler medium, containing varying amounts (0.03% to 1% w/v) of casein hydrolysate. This was followed by a further incubation period of 30h to allow the development of competence. At the end of this incubation, 2ml samples from each culture were centrifuged at 2900xg for 20 min at 4°C and the pellet was resuspended in 10ml Cohen and Wheeler medium containing the same casein hydrolysate concentration as had been used previously. 2ml of this suspension was treated with donor DNA (final concentration 5µg ml^{-1}) and incubated at 37°C for 10h, to allow incorporation of DNA. After this time, 100µl was spread on BGA containing streptomycin (200µg ml^{-1}) and incubated for 4-5 days at 37°C to allow growth of Str^R transformants.

2.2.6 Effect of calcium chloride on transformation.

From the pre-culture, 0.2ml aliquots were transferred to 4 x 1/2" test tubes and calcium chloride (10% or 1% solution) sterilised by membrane filtration, was added to give a range of concentrations from 10^{-1}M to 10^{-4}M. These cells were chilled on ice for 2h after which donor DNA was added to a final concentration of 5µg ml^{-1}. This was followed by a further 40 min on ice, then 50µl of cell suspension was transferred to 2ml of fresh Cohen and Wheeler medium and incubated at 37°C for 24h without shaking. 100µl from each suspension was then spread on BGA containing streptomycin (200µg ml^{-1}) and incubated at 37°C for 4-5 days.

2.2.7 Plasmid transfer procedures.

Donor and recipient E. coli were grown in 25ml amounts of nutrient broth overnight at 37°C, with shaking. The donor culture contained tetracycline to maintain the plasmid (10µg ml^{-1}) or kanamycin (20µg ml^{-1}).

i) Filter matings.

Donor and recipient E. coli strains were mixed in a 1:5 ratio and passed through an Oxoid membrane filter (47mm x 0.45µm pore diameter) under negative pressure. A similar volume of donor and recipient, filtered separately, acted as controls. Filters were transferred to nutrient agar plates and incubated at 37°C overnight. The growth was resuspended, using a sterile glass rod in 2ml of sterile saline (0.85% w/v) from which 10-fold dilutions were made in sterile saline. For selection of transconjugants and determination of recipient viable count, 50µl amounts of each dilution was spread on nutrient agar plates containing antibiotics at the following concentrations : kanamycin - 20µg ml^{-1}, carbenicillin - 50µg ml^{-1}, streptomycin - 200µg ml^{-1}. For determination of chromosomal mobilisation frequency, nutrient agar containing nalidixic acid (40µg ml^{-1}) and streptomycin was used.

For filter matings between E. coli and B. pertussis filters were incubated on BGA and transconjugants were selected on BGA containing antibiotics. The donor:recipient ratio was 1:10 and filters were incubated for 48h. Selection plates were incubated for 4-5 days at 37°C.

ii) Broth matings.

Donor and recipient E. coli were grown as before. From each, a 1 in 20 dilution was made in fresh nutrient broth and incubated for a further 3h at 37°C. Donor and recipient were then mixed in equal volumes and the mixture diluted by transferring 1ml to 1ml of fresh nutrient broth. This was incubated at 37°C for 6h. Transconjugants were selected by spreading 50µl of the mating mixture on nutrient agar containing antibiotics as described previously. On occasion, the mating mixture was supplemented with agar, 0.1% (Oxoid Agar Technical No. 3) to facilitate enhanced plasmid transfer (Groot Obbink and Ackerman, 1980).

iii) Plate matings.

These were carried out by the method of Bradley et al. (1981). Donor and recipient E. coli were mixed initially in various ratios, to determine the optimum ratio for plasmid transfer. 0.3ml of the mating mixture was spread on nutrient agar and allowed to dry at room temperature. The plates were then incubated, surface uppermost, for 4-6h at 37°C. Following incubation, growth was resuspended in 2ml of sterile saline. The suspension was transferred to a sterile 4 x 1/2" test tube and 10-fold dilutions made, from which 50µl of each dilution was spread on NA containing antibiotics as before.

Plate matings which used E. coli K12 as recipient and an auxotrophic donor were modified. Following resuspension of growth from the mating plate, the mixture was centrifuged at 2900xg (MSE Bench Centrifuge). The pellet was resuspended in 1ml of sterile saline and transconjugants of K12 selected by

spreading 50µl of each 10-fold dilution on ECMA containing kanamycin (20µg ml^{-1}) and carbenicillin (50µg ml^{-1}). Mobilisation of streptomycin resistance from the donor was detected by spreading the mating mixture on ECMA containing streptomycin (200µg ml^{-1}) and kanamycin (20µg ml^{-1}).

Plate matings between E. coli and B. pertussis were carried out in a similar fashion. B. pertussis was grown in Cohen and Wheeler medium for 48h and was mixed in a 1:1 ratio with E. coli donor. Mating was performed by spreading 0.4ml of the mixture on BGA containing cephalexin (40µg ml^{-1}) and incubating at 37°C for 24h. A modification of the procedure was that 0.4ml of the 48h recipient B. pertussis was plated on BGA and incubated, surface uppermost, for 48h at 37°C. 0.4ml of an overnight E. coli donor was spread on the B. pertussis lawn, and incubation continued for a further 4-6h at 37°C. The resulting growth was resuspended in 2ml saline and transconjugants were selected on BGA containing kanamycin (20µg ml^{-1}), carbenicillin (50µg ml^{-1}) and streptomycin (200µg ml^{-1}). For plate matings between strains of B. pertussis and between B. pertussis and B. parapertussis, a 1:1 donor/recipient ratio was used, 0.4ml was spread on BGA and transconjugants were selected after 48h incubation. For selection, BGA contained the following antibiotics - kanamycin - 20µg ml^{-1}, carbenicillin - 50µg ml^{-1}, streptomycin - 200µg ml^{-1}, nalidixic acid -40µg ml^{-1}, chloramphenicol - 10µg ml^{-1}, erythromycin - 0.3µg ml^{-1}, rifampicin - 10µg ml^{-1}.

For selection of recombinant prototrophs of B. pertussis CSM containing BSA (0.05% w/v), streptomycin (200µg ml^{-1}) or nalidixic acid (40µg ml^{-1}) was used. Casamino acids was not included.

2.2.8 Determination of plasmid transfer and chromosome mobilisation frequencies and spontaneous mutation frequencies.

Plasmid transfer frequencies were expressed, in all experiments, as the number of transconjugants divided by the total recipient viable count. Chromosome mobilisation frequency was expressed, in all cases, as the number of recombinants carrying the mobilised marker divided by the total viable count.

Spontaneous mutation rates were obtained from the control plates. In all experiments, donor and recipient strains were plated separately on the mating medium and resuspended and spread on selective media as for the test plates. Mutation rates were expressed as a fraction of the total viable count of the donor or recipient. In cases where mutation rates are expressed as being less than a given value, this signifies that in 100µl of an undiluted bacterial suspension from the control plate, less than one colony has grown on the selective medium. Therefore, the spontaneous mutation rate is less than 10 c.f.u. ml^{-1} divided by the total viable count.

2.2.9 Restriction enzyme analysis of DNA.

Plasmid DNA was digested with restriction endonuclease enzymes EcoRI and HindIII. (Bethesda Research Laboratories, Maryland, U.S.A.). The enzymes were obtained from the manufacturer as concentrated solutions (10 units μl^{-1}) and an enzyme mix was made before use consisting of concentrated enzyme (2µl) distilled water (36.5µl) and BRL Core Buffer (14µl). 15µl of this was added to 25µl of plasmid DNA and incubated at 37°C for 2-3h, the final concentration of each enzyme being about 3 units. Restriction fragments were visualised by agarose gel electrophoresis.

Chromosomal DNA prepared from strains of B. pertussis and B. parapertussis was digested with HindIII. An enzyme mix was prepared by adding 2µl

of undiluted enzyme to 18µl of BRL Core Buffer. The digestion mixture contained 10µl of sterile distilled water, 2µl of BRL Core Buffer, 5µl of DNA and 2µl of diluted enzyme. Following incubation at 37°C for 2-3h restriction fragments were visualized by agarose gel electrophoresis.

2.2.10 Agarose gel electrophoresis.

Electrophoresis of DNA was performed in agarose gels. For visualisation of whole plasmids, 0.6% gels were used while electrophoresis of digested plasmid and chromosomal DNA was performed in 0.7% gels. An LKB 2117 multiphor gel system (Bromma, Sweden) was used, consisting of a horizontal gel and continuous buffer system. The running buffer used was GGB which was made up as 20x normal strength. (Tris 96.88g/1, Na acetate 32.8g/1, EDTA 5.84g/1 adjusted to pH 8.3 with acetic acid).

Gels were prepared by adding the appropriate weight of agarose (Sigma, Type IV) to 5ml of GGBx20 and 95ml of distilled water, and steaming for 10 min to dissolve the agarose. Before pouring, ethidium bromide (5mg ml^{-1}) was added to a final concentration of 0.5µg ml^{-1}.

20µl volumes of sample were added to the wells and electrophoresis was carried out at 10mA overnight for whole plasmids, or at 50-60mA for several hours for digested DNA. Following electrophoresis, DNA was visualised by U.V. light, using a long wave transilluminator (Ultra-Violet Products Inc. California, U.S.A.) and gels were photographed using a Polaroid 800 Camera containing Type 47 film with a 1 second exposure time.

2.2.11 Induction of lysogenic bacteriophage from *B. pertussis*.

Attempts to detect the presence of a lysogenic bacteriophage in *B. pertussis* were carried out using an agarose overlay technique. *B. pertussis* strain 134 and *B. parapertussis* 10520 were grown in Cohen and Wheeler medium as described

previously. Mitomycin C (Sigma) was added to B. pertussis at final concentrations of 1, 5 and 10ng ml^{-1} and incubation at 37°C was continued for a further 20 min, 1·5h and 2·5h. Cells were removed by centrifugation (20,000xg for 1 min in Sarstedt Microfuge) and the supernate from each culture was filtered through a 0.45µm filter (Sartorius Minisart NML). As a control the same procedure was carried out without the addition of Mitomycin C. 3ml amounts of agarose 1% (Sigma, Type IV) were dispensed in 4 x 1/2" test tubes, melted before use and maintained at 40°C. To each tube, 0.1ml of maltose, 20% (Koch-Light Laboratories Ltd. Buckinghamshire), 0.1ml of B. parapertussis indicator bacteria and 0.1ml of B. pertussis phage lysate were added, having been pre-warmed to 40°C before addition. The contents of the tube were mixed and poured over the surface of a nutrient agar plate, giving an even overlay which was left to solidify at room temperature. Plates were incubated at 37°C for 24-48h after which time any lytic phage present would produce plaques on the B. parapertussis lawn.

2.3 OTHER MATERIALS AND METHODS

2.3.1 DNase test for B. pertussis

The method of testing for DNase production by B. pertussis was a modification of that of Jeffries et al. (1957). Cyclodextrin Solid Medium was supplemented by the addition of 42g l^{-1} of DNase Test Agar (Difco). Agar Technical No. 3 was omitted from CSM. Test organisms were streaked on the medium and plates were incubated at 37°C for 48h. The plates were then flooded with 1N HCl to precipitate nucleic acids. DNase activity was observed as a zone of clearing of the precipitate round the bacterial growth.

2.3.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Whole cells were prepared for SDS-PAGE by harvesting from 1ml of 48h liquid cultures in a Sarstedt Microfuge. The cells were washed once in saline and resuspended to an optical density of 0.8 at 600nm wavelength (Pye-Unicam SP6-500 Spectrophotometer). 100µl of cell suspension was transferred to 1ml solubilising buffer (125mM Tris-HCl pH 6.8; SDS 4% w/v; β-mercaptoethanol 10% v/v; glycerol 20% v/v; bromophenol blue 0.002% w/v) and heated in a boiling water bath for 5 min. Alternatively, a dense cell suspension was made in solubilising buffer directly from growth on a BGA plate. Stacking gels contained 3-4ml of acrylamide (30% v/v); 5.0ml of 0.5M Tris-HCl pH 6.8; 100 µl of SDS (20% w/v); 25µl of TEMED; 2ml of ammonium persulphate (1% w/v) and 9.5ml of water. Separating gels contained 27.5ml of acrylamide (30% v/v); 28.1ml of 1M Tris-HCl pH 8.8; 375µl of SDS (20% w/v); 19µl of TEMED; 7.5ml of ammonium persulphate (1% w/v) and 11.5ml of distilled water. TEMED is N,N,N',N',-Tetramethyl ethylenediamine. 25-50µl of sample was loaded into each well and electrophoresis was carried out at 15mA for 2½-3h. Gels were immersed in staining /fixing solution for 60-90 min. This contained Coomassie Blue R250 (1.25g), 46ml of glacial acetic acid and 454ml of 50% methanol (v/v). Gels were destained by soaking in several changes of destaining solution containing 50ml methanol; 75ml glacial acetic acid and 875ml of distilled water. For molecular weight comparisons, a mixture of marker proteins (200µg ml^{-1} final concentration) was in each gel. The mixture contained BSA (MW = 66000), ovalbumin (45000), pepsin (34700), trypsinogen (24000), β-lactoglobulin (18400) and lysozyme (14300).

2.3.3 Measurement of phagocytosis by chemiluminescence.

Bacteria were grown on nutrient agar (E. coli) or BGA (B. pertussis) containing streptomycin (200µg ml^{-1}), giving a lawn of bacteria. Following incubation, growth was resuspended in 2ml of Hepes-buffered-saline (HBS) (Appendix 3) and transferred to a universal bottle. The volume was adjusted to 3ml with HBS and cell suspensions were standardised to an Optical Density of 0.8 at 600nm (Pye-Unicam SP6-550 Spectrophotometer).

Polymorphonuclear leucocytes were obtained by injecting New Zealand white rabbits intraperitoneally with 500ml of 0.85% (w/v) saline containing glycogen 0.1% w/v (Sigma). The peritoneal exudate was drained off 5h later and was stored at $4^{o}C$ overnight. The exudate was used within 3 days of isolation. Before use, the phagocytic cells were washed once in divalent cation-free HBS-EDTA (Appendix 3) then in HBS. Contaminating erythrocytes were removed after the first wash by hypotonic lysis. Washed cells were used within 2h of preparation. Cell suspensions contained over 95% neutrophils and cell viability was greater than 95%, as determined by Trypan Blue exclusion. Phagocytosis of whole bacteria was measured by chemiluminescence using an LKB Luminometer connected to a BBC Acorn Microcomputer. Before use bacterial suspensions were diluted 1 in 3 and kept on ice. The neutrophil count was adjusted to 2×10^{6} cells ml^{-1} with HBS. The experimental mixture contained 100µl of test agent (bacteria), 500µl of neutrophil cell suspension, 30µl of HBS and 70µl of luminol (10^{-4}M final concentration) (5-amino-2,3-dihydro-1, 4-phthalazinedione) (Sigma). Before the addition of luminol and test agent, phagocytes were pre-incubated for 5 min at $37^{o}C$. All chemiluminescence studies were carried out at $37^{o}C$. The count time of each sample was 30 sec and duplicate counts were taken over 90 min.

3. RESULTS

3.1 Development of media for growth and selection of recombinant and auxotrophic organisms.

3.1.1 B. pertussis

In developing a gene transfer system, various types of media which are suitable for the selection of transconjugants and recombinants of the organism under study are required. With B. pertussis, development of such media has to take into account the fastidious nature of the organism and its sensitivity to inhibition by fatty acids in the medium. Bordet-Gengou Agar (BGA) is the medium used for routine growth of B. pertussis but is unsuitable for isolation of auxotrophic mutants. Although BGA was used in this study to obtain plasmid carrying transconjugants, it could not be used as a selective medium in the study of mobilisation of donor nutritional markers. Therefore, for these purposes, development of a suitable minimal medium was a priority.

Initial attempts to grow phase I B. pertussis on Cohen and Wheeler medium or Stainer and Scholte medium, solidified by addition of 1% Agar Technical No. 3 (Oxoid) proved unsuccessful. Imaizumi et al. (1983) used a solid medium, based on Stainer and Scholte medium, but containing 1mg ml^{-1} (Heptakis (2-6-O Dimethyl) β-Cyclodextrin (MeβCD), to allow growth of colonies of B. pertussis from small inocula. Therefore, MeβCD was incorporated in Stainer and Scholte Medium at a concentration of 1mg ml^{-1}. The medium was solidified by addition of 1.5% w/v Agar (Oxoid). To determine how efficiently the medium supported growth of B. pertussis 100µl of a series of 10-fold dilutions made from a suspension of approximately 10^9 cells ml^{-1} of strain Tohama was spread on each of the following types of medium :

i. BGA

ii. CSM

iii. CSM containing Casamino acids (CAA) (Casein hydrolysate)

iv. CSM containing CAA and bovine serum albumin (BSA 0.15%)

v. CSM containing BSA alone.

Table 1. Comparison of ability of BGA and CSM to support B. pertussis growth from small inocula. 100 µl aliquots of 10^{-5} and 10^{-7} dilution of a suspension of strain Tohama was spread on each type of medium. Results are the number of cells per ml which are capable of growth on each medium. Efficiency of plating (EOP) is defined as viable count on each type of medium divided by viable count on BGA multiplied by 100.

	c.f.u. ml^{-1} at dilutions of :			
	10^{-5}		10^{-7}	
Medium	cfu ml^{-1}	EOP %	cfu ml^{-1}	EOP %
BGA	1.82×10^{9}	100	2.4×10^{9}	100
CSM	6×10^{6}	0.33	0	0
CSM + Casamino Acids	7.1×10^{8}	39	4.0×10^{8}	16.6
CSM + BSA + CAA	1.44×10^{9}	79	7.0×10^{8}	29.1
CSM + BSA only	9.8×10^{8}	53.8	6.0×10^{8}	25

After 4-5 days incubation of plates at 37°C, the number of colonies on each was counted and the efficiency of plating (EOP) determined. This was expressed as a percentage value of the number of colonies on BGA, for each type of medium (Table 1). CSM without addition of BSA or Casamino Acids supported growth from single colonies at a much lower level than BGA. Addition of Casamino Acids increased the EOP value about 100 fold to about 39%. When BSA was added to the medium in the presence of Casamino Acids, a higher EOP was obtained, and colonies grew at a faster rate, taking about 3 days to develop, as opposed to the 4 or 5 days in the absence of BSA. If CSM was to be used as a minimal medium for B. pertussis, then Casamino Acids had to be omitted since it contained a heterogenous mixture of amino acids which would permit growth of auxotrophs. Therefore, in order to produce good growth of single colonies on a defined medium, CSM containing 0.15% BSA was found to be the medium of choice since it produced a high EOP and colonies grew in 3-4 days. It may be, however, that this concentration of BSA would provide a sufficient amino acid supply to allow an auxotrophic mutant to overcome the mutation and behave as a prototroph. Therefore, the minimum concentration of BSA which would allow growth of B. pertussis was determined. Since no auxotrophic mutant of B. pertussis with which the medium could be tested was available, at this stage, an auxotrophic strain of E. coli which produced small colonies on CSM + CAA was tested in parallel with the B. pertussis prototrophic strain (Table 2). Cultures were grown and standardised as described previously and 100µl of each of a series of 10-fold dilutions was plated on CSM containing a range of BSA concentrations from 0 to 0.2% (w/v). Following incubation, the auxotrophic E. coli strain could not grow on CSM in the absence of Casamino Acids, but B. pertussis growth was supported by a minimum BSA concentration of 0.05%. Lower concentrations were not tested. B. pertussis failed to grow when BSA was omitted from the medium.

Table 2. Determination of BSA requirement of B. pertussis Tohama, and an E. coli auxotroph for growth on CSM. 100 µl aliquots of 10^{-6} and 10^{-7} dilutions of a suspension of B. pertussis Tohama or a 10^{-7} dilution of E. coli JC3272 were spread on CSM containing various BSA concentrations.

Strain	Dilution	Number of colonies at the following BSA concentration (% w/v)				
		0	0.05	0.1	0.15	0.2
B. pertussis Tohama	10^{-6}	0	459	448	488	354
	10^{-7}	0	38	41	44	39
E. coli JC3272	10^{-7}	0	0	0	0	0

CSM containing 0.05% BSA, but without the addition of Casamino Acids was subsequently used in two experimental procedures; a) To isolate B. pertussis auxotrophic mutants and b) To obtain prototrophic recombinants carrying mobilised chromosomal markers, in gene transfer experiments.

3.1.2 B. parapertussis

To allow the selection of plasmid carrying transconjugants of B. parapertussis or strains carrying mobilised antibiotic resistance markers, a suitable selection mechanism was required. Several types of media were studied for their ability to support single colony growth of B. parapertussis while failing to support growth of B. pertussis. The media studied were BGA, nutrient agar, E. coli Minimal Agar (ECMA), Stainer and Scholte Agar (SSA) and MacConkey Agar. From a cell suspension standardised as before, 50µl of 10-fold dilutions were spread on each type of medium. Single colony growth of B. parapertussis was supported by BGA, SSA and MacConkey Agar, but growth on MacConkey Agar was not as efficient as on the other media since only a fraction of the total viable count, by comparison with growth on BGA, was supported by MacConkey Agar. Growth on SSA was more efficient than on MacConkey Agar giving a viable count which was about one-third of that obtained on BGA. However, only very small colonies were supported by this medium. Growth on nutrient agar was at a higher plating efficiency than that of MacConkey Agar while ECMA failed to support growth of B. parapertussis.

It was observed that colonies which grew on MacConkey Agar produced, after about 6 days incubation, outgrowths from the periphery of the colony giving a star-like appearance. To determine if growth on MacConkey Agar gave rise to a variant of B. parapertussis, a single colony from a MacConkey plate was inoculated into Cohen and Wheeler broth and incubated for 48h at 37°C. Cell suspension was standardised as before, and 50µl from the 10^{-6} dilution

was spread on each type of medium under study. The efficiency of plating on each type of medium was determined (Table 3). EOP on MacConkey Agar was much increased, and EOP on SSA and MacConkey was higher than on nutrient agar. Thus, not all B. parapertussis capable of growth on BGA can grow on MacConkey Agar which may select for variants within the population. To test this hypothesis, 50 colonies, originally grown on BGA, were restreaked on MacConkey Agar. Only two were capable of growing on this medium.

MacConkeys Agar, SSA and nutrient agar, therefore, whilst being suitable for use as selective media in plasmid transfer and chromosomal mobilisation studies using B. parapertussis recipients, appear to be selective for variants in the population, which would interfere with accurate determination of transfer and recombination frequencies. A more suitable selection mechanism would be to make use of an intrinsic antibiotic resistance marker in B. parapertussis, with selection of transconjugants and recombinants on BGA containing this antibiotic. Such an intrinsic resistance was subsequently shown to exist in B. parapertussis strain 10520.

3.2 Isolation of antibiotic resistant mutants of B. pertussis.

Using the NTG disk method, nalidixic acid resistant colonies of B. pertussis strain Tohama were obtained (Table 4). That the mutants obtained were B. pertussis was confirmed by their inability to grow on nutrient agar, Gram-stain and agglutination in antiserum. Whole cell SDS-PAGE analysis of Tohama mutants and parent strain revealed differences from strain 18334 (Figure 2). Tohama strains lacked the 28 and 30, KD protein bands while a low molecular weight band, present in Tohama, was reduced in 18334. However, subsequent studies have failed to confirm this. Tohama, however, exhibited normal Phase I properties in being unable to grow on Nutrient Agar, and agglutinating with anti-Phase I antiserum. Growth on nalidixic acid was compared for mutant

Table 3. Efficiency of plating of B. parapertussis 10520 on various media, using colonial variant initially isolated by growth on MacConkey agar. *Efficiency of Plating (EOP) is the viable count on each medium divided by viable count on BGA, expressed as a percentage.

	c.f.u ml^{-1} at dilution of 10^{-6}	
Medium	cfu ml^{-1}	EOP* (%)
BGA	6.64×10^9	100
MacConkey Agar	5.96×10^9	90
Stainer & Scholte Agar	5.04×10^9	75
ECMA	0	0
Nutrient Agar	4.40×10^9	66

Table 4 Isolation of B. pertussis resistant mutants by NTG mutagenesis. Results are the number of resistant colonies of each strain which grew around an NTG soaked paper disk.

Strain	Number of colonies resistant to :			
	Rifampicin	Streptomycin	Nalidixic acid	Chloramphenicol
Tohama	0	0	6	0
L84 I	0	0	7	0
D30042	0	4	14	0
Taberman	0	0	0	0

and parent strains (Table 5). These mutants were designated BPN1 to BPN6. BPN6 was used for subsequent genetic studies.

The procedure was repeated using B. pertussis Taberman, D30042 I and L84I. Nalidixic acid resistant mutants of Taberman were obtained and designated BPT 1 to 8 (Table 5).L84I also yielded nalidixic acid resistant mutants while D30042 yielded streptomycin resistant colonies designated CSP1 to 4. CSP1 was used for further study.

The procedure was repeated using rifampicin, erythromycin and chloramphenicol as selective antibiotics, but no mutants resistant to these antibiotics were obtained.

3.3 Isolation of B. pertussis auxotrophic mutants.

Isolation of auxotrophic mutants of B. pertussis was carried out using strains BPN6, CSP1, Taberman P3 and L84I. The NTG treatment and penicillin enrichment procedure was used and CSM was used to screen for auxotrophic properties and nutritional requirements. However, the procedure proved to be unsuitable for auxotroph isolation using Taberman P3, as CSM did not support growth of either presumptive auxotrophs or of the wild-type prototroph. L84I was unsuitable also, since it was resistant to the concentration of penicillin G which was used in the enrichment procedure.

Using strain CSP1 25 presumptive auxotrophs were obtained from a total of 300 colonies screened. By restreaking each colony on CSM amino acid pool plates 19 of these were subsequently shown to be prototrophic by their ability to grow on all pools and on CSM without addition of amino acids.
The remaining colonies grew only on particular amino acid pools. Mutants 8, 10, 18 and 21 appeared to carry multiple auxotrophic mutations, but mutant 24 grew only on pools 5 and 7 indicating that it was deficient in glycine production (Gly^-). Mutant 11 grew only on pools 8 and 2 indicating that it was

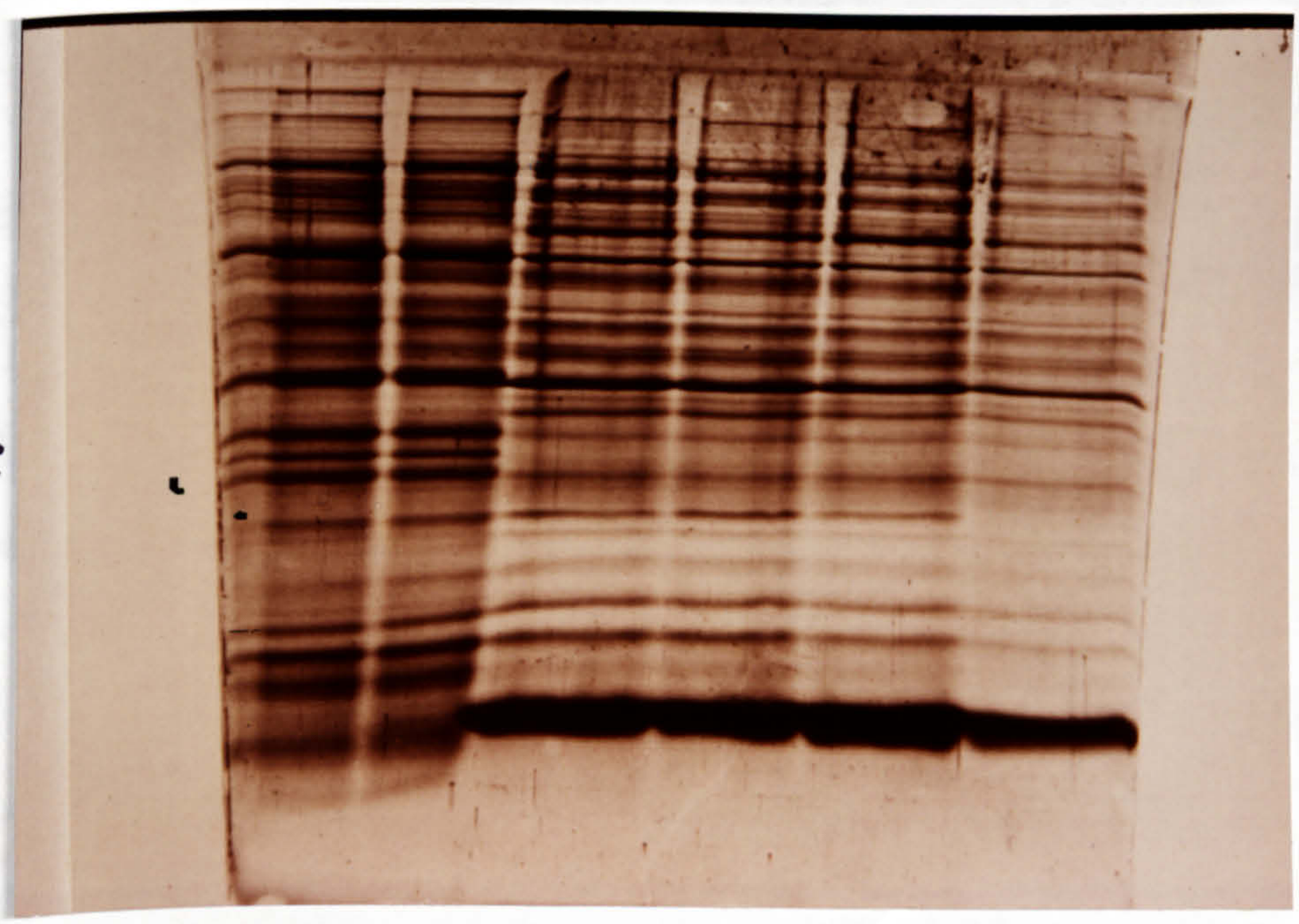
Table 5. Growth of B. pertussis Nal^R mutants on various concentrations of nalidixic acid. + - growth ± - poor growth - no growth.

Strain		Bacterial growth at nalidixic acid concentration of ($\mu g\ ml^{-1}$)				
		20	40	60	80	100
Tohama	1	+	+	±	-	-
	2	+	+	±	-	-
	3	+	+	±	-	-
	4	+	+	±	-	-
	5	+	+	±	-	-
	6	+	+	±	-	-
Parent		-	-	-	-	-
Taberman	1	+	+	±	-	-
	2	+	+	+	-	-
	3	+	+	±	-	-
	4	+	+	+	-	-
	5	+	+	+	-	-
	6	+	+	±	-	-
	7	+	+	±	-	-
	8	+	+	±	-	-
Parent		-	-	-	-	-

Figure 2 : SDS-PAGE analysis of whole cells of Tohama parent, Nal^R mutant and 18334 strains.

Tracks 1 and 2 - 18334

Tracks 3-6 - Tohama parent and mutants BPN 2,4 and 6


28+30k bands
1
2
3
4
5
6

deficient in tryptophan production (Trp^-). Mutant 24 (Gly^-) could not grow in Stainer and Scholte liquid medium without the addition of glycine (0.02%) whilst mutant 11 (Trp^-) would not grow in Stainer and Scholte liquid medium unless tryptophan was present in the medium. These auxotrophs were tested for their ability to grow on CSM in the presence of varying concentrations of BSA, and in the presence and absence of glycine or tryptophan. (Tables 6 & 7). A suspension of about 10^9 cells ml^{-1} was washed once in sterile saline and from a series of 10-fold dilutions, 100µl was spread on BGA, CSM (without any amino acid content) CSM with various BSA concentrations, and CSM containing glycine or tryptophan. Auxotroph 24 (Gly^-) failed to grow unless CSM was supplemented with glycine whilst auxotroph 11 (Trp^-) grew only in the presence of tryptophan. Low concentrations of BSA failed to support growth of auxotrophic mutants. Thus, these auxotrophic mutants were found to be suitable for use in gene transfer studies. The procedure was repeated using Tohama BPN6, a nalidixic acid resistant mutant isolated previously (Section 2). 2 presumptive auxotrophs were isolated and upon characterisation, one showed a requirement for threonine (Thr^-) (Tables 6 & 7) while the other showed a complex nutritional requirement.

In the course of these experiments attempts were made to screen single colonies of B. pertussis for growth requirements by replica plating from BGA onto CSM. However, due to insufficient growth being picked up on the velvet pad, from the small B. pertussis colonies, this proved to be impracticable.

3.4 Screening of B. pertussis and B. parapertussis strains for natural antibiotic resistance.

Although a method for obtaining antibiotic resistant mutants of B. pertussis was available, there was still only a limited range of antibiotic resistant strains which could be used in genetic manipulations. Therefore several strains of B. pertussis and B. parapertussis were screened for naturally occur-

Table 6. - Growth of B. pertussis auxotrophic mutants on CSM in the presence of various BSA concentrations.

Strain	Auxotrophic requirement	Growth in the presence of BSA at the following concentrations (% w/v) :- 0	0.05	0.1	0.15	0.2
CSP 1	Trp^-	-	-	-	-	-
CSP 1	Gly^-	-	-	-	-	-
BPN 6	Thr^-	-	-	-	-	+

+ growth - no growth

Table 7 Growth of auxotrophic mutants of B. pertussis in the presence or absence of amino acids. 100 µl aliquots of a suspension of each mutant were spread on CSM containing the appropriate amino acid.

Auxotrophic mutant	Medium	No. of colonies formed at dilutions of :- 10^{-5}	10^{-6}	10^{-7}
CSP1 Gly^-	CSM	0	0	0
	CSM + gly	186	5	0
	BGA	confluent	102	11
CSP1 Trp^-	CSM	0	0	0
	CSM + trp	49	3	0
	BGA	confluent	171	12
BPN6 Thr^-	CSM	0	0	0
	CSM + thr	142	16	0
	BGA	confluent	218	18

-ring antibiotic resistance using Oxoid Multodisks (Table 8). Laboratory strains such as 18334 and 18323 were found to be sensitive to all antibiotics tested. Tohama exhibited resistance to sulphafurazole, methicillin and streptomycin, although Tohama would not grow on a concentration of 200µg ml^{-1} streptomycin, the concentration at which the antibiotic was used in selective media. Taberman was resistant to 1.5 units of Penicillin G and 2µg ml^{-1} ampicillin, but this concentration was much lower than the 20µg ml^{-1} of carbenicillin used as a selective agent. B. parapertussis strain NCTC 10520 was resistant to several antibiotics at the levels tested, but was sensitive to inhibition by higher levels of the β-lactams (20µg ml^{-1} carbenicillin) and tetracycline (20µg ml^{-1}). However, strain 10520 was resistant to chloramphenicol at a concentration of 20µg ml^{-1}, a property which was utilised in subsequent gene transfer studies. CSP1 was confirmed as being resistant to streptomycin (200µg ml^{-1}).

3.5 Minimum Inhibitory Concentrations (MIC) of various antibiotics on B. pertussis.

Following isolation of antibiotic resistant mutants of B. pertussis, and screening of strains for naturally occurring resistance, a potential genetic recipient strain (44122/7R) was further characterized to determine the MIC of the selective antibiotic, streptomycin (Table 9). In initial transfer of plasmids from E. coli donors, the B. pertussis 44122/7R recipient was required to be resistant to the selective antibiotic, while the donor was required to be sensitive. Therefore, MIC values for E. coli donor strains were also determined (Table 9).

A range of antibiotic resistant strains of B. pertussis was now available and, together with the auxotrophic mutants obtained previously, they could be used to develop a system of gene transfer in B. pertussis.

Table 8 Antibiotic resistant in B. pertussis and B. parapertussis as determined by Multodisk sensitivity tests. Oxoid S3 Multodisks were used - See Appendix 2.

S - The strain is sensitive to inhibition by the antibiotic and a zone of no growth is present round the disk.

R - The strain is resistant to inhibition by the antibiotic and there is no zone of inhibition around the disk.

Species	Strain	Antibiotic Sensitivity							
		C	E	SF	CB	P	PN	S	TE
B. parapertussis	10520	R	S	R	R	R	R	R	R
"	5952	S	S	S	R	R	R	R	S
B. pertussis	44122/7R	S	S	R	S	S	S	R	S
"	18334	S	S	S	S	S	S	S	S
"	18323	S	S	S	S	S	S	S	S
"	Tohama	S	S	R	R	S	S	R	S
"	BPN 6	S	S	R	R	S	S	S	S
"	D30042	S	S	S	S	S	S	S	S
"	CSP1	S	S	R	S	S	S	R	S
"	Taberman	S	S	S	S	R	R	S	S
"	BPT2	S	S	S	S	R	R	S	S
"	P3	S	S	S	S	R	R	R	S
"	L84 I	S	S	S	S	S	S	S	S
"	L84 IV	S	S	R	R	R	R	R	S
"	D30042 IV	S	S	R	R	S	S	R	S
"	11615 IV	S	S	R	R	R	R	S	S

Table 9 - Resistance of E. coli donors and a B. pertussis recipient strain to streptomycin

Organism	Growth in the presence of streptomycin (μg ml^{-1})			
	10	50	100	200
E. coli				
J53-1/R68.45	-	-	-	-
JMP 502	-	-	-	-
JMP 543	+	-	-	-
N7731/R68:45 supdna-G3 R15	-	-	-	-
J53-1/RP4::Tn7	-	-	-	-
B. pertussis				
44122/7R	+	+	+	+

+ - growth

- - no growth

3.6 R plasmid-mediated conjugation and chromosome mobilisation in E. coli.

The Inc-P group broad host-range plasmid R68.45 can promote mobilisation of chromosomal markers within a wide range of Gram-negative bacteria (Holloway, 1979). In this study, R68.45 and several similar plasmids were used to mobilise chromosomal markers between strains of B. pertussis and from B. pertussis to B. parapertussis.

The ability of R68.45 to transfer between strains and to promote exchange of chromosomal markers was first examined in E. coli. The plasmid was transferred from E. coli strain J53-1 to JC3272 by conjugation using the membrane filter method. The frequency of plasmid transfer was high, about 1 recipient cell in every 100 taking up the plasmid. Resistance to nalidixic acid, a chromosomal mutation carried by the donor J53-1, was also transferred, but at a much lower frequency (Table 10).

In plasmid R68.45 chromosome mobilising ability can be stabilised by maintenance of the plasmid in the presence of kanamycin (Haas and Holloway, 1976) which may be linked to the presence of the insertion sequence IS21 on the plasmid (Willetts et al., 1981). To determine if this effect occurred during mobilisation of the donor nalidixic acid resistance marker, conjugation between donor J53-1 and recipient JC3272 strains was carried out after growth of the donor in the presence or absence of kanamycin (10μg ml^{-1}). The experiments were performed as plate matings (Table 11). Transfer of plasmid R68.45 occurred at high frequency and was not affected by the presence or absence of kanamycin in the donor growth medium. However, J53-1 grown in kanamycin mobilised the Nal^R marker at a frequency 60-100 fold greater than J53-1 grown in the absence of kanamycin. Thus, in all subsequent experiments involving R68.45 mediated chromosome mobilisation, kanamycin was incorporated in the donor liquid culture, to mediate maximum mobilisation of chromosomal markers.

Table 10 - R68.45 transfer and mobilisation of a chromosomal marker (Nal^R) from E. coli J53-1 to JC3272.

Selection for plasmid carrying transconjugants was on nutrient agar (NA) containing Kanamycin, carbenicillin and streptomycin. Selection for Nal^R recombinants was on NA containing nalidixic acid and streptomycin. Transfer frequencies are as described in Materials and Methods. Spontaneous mutation rates of donor and recipient to selective antibiotic resistance were $< 8.2 \times 10^{-10}$.

Experiment No.	Plasmid transfer frequency	mean plasmid transfer frequency	Cma frequency	mean Cma frequency
1	9.54×10^{-3}	5.68×10^{-3}	5.0×10^{-9}	5.6×10^{-9}
2	6.12×10^{-3}	($\pm 1.37 \times 10^{-3}$)	6.1×10^{-9}	($\pm 3.3 \times 10^{-10}$)
3	1.38×10^{-3}		5.9×10^{-9}	

Table 11 - Effect of Kanamycin on *cma* ability of R68.45 in *E. coli*

Donors were grown in presence or absence of Kanamycin overnight in nutrient broth. Transfer and *cma* frequencies are as described in Materials and Methods. The marker mobilised was Nal^R. Spontaneous mutation rates of donor and recipient to selective antibiotics resistance were $< 7.8 \times 10^{-10}$.

Donor	Recipient	plasmid transfer frequency	mean transfer frequency (SEM)	*cma* frequency	mean *cma* frequency (SEM)
J53-1	JC3272	4.6×10^{-1}	5.5×10^{-1}	7.1×10^{-8}	7.3×10^{-8}
Kanamycin present		6.2×10^{-1}		6.7×10^{-8}	
		5.8×10^{-1}	($\pm 4.8 \times 10^{-2}$)	8.2×10^{-8}	($\pm 4.4 \times 10^{-9}$)
		1.23×10^{-1}	3.74×10^{-1}	1.56×10^{-9}	2.35×10^{-9}
		3.1×10^{-1}	($\pm 1.6 \times 10^{-1}$	3.1×10^{-9}	
Kanamycin absent		6.9×10^{-1}		2.4×10^{-9}	($\pm 4.4 \times 10^{-10}$)

The mating procedure was found to be unsuitable in attempting to transfer plasmid R68.45 from E. coli J53-1 to B. pertussis 44122/7R. This was due to the negative pressure passing through the membrane filter drawing in atmospheric contaminants which grew on the BGA used for incubation of the filter. Therefore, a contamination-free system was developed based on the plate mating method of Bradley et al., (1981). To determine how mating procedure affected the degree to which plasmid transfer occurred, three systems were compared with the filter mating system using E. coli donor and recipient. These systems were :-

i. Plate mating

ii. Mating in nutrient broth (Gibco)

iii. Mating in nutrient broth containing 0.1% w/v agar (Agar Technical. No.3, Oxoid).

It was found that none of the three procedures produced an efficiency of plasmid transfer equal to that of the filter mating system. Of the three methods tested (Table 12), the plate mating system gave rise generally to a slightly higher transfer frequency than the broth mating systems. The presence of agar in the broth mating medium failed to enhance plasmid transfer, and may actually have reduced it. Thus plasmid transfer between strains of E. coli was mediated most efficiently by the filter mating system, but this method was not suitable for use with B. pertussis due to contamination of the filter. Therefore, all subsequent plasmid transfers which involved B. pertussis, were carried out using the plate mating method, which was much less susceptible to contamination yet produced efficient plasmid transfer.

3.7 Determination of optimal donor : recipient ratio in E. coli.

This was carried out in order to determine the donor : recipient ratio which gave maximum plasmid transfer and chromosome mobilisation. 1ml of

Table 12 - Comparison of mating methods in E. coli. In all cases the donor was J53-1 (R68.45) and the recipient was JC3272. Transfer frequencies are as described in Materials and Methods. Spontaneous mutation rates of donor and recipient to selective antibiotic resistance was $< 5.4 \times 10^{-10}$

Method of mating	plasmid transfer frequency	mean (SEM)
Filter	5.24×10^{-2}	5.15×10^{-2}
	5.19×10^{-2}	
	5.03×10^{-2}	$(\pm 6.3 \times 10^{-4})$
Broth	9.9×10^{-4}	1.99×10^{-3}
	1.99×10^{-3}	
	3.01×10^{-3}	$(\pm 5,8 \times 10^{-4})$
Broth containing 0.1% agar	6.75×10^{-4}	7.48×10^{-3}
	5.9×10^{-4}	
	9.8×10^{-4}	$(\pm 1.18 \times 10^{-4})$
Plate	2.57×10^{-3}	2.81×10^{-3}
	2.75×10^{-3}	
	3.11×10^{-3}	$(\pm 1.5 \times 10^{-4})$

donor E. coli J53-1 was mixed with 1, 5 or 10ml of recipient strain JC3272, and plate matings were carried out using nutrient agar as the mating medium (Table 13). The donor : recipient ratio had little effect on the frequency of plasmid transfer, but a ratio of 1:10 produced a 10-fold reduction in chromosome mobilisation frequency, compared to a 1:1 or 1:5 ratio. Therefore, in all subsequent plasmid transfer and chromosome mobilisation studies, a donor: recipient ratio of 1:1 was used as the mating mixture.

3.8 Plasmid R68.45 transfer from E. coli to B. pertussis.

Plasmid R68.45 was transferred from E. coli J53-1 to B. pertussis 44122/7R by conjugation using the plate mating method and a 1:1 donor:recipient ratio (Table 14). Single colony transconjugants of B. pertussis which exhibited plasmid-coded antibiotic resistance were obtained following incubation of BGA selection plates for 4-5 days at 37°C. In the first few attempts at transferring the plasmid, the E. coli donor was found to rapidly outgrow B. pertussis on the mating medium during overnight incubation of the mating mixture. This gave rise to spontaneous streptomycin resistant mutants of E. coli which grew on the selection medium. Thus, in order to reduce overgrowth of E. coli cephalexin (40µg ml^{-1}) was incorporated in the mating medium which reduced donor growth but still allowed selection of B. pertussis transconjugants at a similar frequency (Table 14). A further modification of the plasmid transfer system, used a mating period of 4-6h as opposed to overnight (Materials and Methods, Section 2-6). This became the incubation period of choice since E. coli growth was further reduced but still allowed plasmid transfer to take place.

B. pertussis transconjugants showed resistance to kanamycin, carbenicillin and tetracycline. R68.45 was stably maintained following several subcultures on BGA after which, resistance to the antibiotics continued to be exhibited.

Table 13 - Determination of optimal donor : recipient ratio for plasmid transfer and mobilisation of Nal^R from E. coli J53-1 to E. coli JC3272. Transfer and cma frequencies are as described in Materials and Methods. Transconjugants were selected on NA containing selective antibiotics. Spontaneous mutation frequency of donor and recipient to selective antibiotics was $<6.3 \times 10^{-10}$.

Donor : recipient ratio	plasmid transfer frequency	mean plasmid transfer frequency (SEM)	cma frequency	mean cma frequency (SEM)
1 : 1	1.3×10^{-1}	2.1×10^{-1}	1.7×10^{-8}	1.9×10^{-8}
	2.3×10^{-1}		1.8×10^{-8}	($\pm 1.5 \times 10^{-9}$)
	2.7×10^{-1}	($\pm 4.1 \times 10^{-2}$)	2.2×10^{-8}	
1 : 5	1.9×10^{-1}	2.6×10^{-1}	1.0×10^{-8}	1.53×10^{-8}
	4.3×10^{-1}		1.6×10^{-8}	($\pm 2.9 \times 10^{-9}$)
	1.6×10^{-1}	($\pm 8.5 \times 10^{-2}$)	2.0×10^{-8}	
1 : 10	1.7×10^{-1}	1.9 x 10-1	2.4×10^{-9}	2.03×10^{-8}
	2.1×10^{-1}		1.6×10^{-9}	
	1.9×10^{-1}	($\pm 1.1 \times 10^{-2}$)	2.1×10^{-9}	($\pm 2.3 \times 10^{-9}$)

Table 14 - Transfer of plasmid R68.45 from E. coli J53-1 to B. pertussis 44122/7R in the presence or absence of cephalexin (40 $\mu g\ ml^{-1}$).

Plasmid transfer frequency is as described on BGA containing selective antibiotics. Spontaneous mutation rates of donor and recipient to Str^R and Km^R, Cb^R was $< 5.7 \times 10^{-10}$.

cephalexin present during mating	plasmid transfer frequency	mean transfer frequency (SEM)
NO	2.01×10^{-4}	2.37×10^{-4}
	2.57×10^{-4}	($\pm 1.8 \times 10^{-5}$)
	2.54×10^{-4}	
YES	2.03×10^{-4}	2.50×10^{-4}
	3.01×10^{-4}	($\pm 2.8 \times 10^{-5}$)
	2.48×10^{-4}	

MIC values of each antibiotic were compared for the parent strain 44122/7R and for plasmid carrying transconjugants, designated 44122/7RP. The MIC of tetracycline was less than 2.5µg ml^{-1} and greater than 20µg ml^{-1} for the parent and transconjugants respectively while the MIC of kanamycin was less than 20µg ml^{-1} and greater than 80µg ml^{-1} for parent and transconjugants respectively. MIC of carbenicillin was less than 10µg ml^{-1} for the parent and 100µg ml^{-1} for the transconjugants (Table 15). Thus it was possible for B. pertussis to inherit and stably maintain an R plasmid.

Transfer of R68.45 from B. pertussis 44122/7RP to an E. coli recipient JC3272 was also studied, to determine the ability of B. pertussis to behave as a genetic donor. It was possible, in this case, to use a donor which was resistant to the same antibiotics as the recipient, since the selection procedure was based on the inability of the donor B. pertussis to grow on nutrient agar. Transconjugants of E. coli grew overnight on nutrient agar containing kanamycin, carbenicillin and streptomycin following plate mating on BGA containing cephalexin (Table 16). The frequency of plasmid transfer to E. coli from B. pertussis was approximately 100-fold lower than from E. coli to B. pertussis, suggesting that the ability of B. pertussis to act as a genetic donor was reduced compared to E. coli.

To show that transfer of plasmid-borne antibiotic resistance between E. coli and B. pertussis was due to conjugation and transfer of DNA by cell to cell contact, rather than by genetic transformation, the frequency of transfer of plasmid R68.45 was determined in the presence and absence of deoxyribonuclease (DNase II, Sigma), at a concentration of 100µg ml^{-1}, in the mating medium. Donor E. coli and recipient B. pertussis strains were incubated together for 5h on BGA containing DNase II. Following selection of transconjugants, no difference was found in the plasmid transfer frequency in the presence of DNase, indicating that naked plasmid DNA was not transforming B. pertussis

Table 15 - Differences in antibiotic MIC values due to the presence of R68.45 in B. pertussis 44122/7R. Growth is denoted by + and no growth by - .

Kanamycin	Antibiotic concentration µg ml^{-1}						
Strain	20	30	40	50	60	70	80
44122/7R	-	-	-	-	-	-	-
44122/7RP	+	+	+	+	+	+	+

Carbenicillin	Antibiotic concentration µgml^{-1}						
Strain	10	20	50	100	120	150	200
44122/7R	-	-	-	-	-	-	-
44122/7RP	+	+	+	-	-	-	-

Tetracycline	Antibiotic concentration µg ml^{-1}						
Strain	2.5	1	5	10	20	25	30
44122/7R	-	-	-	-	-	-	-
44122/7RP	+	+	+	+	-	-	-

Table 16 - Transfer of plasmid R68.45, by conjugation, from B. pertussis to E. coli. Transconjugants were selected on NA containing selective antibiotics. Plasmid transfer frequency is as described in Materials and Methods. Spontaneous mutation frequencies were not determined.

Donor (plasmid)	Recipient	Experiment Number	plasmid transfer frequency	mean transfer frequency (SEM)
B. pertussis 44122/7RP (R68.45)	E. coli JC3272	1	3.88×10^{-6}	3.74×10^{-6} ($\pm 6.4 \times 10^{-7}$)
		2	1.66×10^{-6}	
		3	2.77×10^{-6}	
		4	3.33×10^{-6}	
		5	4.65×10^{-6}	
		6	6.20×10^{-6}	

recipient cells and that transfer occurs by cell to cell contact (Table 17).

To determine if plasmid R68.45 was capable of transferring between strains of B. pertussis, 44122/7RP was used as the donor strain with Taberman R1 (resistant to rifampicin Rif^R) as recipient. Selection for plasmid carrying transconjugants of R1 was carried out on BGA containing kanamycin, carbenicillin and rifampicin. It was found that R68.45 was capable of transferring between the two strains at high frequency (Table 18). However, a relatively high frequency of spontaneous mutation to rifampicin resistance in the donor strain was observed which precluded use of this particular donor/recipient combination in studies on mobilisation of the chromosomal streptomycin resistance marker.

3.9 Transfer of other plasmids to E. coli and B. pertussis

Since R68.45 was transferred by conjugation from E. coli to B. pertussis several similar R plasmids were studied for their ability to transfer to B. pertussis. These plasmids were RP1, RP1::Tn501, R68.45supdnaG315 and RP4::Tn7. RP1 and RP4 are similar to R68.45 and also encode resistance to kanamycin (Km^R), carbenicillin (Cb^R) and tetracycline (Tc^R), R68.45supdnaG315 is a mutant of R68.45. The transposon Tn501 encodes resistance to mercury (Hg^R) while Tn7 encodes resistance to trimethoprim (Tp^R) and streptomycin (Sm^R) in E. coli. Initially, these plasmids were tested for their ability to transfer, by conjugation, between strains of E. coli using JC3272 as recipient. However, since Tn7 encodes streptomycin resistance in E. coli, plasmid RP4::Tn7 could not be compared using this system. The E. coli hosts in which the plasmids were present initially were tested for their resistance to streptomycin, to determine if E. coli JC3272 and subsequently B. pertussis 44122/7R could be used as recipients. All donor strains except J53-1 (RP4::Tn7) were sensitive to streptomycin at the concentration at which the antibiotic is used for trans-

Table 17 - Plasmid transfer from E. coli to B. pertussis in the presence of DNase Plasmid transfer is as defined in Materials and Methods. Spontaneous mutation rates of donor to Str^R and recipient to Km^R were $< 2.54 \times 10^{-10}$. Transconjugants were selected on BGA containing selective antibiotics.

Donor (plasmid)	recipient	DNase	Exp. No.	plasmid transfer frequency	mean transfer frequency (SEM)
E. coli J53-1 (R68.45)	B. pertussis 44122/7R	present	1	1.21×10^{-4}	1.34×10^{-4}
			2	1.44×10^{-4}	$(\pm 6.8 \times 10^{-6})$
			3	1.38×10^{-4}	
		absent	1	1.10×10^{-4}	1.28×10^{-4}
			2	1.49×10^{-4}	
			3	1.27×10^{-4}	$(\pm 1.1 \times 10^{-5})$

Table 18 - Transfer of plasmid R68.45 between strains of B. pertussis

Transconjugants were selected on BGA containing streptomycin and rifampicin. Spontaneous mutation rate of the donor to Rif^R was 3.6×10^{-7} and of the recipient to Str^R 2.9×10^{-9}.

Donor (plasmid)	Recipient	Experiment Number	plasmid transfer frequency	mean transfer frequency (SEM)
44122/7RP (R68.45)	Taberman R1	1	2.14×10^{-3}	2.62×10^{-3}
		2	2.96×10^{-3}	($\pm 2.4 \times 10^{-4}$)
		3	2.75×10^{-3}	

conjugant selection (200µg ml^{-1}). Plasmids were transferred to recipient E. coli strains using the plate mating method. Plasmids RP1 and RP1::Tn501 were capable of transferring to JC3272 at high frequencies (Table 19). R68.45supdnaG315 however, failed to transfer between E. coli strains. With plasmid RP1::Tn501, the frequency of transfer of mercury resistance was similar to the plasmid transfer frequency, showing clearly that Tn501 was carried on the plasmid into the recipient cell.

Having shown the plasmids to be conjugative, with the exception of R68.45supdnaG315 and RP4::Tn7, they were then transferred by plate mating to B. pertussis 44122/7R (Table 20). Transconjugants were selected on BGA containing streptomycin, kanamycin and tetracycline. Although R68.45supdnaG315 failed to transfer efficiently to E. coli JC3272, its ability to transfer to B. pertussis 44122/7R was determined, as was the ability of RP4::Tn7. Plasmids RP1 and RP1::Tn501 transferred to B. pertussis at relatively high frequencies, but RP1 transferred at about 10 times the frequency of RP1::Tn501. Plasmid RP4::Tn7 was also found to transfer at high frequencies and no resistant colonies of the E. coli donor grew on selection plates, even though the donor carried Tn7. Plasmid R68.45supdnaG315 was found to transfer at low frequency to B. pertussis. It was not used in subsequent studies on chromosome mobilisation in B. pertussis. In transfer of RP1::Tn501, the frequency of inheritance of the transposon was determined by direct selection on BGA containing streptomycin and $HgCl_2$ (10µgml^{-1}) in the absence of kanamycin or carbenicillin (Table 20). The frequency of Tn501 transfer was similar to the frequency of plasmid transfer (Table 20) indicating that all transconjugants had acquired Tn501 on the plasmid. However, when 60 colonies were restreaked on BGA containing kanamycin and carbenicillin, all failed to grow, indicating that although the transposon was stably inherited, the plasmid was lost. By contrast, transconjugants which were initially selected on BGA containing kanamycin and carbenicillin were capable of growth

Table 19 Conjugative ability of other R-plasmids in *E. coli*. Recipient was *E. coli* JC3272. Transconjugants were selected on NA containing kanamycin, carbenicillin and streptomycin. Plasmid transfer frequency is as defined in Materials and Methods. Transposon transfer frequency is defined as the number of mercury resistant recipients divided by total number of recipients. Spontaneous mutation rate of donor and recipient to selective antibiotic resistance was $< 3.1 \times 10^{-10}$.

Donor (plasmid)	plasmid transfer frequency	mean transfer frequency (SEM)
JMP 502	8.0×10^{-1}	7.65×10^{-1}
(RP1)	7.3×10^{-1}	$(\pm 3.5 \times 10^{-2})$
JMP 543	7.6×10^{-1}	7.25×10^{-1}
(RP1::Tn501)	6.9×10^{-1}	$(\pm 3.5 \times 10^{-2})$
N7731	1.3×10^{-5}	-
R68.45 supdna G315		

Donor (plasmid)	Transposon transfer frequency	mean transfer frequency (SEM)
JMP 543	6.4×10^{-1}	8.0×10^{-1}
(RPl :: Tn 501)	9.6×10^{-1}	$(\pm 1.6 \times 10^{-1})$

Table 20 - Plasmid and transposon transfer to B. pertussis from E. coli donors. Selection of transposon carrying recipients was on BGA containing streptomycin and $HgCl_2$. Selection of transconjugants was on BGA containing streptomycin, kanamycin and carbenicillin. Plasmid transfer frequencies are as defined in Materials and Methods. Spontaneous mutation rates of donor and recipient to selective antibiotics. resistance was $< 7.8 \times 10^{-9}$.

E. coli (plasmid)	B. pertussis Recipient	Exp. No.	plasmid transfer frequency	mean plasmid transfer frequency (SEM)
JMP 502 (RP1)	44122/7R	1	4×10^{-2}	3.12×10^{-2} ($\pm 8.7 \times 10^{-3}$)
		2	2.25×10^{-2}	
JMP 543 (RP1::Tn501)	44122/7R	1	5.95×10^{-3}	3.8×10^{-3} ($\pm 2.15 \times 10^{-3}$)
		2	1.65×10^{-3}	
NY 731 (R68.45 supdna G315)	44122/7R	1	2.0×10^{-6}	4.0×10^{-6} ($\pm 2.0 \times 10^{-6}$)
		2	6.0×10^{-6}	
J53-1 (RP4::Tn7)	44122/7R	1	8.23×10^{-4}	1.28×10^{-3} ($\pm 4.6 \times 10^{-4}$)
		2	1.75×10^{-3}	

E. coli donor (plasmid)	B. pertussis recipient	Exp. No.	Transposon transfer frequency	mean transposon transfer freq. (SEM)
JMP 543 (RP1::Tn 501)	44122/7R	1	7.15×10^{-3}	5.01×10^{-3} ($\pm 1.15 \times 10^{-3}$)
		2	3.2×10^{-3}	
		3	4.7×10^{-3}	

on $HgCl_2$ and stably maintained the plasmid-borne antibiotic resistance.

3.10 Chromosome mobilisation in E. coli.

Strains of B. pertussis 44122/7R which carried the various plasmids were ultimately used as genetic donors, to promote mobilisation of chromosomal markers between strains of B. pertussis. However, prior to studies on B. pertussis, plasmids were first compared for their abilities to mobilise chromosomal markers between strains of E. coli. The marker chosen for use, in this comparison, was Str^R but before comparing mobilisation of Str^R by R68.45, RP1 and RP1::Tn501, it was necessary to transfer the plasmids to a suitable host strain (JC3272) which carried a high level of streptomycin resistance. This was a simple procedure with R68.45 since the original host (J53-1) was Str^S which allowed direct plasmid transfer to JC3272 with selection on NA containing kanamycin, carbenicillin and streptomycin. However, since the original hosts of RP1 and RP1::Tn501 - JMP502 and JMP543 respectively - exhibited a low level of streptomycin resistance, the plasmids were transferred initially to E. coli K12, a Str^S prototroph. Transconjugants were selected on ECMA containing kanamycin and carbenicillin following washing of the bacteria in saline. This procedure selected for plasmid-carrying E. coli K12 transconjugants on the basis that auxotrophic donors failed to grow on ECMA while prototrophic K12 transconjugants grew on ECMA in the presence of the antibiotics. Plasmids RP1 and RP1::Tn501 transferred at high frequency (10^{-2} to 10^{-1} per recipient). Single colony transconjugants were confirmed as being E. coli K12 by their ability to grow, upon subculture, on ECMA containing the selective antibiotics but being sensitive to streptomycin. From the K12 transconjugants, a single colony donor was used to transfer the plasmids to JC3272. By selecting on nutrient agar containing streptomycin, kanamycin and carbenicillin high frequency plasmid transfer to JC3272 was

Table 21 - Comparison of the ability of plasmids to mediate Str^R mobilisation in E. coli. Plasmid transfer and cma frequencies are as defined in Materials and Methods. Transconjugants and recombinants of E. coli K12 were selected on ECMA containing selective antibiotics. Spontaneous mutation rates of donor and recipients to prototrophy and Str^R was $< 6.9 \times 10^{-9}$. In all cases, the recipient was E. coli K12.

Donor (plasmid)	Exp. No.	plasmid transfer frequency	mean transfer frequency (SEM)	cma frequency	mean cma frequency (SEM)
JC 3272	1	2.62×10^{-1}	2.85×10^{-1}	2.32×10^{-5}	3.16×10^{-5}
(RP1)	2	3.09×10^{-1}	($\pm 1.19 \times 10^{-1}$)	4.0×10^{-5}	($\pm 1.25 \times 10^{-5}$)
JC 3272	1	2.7×10^{-1}	5.91×10^{-1}	1.57×10^{-6}	2.52×10^{-6}
(RP1::Tn 501)	2	6.3×10^{-1}	($\pm 1.75 \times 10^{-1}$)	3.94×10^{-6}	($\pm 7.2 \times 10^{-7}$)
	3	8.71×10^{-1}		2.05×10^{-6}	
JC 3272	1	6.36×10^{-2}	9.74×10^{-2}	8.42×10^{-7}	2.85×10^{-6}
(R68 · 45)	2	1.37×10^{-1}	($\pm 2.14 \times 10^{-2}$)	1.6×10^{-6}	($\pm 1.65 \times 10^{-6}$)
	3	9.18×10^{-2}		6.13×10^{-6}	

obtained. Thus, JC3272 donors carrying plasmids RP1, RP1::Tn501 and R68.45 were now available for use in comparing the relative ability of each plasmid to promote chromosome mobilisation in E. coli.

Mobilisation of Str^R by plasmids R68.45, RP1 and RP1::Tn501 was compared by transferring the marker from the E. coli JC3272 transconjugants to E. coli K12 with selection on ECMA containing kanamycin and carbenicillin, for plasmid transfer, and on ECMA containing streptomycin (200µg ml^{-1}) for Str^R mobilisation (Table 21). Since Tn7 encodes streptomycin resistance in E. coli (Barth et al., 1976) the ability of plasmid RP4::Tn7 to mobilise a chromosomal marker was compared to that of R68.45 using nalidixic acid resistance which is present in E. coli J53-1. Plasmid carrying transconjugants of the JC3272 recipient strain were selected on NA containing kanamycin, carbenicillin and streptomycin while Nal^R recombinants were selected on NA containing nalidixic acid and streptomycin (Table 22).

Mobilisation of the Str^R marker was mediated most efficiently by RP1 while R68.45 and RP1::Tn501 were approximately 10-fold less efficient (Table 21). All three plasmids yielded transconjugants at a high frequency.

In experiments to compare mobilisation of the J53-1 Nal^R chromosomal marker, RP4::Tn7 was found to be slightly more efficient than R68.45 (Table 22), although the frequencies were generally lower than those obtained with the other plasmids during mobilisation of Str^R (Table 21). Plasmid transfer frequencies were similar in all cases. Thus, all the plasmids were shown to be capable of promoting mobilisation of Str^R or Nal^R chromosomal markers between strains of E. coli.

Table 22 - Comparison of plasmid ability to mobilise Nal^R between strains of E. coli. Transconjugants and recombinants were selected on NA containing selective antibiotics. Plasmid transfer and cma frequencies are as defined in Materials and Methods. Spontaneous mutation rates of donor and recipient to Str^r and nal^R were $< 5.8 \times 10^{-10}$.

Donor (plasmid)	Recipient	Exp. No.	plasmid transfer frequency	mean transfer frequency (SEM)	cma frequency	mean cma frequency (SEM)
J53-1	JC3272	1	2.56×10^{-1}	5.54×10^{-1}	8.16×10^{-8}	7.18×10^{-8}
(R68,45)		2	8.53×10^{-1}	($\pm 2.98 \times 10^{-1}$)	6.21×10^{-8}	($\pm 9.7 \times 10^{-9}$)
J53-1	JC3272	1	4.64×10^{-1}	3.95×10^{-1}	8.13×10^{-7}	4.79×10^{-7}
(RP4::Tn7)		2	3.27×10^{-1}	($\pm 6.8 \times 10^{-2}$)	1.45×10^{-7}	($\pm 3.34 \times 10^{-7}$)

3.11 Effect of antigenic modulation on plasmid transfer to B. pertussis.

Previous workers mated E. coli donors with B. pertussis recipients in the presence of 10mM $MgSO_4$ in the mating medium (Weiss and Falkow, 1982). To determine if high concentrations of $MgSO_4$ had an effect on plasmid transfer frequency, the plasmids RP1, RP1::Tn501 and RP4::Tn7 were transferred from E. coli JC3272 to B. pertussis BPT2 in the presence or absence of 20mM $MgSO_4$ (Table 23). The presence of $MgSO_4$ in the mating medium had no effect on the transfer of any of the plasmids from E. coli to B. pertussis. To determine if B. pertussis recipients mated with E. coli donors in the presence of $MgSO_4$ were more resistant to the inhibitory effect of fatty acids excreted by the donor, the viable count of B. pertussis in matings carried out in the presence of $MgSO_4$ was compared to the viable count of B. pertussis on control plates without the addition of the E. coli donor. No differences in viable count were observed in the presence of $MgSO_4$, suggesting that E. coli did not have an inhibitory effect on B. pertussis.

3.12 Visualisation of plasmid DNA in agarose gels.

In addition to confirming the presence of plasmids in E. coli and B. pertussis by screening for plasmid encoded resistance, it was possible to visualise plasmid DNA in agarose gels. Using the boiling method of Holmes and Quigley (1981), whole plasmids were visualised following plasmid isolation from E. coli (Figure 3) and from B. pertussis (Figure 4). The isolation procedure was satisfactory for use with E. coli, but was not as suitable for plasmid isolation from B. pertussis. Plasmid DNA was digested with the restriction enzymes EcoRI and HindIII. Plasmids obtained from E. coli digested well, yielding the 13 and 25 Md fragment characteristic of R68.45 or RP1 digestion. With RP4::Tn7, digestion yielded additional bands, due to the presence of restriction sites within Tn7 (Figure 3). Unfortunately digestion

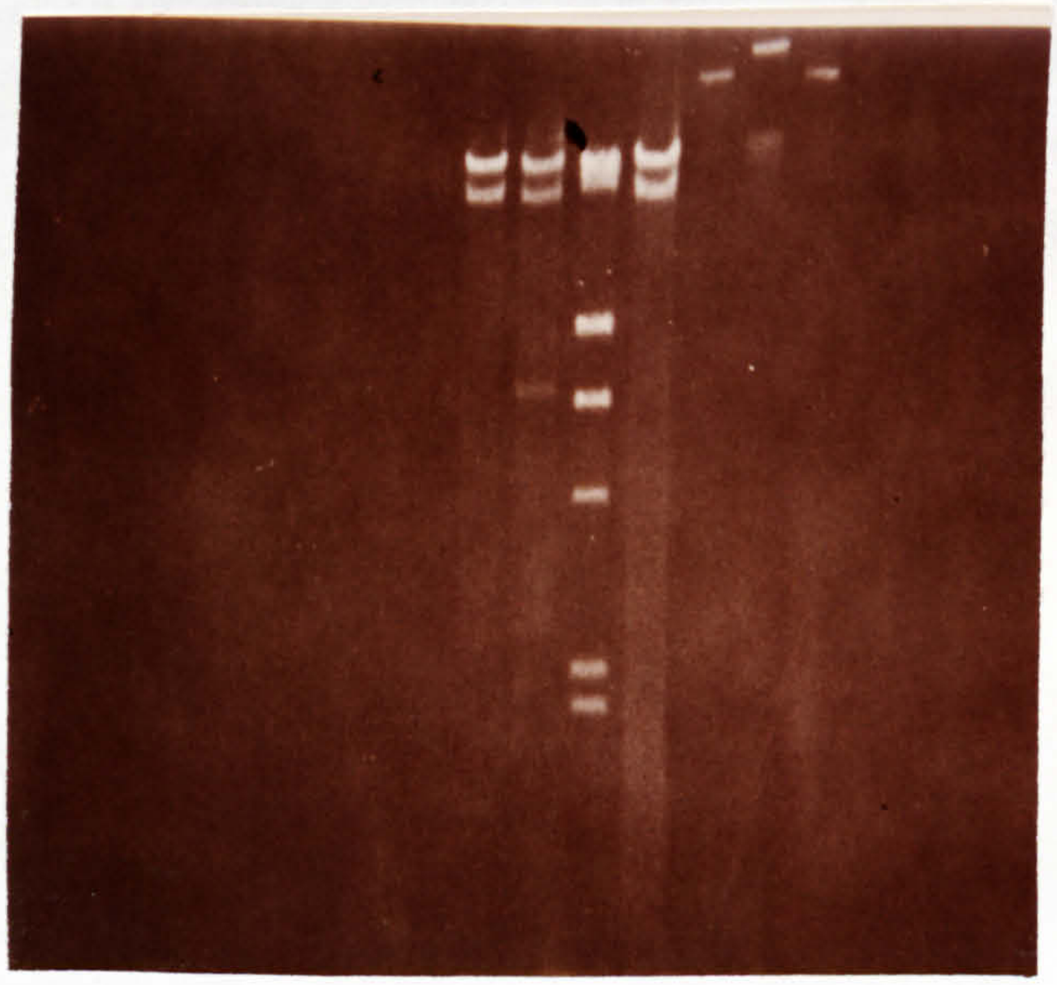
Table 23 - Effect of 20mM $MgSO_4$ in the BGA mating medium on plasmid transfer from E. coli to B. pertussis. Transconjugants were obtained on BGA containing selective antibiotics. Spontaneous mutation frequencies of the donor and recipient to Nal^R and Str^R were $< 2.9 \times 10^{-10}$. Transfer frequencies are as defined in Materials and Methods. Results are means of two observations. Values in brackets are recipient viable counts.

Donor E. coli (plasmid)	Recipient B. pertussis	plasmid transfer frequency $MgSO_4$ present	$MgSO_4$ absent
JC 3272 (RP1 :: Tn501)	BPT 2	3.30×10^{-5} (4.95×10^9)	1.58×10^{-5} (2.71×10^9)
JC 3272 (RP1)	BPT 2	8.43×10^{-4} (6.02×10^9)	1.13×10^{-3} (1.8×10^9)
JC 3272 (RP4::Tn7)	BPT 2	1.26×10^{-6} (1.55×10^9)	9.34×10^{-7} (4.28×10^9)
JC 3272 (R68:45)	BPT 2	1.3×10^{-3} (3.6×10^9)	9.3×10^{-4} (1.82×10^9)
none	BPT 2	- (9.6×10^9)	- (1.16×10^{10})

Figure 3 : Visualisation of whole plasmids from E. coli and plasmids digested by restriction enzymes HindIII and EcoRI in agarose gels.

Tracks 1 to 4. EcoRI and HindIII digestion of RP1, ~~RP1::Tn501~~ RP4::Tn7, λDNA and R68.45.

Tracks 5 to 7. Whole plasmids RP1, ~~RP1::Tn501~~ RP4::Tn7 and R68.45.



1 2 3 4 5 6 7

Figure 4 : Visualisation of whole plasmids, prepared from B. pertussis, in agarose gels.

Tracks 3, 2, 1 R68.45, RP4::Tn7, RP1.


3 2 1
chromosomal fragments.

of plasmid DNA from B. pertussis, which should be modified at HindIII sites, did not allow visualisation of restriction fragments in agarose gels.

3.13 R-plasmid mediated chromosome mobilisation in B. pertussis.

3.13.1 Antibiotic resistance

Initial experiments to mobilise a chromosomal marker in B. pertussis used plasmid R68.45 to mediate transfer of the Str^R marker from 44122/7R. The recipient strain chosen was Taberman R1. Although plasmid transfer occurred at high frequency, masking any mobilisation which may have occurred. A similar effect was observed with plasmid RP1::Tn501. A suitable donor/recipient system was observed with plasmid RP1::Tn501. A suitable donor/recipient system was therefore required which would reduce the levels of spontaneous mutation to selective antibiotics. Mobilisation of the 44122/7R Str^R marker was therefore studied using, as recipients, a phosphonomycin resistant mutant of Taberman and nalidixic acid resistant mutants of Tohama (BPN6) and Taberman (BPT2). Donor strains of B. pertussis carrying plasmids were tested for resistance to phosphonomycin, as were each of the three resistant mutants available (P1, P2 and P3) and the Taberman parent strain (Table 24). Mutant P3 was found to be the most resistant strain (MIC > 50µg ml^{-1}). Thus P3 was chosen as a recipient strain in plasmid transfer and chromosome mobilisation experiments (Table 25). Plasmids RP1::Tn501, RP1 and R68.45 were used to attempt mobilisation of the Str^R marker. Although plasmid transfer occurred at relatively high frequency, the frequency of spontaneous mutation of the donor to phosphonomycin resistance was also found to be high, preventing adequate detection of mobilisation of the donor Str^R marker.

The use of nalidixic acid resistant strains of B. pertussis as recipients

Table 24 - Characterisation of phosphonomycin resistant B. pertussis strains

+ - growth - no growth.

Strain	Growth in the presence of the following phosphonomycin concentrations :-		
	1	10	50
p 1	+	+	-
p 2	+	+	-
p 3	+	+	+
Taberman	+	-	-
44122/7RP	+	-	-

was found to be more suitable for studies on chromosome mobilisation. Transfer of R68.45 was similar using either BPN6 or BPT2 as recipients (Table 26). RP1::Tn501 and RP4::Tn7 transfer frequencies were higher than R68.45. R68.45 mobilised the donor Str^R marker at a level approximately 1000-fold higher than the spontaneous mutation frequency regardless of the recipient strain used. RP1::Tn501 failed to mediate mobilisation of the Str^R marker to either of the Nal^R recipients but RP4::Tn7 mediated mobilisation of the marker at high frequency, approximately 100-fold greater than the cma using R68.45.

3.13.2 Mobilisation of prototrophic markers.

Single colony transconjugants of BPN6 (R68.45) and BPN6 (RP1::Tn501), obtained in the previous experiments, were used as genetic donors to mobilise nutritional markers from the prototrophic BPN6 donor to an auxotrophic recipient which had been isolated as previously described. The recipient used was the glycine auxotroph of CSP1. Selection for plasmid-carrying transconjugants was carried out on BGA containing kanamycin, carbenicillin and streptomycin, to which the recipient was resistant, and selection for mobilisation of the donor prototrophic Gly^+ marker was carried out on CSM containing streptomycin. To test for linkage between the Gly^+ marker and the Nal^R marker, co-inheritance of the donor Nal^R was determined by selecting for recombinants carrying this marker on CSM containing nalidixic acid and streptomycin. Using BPN6 donors it was not possible to obtain mobilisation of the Gly^+ marker. However recombinants were obtained which were Nal^R. Since only prototrophs will grow on CSM, any Nal^R recombinant must also inherit the Gly^+ marker, indicating that linked transfer of Gly^+ and Nal^R has occurred. R68.45 transferred plasmid-borne markers at a low frequency to CSP1 while RP1::Tn501 transferred at higher frequency (Table 27).

Table 25 - Plasmid transfer and mobilisation of Str^R to a phosphonomycin resistant strain of B. pertussis . Spontaneous mutation rate of donor 44122/7R to phosphonomycin resistance was 7.38×10^{-6} and spontaneous mutation rate of P3 to Str^R was 6.9×10^{-8}. Plasmid and cma frequencies are as defined in Materials and Methods. Results are the mean of at least two observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	cma frequency (SEM)
44122/7RP (R68.45)	Taberman P3	9.93×10^{-4} ($\pm 2.82 \times 10^{-5}$)	1.19×10^{-4} ($\pm 2.43 \times 10^{-5}$)
44122/7RP (RP1)	Taberman P3	6.25×10^{-4} ($\pm 1.14 \times 10^{-4}$)	9.8×10^{-5} ($\pm 7.32 \times 10^{-6}$)
44122/7RP (RP1:: Tn501)	Taberman P3	3.7×10^{-4} ($\pm 9.61 \times 10^{-5}$)	9.2×10^{-5} ($\pm 1.21 \times 10^{-5}$)

Table 26 - Plasmid transfer and mobilisation of chromosomal Str^R between B. pertussis strains using Nal^R recipients.

Transfer and cma frequencies are as defined in Materials and Methods. Transconjugants and recombinants were selected on BGA containing antibiotics. Spontaneous mutation frequencies of donor and recipient to Nal^R and Str^R were $< 8.5 \times 10^{-10}$.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	cma frequency (SEM)
44122/7RP (R68:45)	BPN 6	5.45×10^{-5} ($\pm 1.2 \times 10^{-5}$)	1.83×10^{-7} ($\pm 5.7 \times 10^{-8}$)
44122/7RP (RP1::Tn 501)	BPN 6	2.98×10^{-3} ($\pm 9.83 \times 10^{-4}$)	$< 1.6 \times 10^{-10}$
44122/7RP (RP4::Tn7)	BPN 6	8.76×10^{-4} ($\pm 1.32 \times 10^{-4}$)	7.93×10^{-6} ($\pm 1.95 \times 10^{-6}$)
44122/7RP (R68.45)	BPT 2	8.03×10^{-5} ($\pm 1.3 \times 10^{-6}$)	7.43×10^{-8} ($\pm 3.2 \times 10^{-8}$)
44122/7RP (RP1 :: Tn501)	BPT 2	1.59×10^{-3} ($\pm 2.45 \times 10^{-4}$)	$< 6.0 \times 10^{-9}$
44122/7RP (RP4::Tn 7)	BPT 2	9.23×10^{-4} ($\pm 1.9 \times 10^{-4}$)	7.15×10^{-6} ($\pm 8.7 \times 10^{-7}$)

Table 27 - Mobilisation of a nutritional marker (Gly^+) and antibiotic resistance (Nal^R) between strains of B. pertussis

Plasmid transfer and cma frequencies are as defined in Materials and Methods. Transconjugants and Nal^R recombinants were selected on BGA containing antibiotics. Prototrophic recombinants were selected on CSM containing streptomycin. Spontaneous mutation rate of the recipient to gly^+ was 1.9×10^{-9} and to Nal^R 6.4×10^{-10}. Spontaneous mutation rates of donor and recipient to Str^R and Nal^R was $< 6.3 \times 10^{-10}$. Results are the means of two observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	cma frequency (SEM)
BPN 6 (R68:45)	CSP1 (Gly^-)	1.64×10^{-6} ($\pm 8.2 \times 10^{-7}$)	2.13×10^{-9} (for Nal^R) ($\pm 7.8 \times 10^{-10}$)
			1.06×10^{-9} (for Gly^+)
BPN 6 (RPN::Tn501)	CSP 1 (Gly^-)	2.48×10^{-3} ($\pm 6.5 \times 10^{-4}$)	8.6×10^{-10} (for Nal^R)
			8.6×10^{-10} (for Gly^+)

As an alternative, a marker from 44122/7R transconjugants was studied using the threonine requiring auxotroph of BPN6 as recipient. Three plasmids were compared for their ability to mobilise the Thr^+ marker from 44122/7R donors (Table 28). RP1 transferred to BPN6 (Thr^-) at high frequency while R68.45 and RP1::Tn501 transferred at a lower frequency. Mobilisation of Thr^+ chromosomal marker from 7R donors was found to be mediated most efficiently by RP1. Cma frequencies produced by RP1::Tn501 and R68.45 were lower and doubt must be cast on the ability of RP1::Tn501 and R68.45 to mobilise this marker due to the high spontaneous mutation rate of the recipient to Thr^+ which was found to be 2.7×10^{-6}.

Mobilisation of Nal^R and a prototrophic marker from another donor, BPT2 was also examined. Plasmid-carrying transconjugants of BPT2 were obtained in the antigenic modulation experiment (Table 23). As a suitable recipient for Nal^R mobilisation 44122/7R was used, since it was Str^R and showed no resistance to nalidixic acid. Plasmids used to mediate mobilisation of the Nal^R marker were R68.45, RP1::Tn501 and RP4::Tn7, the last named having mediated mobilisation of the 44122/7R Str^R marker at high frequencies (Table 26). However, it was only possible to use this plasmid if the transposon did not encode streptomycin resistance, as it does in E. coli. Therefore, the BPT2/RP4::Tn7 donor strain was checked for its ability to grow on streptomycin, and was found to be sensitive. It was subsequently used as a genetic donor in experiments using a Str^R recipient. Transfer of plasmids from BPT2 to 44122/7R was found to occur at high frequency (Table 29). Mobilisation of the Nal^R marker occurred at a frequency between 10^{-8} and 10^{-7} with R68.45 and RP1::Tn501, but with RP4::Tn7 the frequency of mobilisation was increased 100 to 1000 fold.

Co-transfer of Nal^R and a prototrophic nutritional marker was studied using the glycine and tryptophan - requiring auxotrophs of CSP1. These strains, being streptomycin resistant, were suitable recipients. Recombinant

Table 28 - Mobilisation of Thr^+ between strains of B. pertussis

Transfer and cma frequencies are as defined in Materials and Methods. Transconjugants were selected on BGA containing antibiotics. Recombinants were selected on CSM containing nalidixic acid. Spontaneous mutation rate of the recipient to prototrophy was 2.7×10^{-6}. Spontaneous mutation rate of donor and recipient to Nal^R and Str^R was $<6.9 \times 10^{-9}$. Results are the means of at least two observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	cma frequency (SEM)
44122/7R (RP1)	BPN 6 (Thr^-)	1.96×10^{-3} ($\pm 5.42 \times 10^{-4}$)	2.39×10^{-5} ($\pm 8.63 \times 10^{-6}$)
44122/7R (RP1::Tn 501)	BPN 6 (Thr^-)	8.42×10^{-4} ($\pm 1.21 \times 10^{-4}$)	2.78×10^{-6} ($\pm 7.54 \times 10^{-7}$)
44122/7RP (R68·45)	BPN 6 (Thr^-)	5.21×10^{-4} ($\pm 7.6 \times 10^{-5}$)	7.38×10^{-7} ($\pm 2.12 \times 10^{-7}$)

Table 29 - Mobilisation of Nal^R between strains of B. pertussis.

Plasmid transfer and cma frequencies are as defined in Materials and Methods. Transconjugants and Nal^R recombinants were selected on BGA containing antibiotics. Spontaneous mutation rates of donor and recipient to resistance to the selective antibiotics was $< 4.2 \times 10^{-10}$. Results are the means of at least two observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	cma frequency (SEM)
BPT 2 (R68·45)	44122/7R	2.89×10^{-2} ($\pm 5.6 \times 10^{-3}$)	5.67×10^{-8} ($\pm 1.8 \times 10^{-8}$)
BPT 2 (RP1 :: Tn 501)	44122/7R	1.1×10^{-1} ($\pm 5.0 \times 10^{-3}$)	4.01×10^{-8} ($\pm 8.0 \times 10^{-9}$)
BPT 2 (RP4:: Tn 7)	44122/7R	3.2×10^{-2} ($+1.6 \times 10-2$)	3.82×10^{-5} ($\pm 1.6 \times 10^{-6}$)

prototrophs were selected on CSM containing streptomycin, while recombinants carrying the mobilised Nal^R marker were selected on CSM containing streptomycin and nalidixic acid. Plasmids transferred to CSP1 (Trp^-) at a high frequency in all cases whilst mobilisation of the Nal^R marker was mediated at high frequency by R68.45 and RP4::Tn7 and at a lower frequency by RP1::Tn501 (Table 30).

In all cases the cma frequency of the Nal^R marker was markedly higher than the spontaneous mutation frequency of the recipient. It was not possible to evaluate mobilisation of the Trp^+ marker, due to a high spontaneous mutation of the recipient to Trp^+ (Table 30). Using the CSP1 (Gly^-) auxotroph as recipient, plasmid transfer again occurred at high frequency. The spontaneous mutation frequency of CSP1(Gly^-) to Gly^+ was low, about 10^{-10}, which allowed selection of recombinants carrying the mobilised Gly^+ marker at frequencies of about 10^{-5} with R68.45 and RP4::Tn7, but at less than 10^{-8} with RP1::Tn501 (Table 30). Interestingly, mobilisation of the Nal^R marker also occurred with R68.45 and RP4::Tn7 when selection was made for prototrophic Nal^R recombinants. Roughly half of the prototrophic recombinants were Nal^R with RP4::Tn7 and roughly one fifth with R68.45. These results suggest linkage of the Nal^R and Gly^+ markers.

3.14 Plasmid transfer and chromosome mobilisation to an avirulent B. pertussis mutant.

B. pertussis strain BP347, a Tn5-induced transposon insertion mutant which lacks the ability to produce haemolysin (Hly^-) F-HA, Pertussis toxin and dermonecrotic toxin, was used as a recipient to determine if it was possible to restore haemolytic activity from a donor strain, 44122/7R by mobilising the Hly^+ genes. Plasmids used in this study were RP1, RP1::Tn501 and RP4::Tn7 (Table 31). Plasmid transfer was lower in these crosses than

Table 30 - Co-transfer of Nal^R and nutritional markers between strains of B. pertussis. Transconjugants were selected on BGA containing kanamycin, carbenicillin and streptomycin. Prototrophic recombinants were selected on CSM containing streptomycin and prototrophic recombinants resistant to nalidixic acid were selected separately on CSM containing streptomycin and nalidixic acid. Spontaneous mutation rate of CSP.1 to Trp$^+$ was 2.7×10^{-6} and to Nal^R was 4.1×10^{-10}. Spontaneous mutation rate of CSP 1 to Gly$^+$ was 3.8×10^{-9} and to Nal^R was 2.7×10^{-10}. Spontaneous mutation rate of the donor to Str^R was 3.5×10^{-10}. Frequencies are as defined in Materials and Methods. Results are means of at least two observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	Nal^R Cma frequency (SEM)	Gly$^+$ prototrophic Cma frequency (SEM)
BPT 2 (RP4 :: Tn7)	CSP 1 (Trp$^-$)	7.54×10^{-3} ($\pm 1.1 \times 10^{-3}$)	5.84×10^{-7} ($\pm 5.6 \times 10^{-8}$)	ND
BPT 2 (R68.45)	CSP 1 (Trp$^-$)	1.50×10^{-2} ($\pm 6.2 \times 10^{-3}$)	2.82×10^{-7} ($\pm 4.7 \times 10^{-8}$)	ND
BPT 2 (RP1:: Tn 501)	CSP 1 (Trp$^-$)	4.27×10^{-2} ($\pm 7.1 \times 10^{-3}$)	8.29×10^{-9} ($\pm 1.1 \times 10^{-9}$)	ND
BPT 2 (RP4:: Tn 7)	CSP 1 (Gly$^-$)	5.27×10^{-2} ($\pm 1.0 \times 10^{-3}$)	4.8×10^{-6} ($\pm 7.5 \times 10^{-7}$)	8.0×10^{-6} ($\pm 4.2 \times 10^{-7}$)
BPT 2 (R68·45)	CSP 1 (Gly$^-$)	7.06×10^{-2} ($\pm 2.0 \times 10^{-3}$)	1.15×10^{-6} ($\pm 3.1 \times 10^{-7}$)	9.8×10^{-6} ($\pm 6.1 \times 10^{-7}$)
BPT 2 (RP1:: Tn 501)	CSP 1 (Gly$^-$)	1.32×10^{-1} ($\pm 3.5 \times 10^{-3}$)	7.19×10^{-9} ($\pm 9.8 \times 10^{-10}$)	8.0×10^{-8}

Table 31 - Plasmid transfer and mobilisation of Str^R to a Tn5-induced transposon insertion mutant of B. pertussis. Spontaneous mutation rate of donor and recipient to resistance to selective antibiotics was $< 3.5 \times 10^{-10}$. Transconjugants were selected on BGA containing kanamycin, carbenicillin and nalidixic acid. Recombinants were selected on BGA containing streptomycin and nalidixic acid. Results are means of three observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)	Str^R cma frequency (SEM)
44122/7RP (R68:45)	BP 347	2.9×10^{-5} ($\pm 3.7 \times 10^{-6}$)	4.9×10^{-8} ($\pm 9.8 \times 10^{-9}$)
44122/7R (RP1::Tn501)	BP 347	6.3×10^{-6} ($\pm 1.4 \times 10^{-6}$)	$< 10^{-9}$
44122/7R (RP4::Tn7)	BP 347	9.23×10^{-4} ($\pm 1.2 \times 10^{-4}$)	7.15×10^{-6} ($\pm 8.7 \times 10^{-7}$)

was obtained previously with other recipients and no Hly^+ colonies were obtained from a total of about 1000 transconjugant colonies examined for haemolytic activity from each plasmid. However, mobilisation of the donor streptomycin resistance marker occurred. RP4::Tn7 was most efficient at mobilising this marker, while R68.45 mobilised at about a 100-fold lower frequency. RP1::Tn501 failed to mediate mobilisation of the Str^R marker. No Str^R recombinants were haemolytic.

3.15 Plasmid transfer and chromosome mobilisation between B. pertussis and B. parapertussis.

Initial attempts to mobilise chromosomal markers from B. pertussis to the closely related B. parapertussis used, as a selective mechanism, the inability of Phase I B. pertussis to grow on nutrient agar. Donor B. pertussis were transconjugants of 44122/7R containing plasmids R68.45, RP1::Tn501 or RP1. Although some degree of plasmid transfer was apparent, control plates indicated that spontaneous mutation of B. parapertussis to Str^R was very high, thus obscuring any mobilisation which may have been taking place. A high frequency of spontaneous resistance to carbenicillin was also observed although resistance to kanamycin did not appear. In addition, a high frequency of spontaneous mutation of the donor to Gna^+ (ability to grow on nutrient agar) was also observed. In order to overcome spontaneous mutation of the donor to Gna^+ the selection medium was changed to MacConkey Agar, containing kanamycin and tetracycline or streptomycin to select for Str^R mobilisation. A single colony of B. parapertussis which could grow on MacConkey Agar and was sensitive to streptomycin, kanamycin and carbenicillin was isolated by testing 30 colonies grown on unsupplemented MacConkey Agar for their resistance. This colony was used as the recipient B. parapertussis.

All plasmids in the donor B. pertussis 44122/7R strains transferred to

Table 32 - Plasmid transfer and chromosome mobilisation of Str^R from *B. pertussis* to *B. parapertussis* with selection of recombinants and transconjugants on nutrient agar. Control plates show spontaneous mutation rate of donor to ability to grow on nutrient agar (Gna^+) and mutation rates of recipient to selective antibiotics. Transfer and recombination frequencies are as defined in Materials and Methods. Results are means of two observations.

Donor (plasmid)	Recipient *B. parapertussis*	plasmid transfer frequency (SEM)	Str^R *Cma* frequency (SEM)
44122/7R (RP1::Tn501)	10520	3.06×10^{-4} ($\pm 6.9 \times 10^{-5}$)	4.03×10^{-4} ($\pm 8.2 \times 10^{-5}$)
44122/7R (RP1)	10520	6.8×10^{-2} ($\pm 9.5 \times 10^{-3}$)	1.23×10^{-2} ($\pm 3.7 \times 10^{-3}$)
44122/7RP (R68:45)	10520	9.1×10^{-3} ($\pm 1.2 \times 10^{-3}$)	9.78×10^{-3} ($\pm 1.0 \times 10^{-3}$)

Control plates			
mutation rate of *B. pertussis* donor to Gna^+	mutation rate of recipient to Cb^R	mutation rate of recipient to Str^R	mutation rate of recipient to to Km^R
9.15×10^{-4}	2.36×10^{-3}	9.29×10^{-4}	$< 7.0 \times 10^{-7}$

B. parapertussis at high frequency and R68.45 transferred at a frequency about 5 times that of RP1 (Table 33). By contrast, mobilisation of the Str^R marker was mediated at the highest frequency by RP1.

Although use of MacConkey Agar was suitable for selection of B. parapertussis recombinants, an alternative selection mechanism, which used BGA for selection, was used. This was due mainly to problems associated with the selection of B. parapertussis variants on MacConkey Agar. Antibiotic sensitivity tests (Table 8) showed B. parapertussis 10520 to be resistant to chloramphenicol (10µg ml^{-1}). The frequency of mobilisation of the Nal^R marker from BPT2 donors to B. parapertussis was compared by selection of recombinants on BGA containing chloramphenicol and nalidixic acid. This method of selection was compared to selection on nutrient agar containing chloramphenicol and nalidixic acid. The plasmids used in these studies were R68.45, RP1::Tn501 and RP4::Tn7 (Table 34). Transfer frequencies of the plasmids differed depending on the selection medium used. RP4::Tn7 was more efficient at promoting chromosome mobilisation. Plasmid transfer frequency, of both plasmids, was higher when transconjugants were selected on BGA, but the frequency of mobilisation was marginally improved by recombinant selection on NA.

3.16 Transposon mutagenesis in B. pertussis.

By introducing plasmids into B. pertussis which failed to replicate and carried a transposon, the transposon could be delivered to the bacterial chromosome causing insertion mutations which, in previous reports were detected by the loss of a biological property (Weiss and Falkow, 1983). Two plasmids which were suitable delivery vehicles of transposons to B. pertussis pUW964 and pUW942 (described in Materials and Methods, Section 2.1.1). They were used, in this work, to deliver Tn5 and Tn501 respectively, to the chromosome

Table 33 - Plasmid transfer and chromosome mobilisation of streptomycin resistance from B. pertussis to B. parapertussis, with selection on MacConkey agar. Spontaneous mutation rates of donor and recipient to selective antibiotic resistance were $< 5.1 \times 10^{-10}$. Results are means of two observations. Transfer frequencies are as defined in Materials and Methods.

Donor (plasmid)	recipient B. parapertussis	plasmid transfer frequency (SEM)	cma frequency (SEM)
44122/7RP (R68.45)	10520	3.15×10^{-3} ($\pm 7.8 \times 10^{-4}$)	3.9×10^{-7} ($\pm 5.7 \times 10^{-8}$)
44122/7R (RP1)	10520	5.9×10^{-4} ($\pm 8.1 \times 10^{-5}$)	4.06×10^{-6} ($\pm 6.3 \times 10^{-7}$)
44122/7R (RP1:: Tn501)	10520	2.58×10^{-3} ($\pm 9.6 \times 10^{-4}$)	2.69×10^{-7} ($\pm 5.1 \times 10^{-8}$)

Table 34 - Transfer of plasmids, and chromosome mobilisation of Nal^R from B. pertussis to B. parapertussis with selection on BGA and NA. Plasmid transfer and cma frequencies are as defined in Materials and Methods. Spontaneous mutation rates of donors and recipient to antibiotic resistance were $< 2.9 \times 10^{-10}$. Results are the means of at least two observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)		cma frequency (SEM)	
		on BGA	on NA	on BGA	on NA
BPT 2 (RP4:: Tn7)	10520	6.22×10^{-3} ($\pm 5.8 \times 10^{-4}$)	2.55×10^{-4} ($\pm 3.1 \times 10^{-5}$)	1.88×10^{-7} ($\pm 7.2 \times 10^{-8}$)	1.17×10^{-6} ($\pm 8.1 \times 10^{-7}$)
BPT 2 (R68:45)	10520	9.52×10^{-5} ($\pm 1.1 \times 10^{-5}$)	2.24×10^{-3} ($\pm 7.2 \times 10^{-4}$)	3.65×10^{-8} ($\pm 9.1 \times 10^{-9}$)	4.51×10^{-7} ($\pm 9.8 \times 10^{-8}$)
BPT 2 (RP1:: Tn501)	10520	4.82×10^{-3} ($\pm 6.7 \times 10^{-4}$)	3.21×10^{-4} ($\pm 8.4 \times 10^{-5}$)	6.11×10^{-8} ($\pm 1.9 \times 10^{-8}$)	1.92×10^{-7} ($\pm 4.7 \times 10^{-8}$)

of B. pertussis BPT2. Since the original host E. coli strain of pUW964 was Nal^R, which precluded use of nalidixic acid as the selective antibiotic, the plasmid was transferred to JC3272, via an intermediate Str^S host (K12) since the original host strain of this plasmid was also Str^R (Table 35). The plasmids transferred at high frequencies between E. coli strains. Using JC3272 carrying pUW964 as donor and B. pertussis BPT2 as recipient single colony transposon insertion mutants were obtained by selection on BGA containing kanamycin and nalidixic acid. It was found that the initial concentration of kanamycin used (10µg ml^{-1}) for selection of transconjugants was inhibitory to growth. By selecting on 5µg ml^{-1} kanamycin, single colonies of B. pertussis expressing Tn5 kanamycin resistance were capable of growth. (Table 36). Transposon transfer frequency was low, but was still about 1000 times the spontaneous mutation frequency. However, it was not possible to study these mutants further as, upon restreaking on kanamycin, they failed to grow. Plasmid pUW942, carrying Tn501 was transferred directly from its original Nal^S host to B. pertussis BPT2, by selection on BGA containing $HgCl_2$ and nalidixic acid (Table 37). After the plasmid transferred, 50 colonies which grew in the presence of $HgCl_2$ were tested for loss of kanamycin and carbenicillin resistance, which would indicate loss of the plasmids. None of the colonies tested were resistant to kanamycin and carbenicillin but continued to exhibit resistance to mercury, indicating that all carried Tn501 and were transposon insertion mutants. The 50 colonies were then tested for their nutritional characteristics, by streaking on CSM. None of the colonies grew on CSM, indicating that all carried auxotrophic mutations. However, further characterisation of nutritional requirements by streaking on CSM amino acid 'pool' plates failed to show auxotrophic requirements as all mutants grew on all of the amino acid pools. All mutants were tested for their haemolytic ability on BGA. All colonies were haemolytic as was the parent BPT2.

Table 35 - Transfer of the chimeric plasmid pUW 964 to E. coli JC3272 via K12 intermediate, with selection on ECMA and NA.

HB 101 reversion frequency to prototrophy was $< 6.0 \times 10^{-8}$, K12 reversion to selective antibiotic resistance was $< 7.2 \times 10^{-8}$. JC 3272 reversion to kanamycin and carbenicillin resistance was $< 3.8 \times 10^{-10}$. Transfer frequencies are defined in Materials and Methods. Results are means of 3. observations.

Donor (plasmid)	Recipient	plasmid transfer frequency (SEM)
HB101 (pUW 964)	K12	1.8×10^{-2} ($\pm 1.2 \times 10^{-3}$)
K12 (pUW 964)	JC 3272	9.35×10^{-4} ($\pm 1.1 \times 10^{-4}$)

Table 36 - Transfer of the suicide plasmid pUW 964 from E. coli to B. pertussis Transposon-containing recipients were selected on BGA containing nalidixic acid and kanamycin. Spontaneous mutation of recipient to Km^R was $< 9.5 \times 10^{-10}$, spontaneous mutation of donor to Nal^R was $< 1.7 \times 10^{-10}$. Transposon inheritance frequency is defined as the number of Km^R recipients divided by total number of recipients.

Donor (plasmid)	Recipient	Expt. no	transposon inheritance frequency	mean inheritance frequency (SEM)
E. coli JC3272	B. pertussis	1	4.07×10^{-7}	4.15×10^{-7}
(pUW 964)	BPT 2	2	3.79×10^{-7}	
		3	4.65×10^{-7}	$(\pm 2.58 \times 10^{-8})$

Transposon-induced mutagenesis by $Tn5$ in B. parapertussis was also studied (Table 38). By selecting for B. parapertussis 10520 transposon insertion mutants on SSA supplemented with BSA (0.05%) and containing chloramphenicol and kanamycin, pinhead colonies grew. Transposition apparently occurred at high frequency and colonies of B. parapertussis produced a distinctive brown halo. However, it was not possible to restreak the colonies on BGA containing low levels of antibiotics due to overgrowth of the donor strain carried over from the original selection plates.

3.17 Restriction modification in B. pertussis.

Chromosomal DNA from several strains of B. pertussis and B. parapertussis was digested with the restriction enzymes HindIII and EcoRI. Digestion patterns were visualised by ethidium bromide staining in agarose gel electrophoresis (Figure 5). DNA was found to be susceptible to digestion with EcoRI but not HindIII. The activity of the HindIII was checked by digestion of methylated and non-methylated bacteriophage λ (lambda) DNA, and was found to be capable of digesting non-methylated DNA but not methylated DNA, while EcoRI digested both types. Therefore, it appeared that B. pertussis and B. parapertussis DNA was methylated at sites of HindIII digestion.

3.18 Bacteriophage studies.

3.18.1 Induction of a lysogenic bacteriophage from B. pertussis.

The possibility that virulent strains of B. pertussis may harbour a lysogenic bacteriophage which, upon lytic infection of B. parapertussis, can cause a change of serotype has already been considered (See Introduction, Section 4.2). B. pertussis strain 134 was studied to determine if such a bacteriophage could be detected either by spontaneous release, or by induction from the lysogenic

Table 37 - Transfer of the suicide plasmid pUW942 from E. coli to B. pertussis. Transposon containing recipients were selected on BGA containing mercuric chloride and nalidixic acid. Spontaneous mutation of recipient to Hg^R was $< 4.1 \times 10^{-10}$ while spontaneous mutation of the donor to Nal^R was $< 6.1 \times 10^{-10}$. Transposon inheritance frequency is the number of Hg^R recipients divided by total number of recipients.

Donor (plasmid)	Recipient	Exp. No.	transposon inheritance frequency	mean inheritance frequency (SEM)
E. coli UW937 (pUW942)	B. pertussis BPT 2	1	4.0×10^{-6}	4.15×10^{-6}
		2	3.27×10^{-6}	
		3	5.19×10^{-6}	($\pm 5.6 \times 10^{-7}$)

Table 38 - Transfer of suicide plasmid pUW 964 from E. coli to B. parapertussis by selection on SSA containing chloroamphenicol . Transposon transfer frequency is defined as the number of Km^R B. parapertussis recipients divided by total number of recipients. Spontaneous mutation frequency of the recipient to Km^R was $< 3 \times 10^{-9}$, while spontaneous mutation of the donor to Cm^R was $< 6.2 \times 10^{-10}$.

Donor (plasmid)	Recipient	transposon transfer frequency	mean transfer frequency (SEM)
E. coli	B. parapertussis	2.1×10^{-2}	1.84×10^{-2}
HB101 (pUW 964)	10520	1.57×10^{-2}	$(\pm 2.6 \times 10^{-3})$

Figure 5 : Digestion by restriction enzymes EcoRI and HindIII of chromosomal DNA from B. pertussis and B. parapertussis.

Tracks 1 and 2. B. parapertussis 10520 DNA digested with EcoRI (Track 1) and HindIII (Track 2).

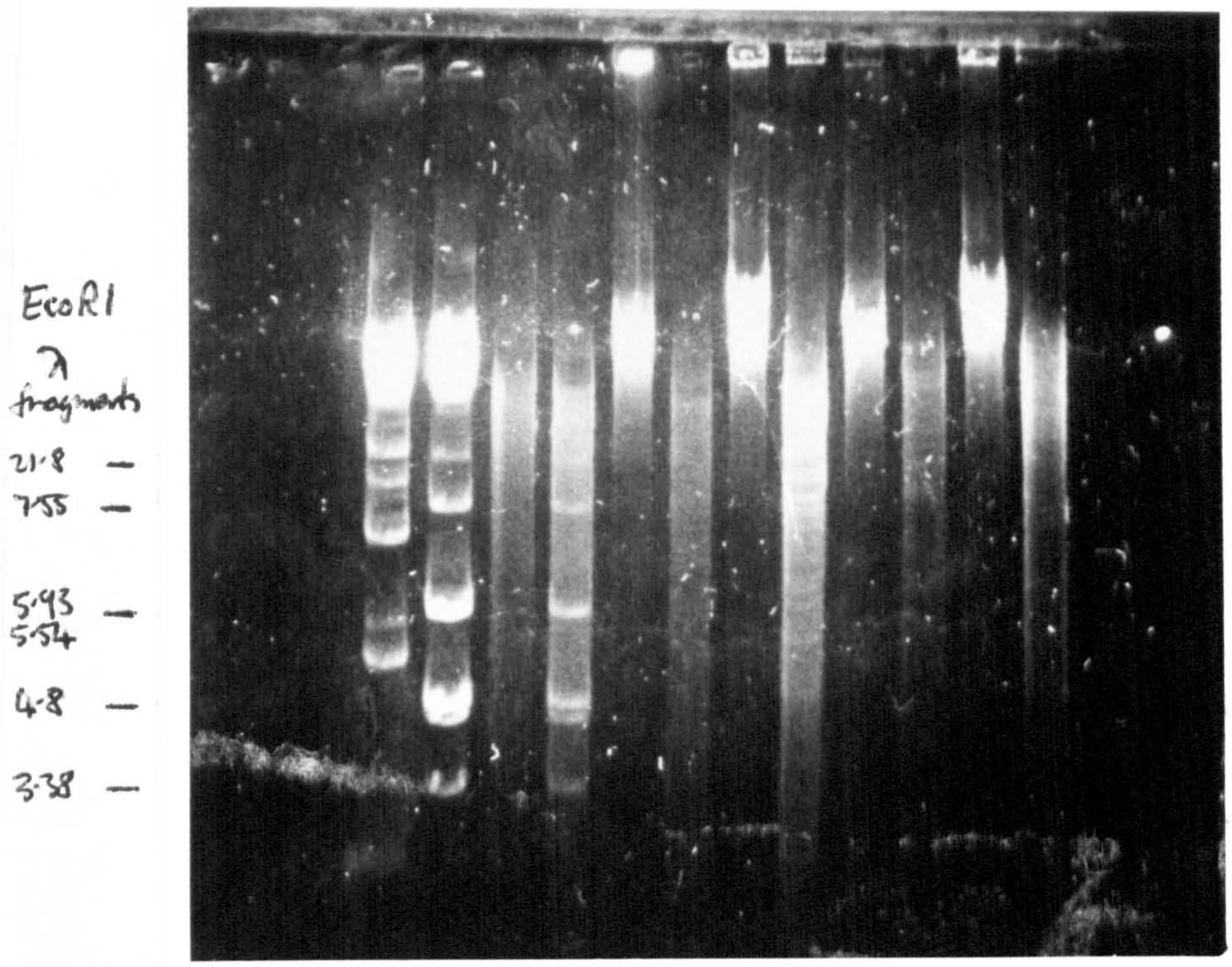
Tracks 3 and 4. B. pertussis 44122/7R DNA digested with EcoRI and HindIII respectively.

Tracks 5 and 6. 18334 DNA digested as above.

Tracks 7 and 8. L84 Phase I DNA digested as above.

Tracks 9 and 10. Methylated λ phage DNA digested as above.

Tracks 11 and 12. Non-methylated λ phage DNA digested as above.


EcoR1
λ
fragments
21·8
7·55
5·93
5·54
4·8
3·38
12 11 10 9 8 7 6 5 4 3 2 1

state using mitomycin C. A repeat of the experiment of Lapaeva et al. (1981) was carried out, in which varying concentrations of mitomycin C were added to cultures of B. pertussis 134. Phage lysate (see Materials and Methods, Section 2.2.10) was added to B. parapertussis indicator bacteria on which any phage present should be seen as a lytic or lysogenic plaque. However, no plaques were visible on the B. parapertussis lawn, suggesting that there was no release of a bacteriophage from B. pertussis either spontaneously or following mitomycin C treatment. It was not possible to use B. pertussis as the indicator organism due to its inability to grow in the overlay medium.

3.18.2 Replication of bacteriophage Mu in B. pertussis.

Bacteriophage Mu cts 62 , a temperature-sensitive derivative of Mu, which forms viable phage particles at non-permissive temperatures (42°C) was introduced into B. pertussis 44122/7R by conjugation. The Mu prophage was delivered to B. pertussis on plasmid RP4 and transconjugants were selected on BGA containing streptomycin, kanamycin and carbenicillin (Table 39). To determine if B. pertussis was capable of supporting the lytic cycle of bacteriophage growth, 100ml of an exponential culture of B. pertussis 44122/7R (RP4::Mucts62) grown at 37°C was further incubated at 39°C or 42°C for 1-2h. Culture supernate was examined for the presence of phage by adding 100µl of each of a series of 10-fold dilutions of the lysate to an indicator organism - in this case E. coli K12. The phage and bacterial suspensions were incorporated in 3ml agarose overlays. Following incubations, plaques were visible on the E. coli lawn (Table 40) indicating that Mu had been induced via the lytic cycle in B. pertussis which was induced at 39°C and at 42°C. About 1 x 10^4 viable phage particles were produced per ml of B. pertussis culture.

Table 39 - Transfer of plasmid RP4 :: Mu cts 62 from E. coli to B. pertussis by selection on BGA. Plasmid transfer frequency is as defined in Materials and Methods. Spontaneous resistance of donor and recipient to selective antibiotics occurred at frequencies less than 9.7×10^{-10}.

Donor (plasmid)	Recipient	Exp. No.	plasmid transfer frequency	mean transfer frequency (SEM)
E. coli	B. pertussis	1	1.0×10^{-3}	1.30×10^{-3}
J53-1	44122/7R	2	1.4×10^{-3}	$(\pm 1.5 \times 10^{-4})$
(RP4::Mucts62)		3	1.52×10^{-3}	

Table 40 - Induction of a temperature-sensitive mutant of bacteriophage Mu from B. pertussis 44122/7R (RP4::Mu cts 62) determined by plaque formation on E. coli K12 indicator bacteria in agarose overlays.

Lysate dilution	incubation temperature	plaque-forming units (p.f.u.) per ml of lysate
neat	42°C	1.21×10^4
10^{-1}	42°C	9.0×10^3
neat	39°C	1.43×10^4
10^{-1}	39°C	9.0×10^3
neat	37°C	0

3.19 Effect of plasmid presence on stimulation of neutrophils by B. pertussis.

The presence of a plasmid-encoded protein, the product of the traT gene, on the surface of E. coli cells carrying plasmid R6.5, enhances resistance of the bacterium to phagocytosis (Aquero et al., 1984). Since plasmids such as R68.45 and RP1 also encode similar transfer proteins, these plasmids were examined for their ability to enhance resistance to phagocytosis in B.pertussis. Initially, E. coli JC3272 strains carrying various plasmids were compared. In several independent observations, it was found that stimulation of phagocytes, as measured by chemiluminescence, (Allen et al., 1976) was reduced by the presence of R68.45 and RP1. Although the degree to which chemiluminescence was reduced varied between experiments, RP1 and R68.45 exhibited a similar effect in each experiment. The presence of RP1::Tn501 in E. coli was found to greatly enhance the neutrophil chemiluminescent response (Figure 6). In B. pertussis 44122/7R phagocytosis was not found to be significantly affected by plasmid presence. The presence of Tn501 on the plasmid RP1 appeared to slightly reduce the initial high level of chemiluminescence but had no effect thereafter (Figure 7). However, when B. pertussis BPT2 was studied (Figure 8), it was found that the presence of R68.45 caused a delay in neutrophil stimulation but did not have an effect on the levels of stimulation. The presence of Tn501 on the plasmid was found to enhance chemiluminescence significantly. Plasmid RP4::Tn7 was found to dramatically increase the levels of chemiluminescence with BPT2 but was not tested in 44122/7R.

Whole cell SDS-PAGE was carried out on E. coli JC3272 and B. pertussis 44122/7R strains which carried the plasmids. Any major change in protein profiles due to plasmid or transposon coded proteins would be observed. However, no change in whole cell profiles was discernible with either E. coli or B. pertussis transconjugants (Figure 9).

Figure 6 : Effect of plasmid presence on neutrophil chemiluminescent response to E. coli JC3272 wild type and E. coli JC3272 containing plasmids R68.45, RP1 and RP1::Tn501.

····· Control - no bacteria added.

—— JC3272 (RP1::Tn501)

------ JC3272

—·—·—·— JC3272 (RP1)

——— JC3272 (R68.45)

Chemiluminescence is measured in arbitrary units.

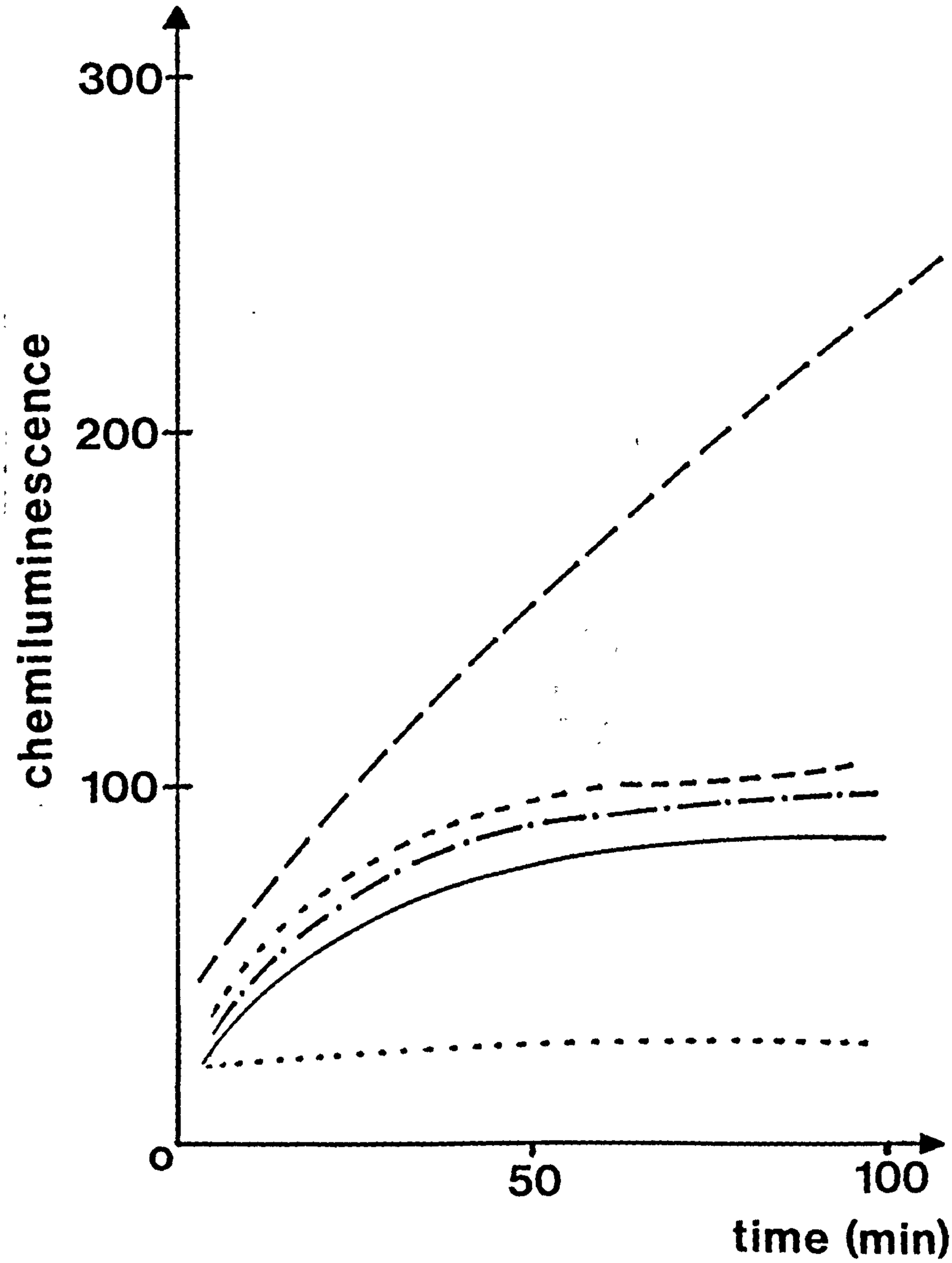

300
200
100
0
50
100
chemiluminescence
time (min)

Figure 7 : Effect of plasmid presence on neutrophil chemiluminescent response to B. pertussis 44122/7R wild type and B. pertussis 44122/7R containing plasmids RP1, R68.45 and RP1::Tn501.

..... Control - no bacteria added.

—————— 44122/7R (RP1)

------- 44122/7RP (R68.45)

—·—·—·— 44122/7R

———————— 44122/7R (RP1::Tn501)

Chemiluminescence is measured in arbitrary units.

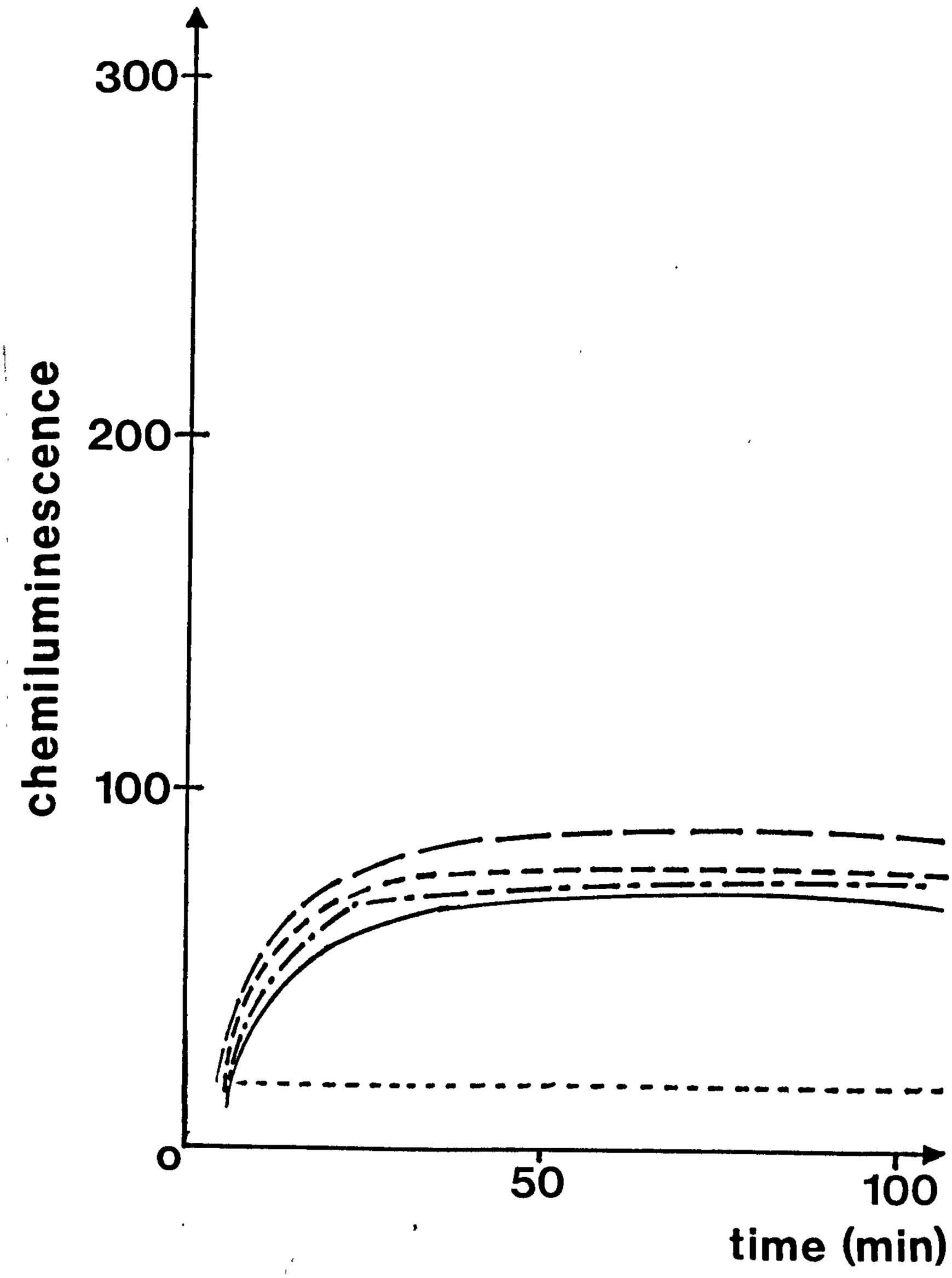
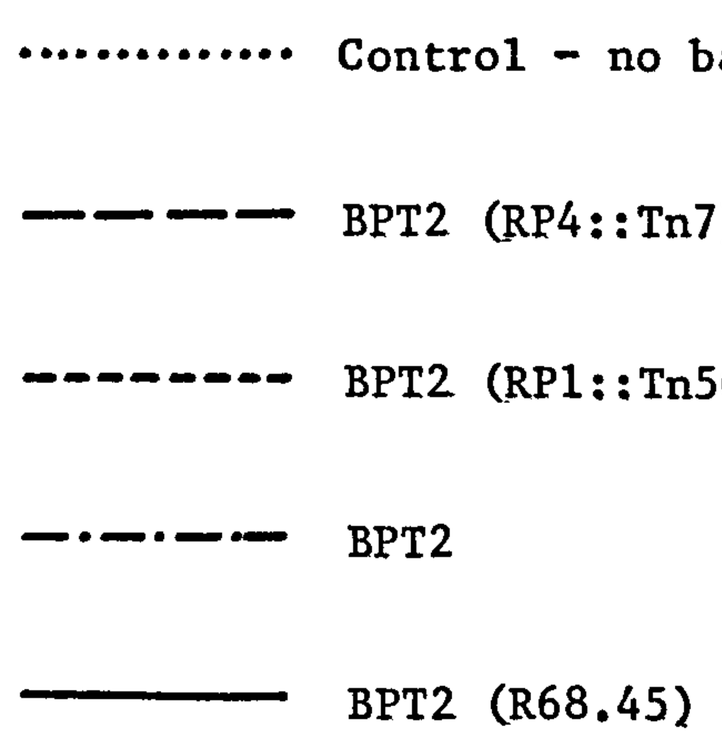

300
200
100
0
chemiluminescence
50
100
time (min)

Figure 8 : Effect of plasmid presence on neutrophil chemiluminescent response to B. pertussis BPT2 wild type and B. pertussis BPT2 containing plasmids R68.45, RP1::Tn501 and RP4::Tn7.



Chemiluminescence is measured in arbitrary units.

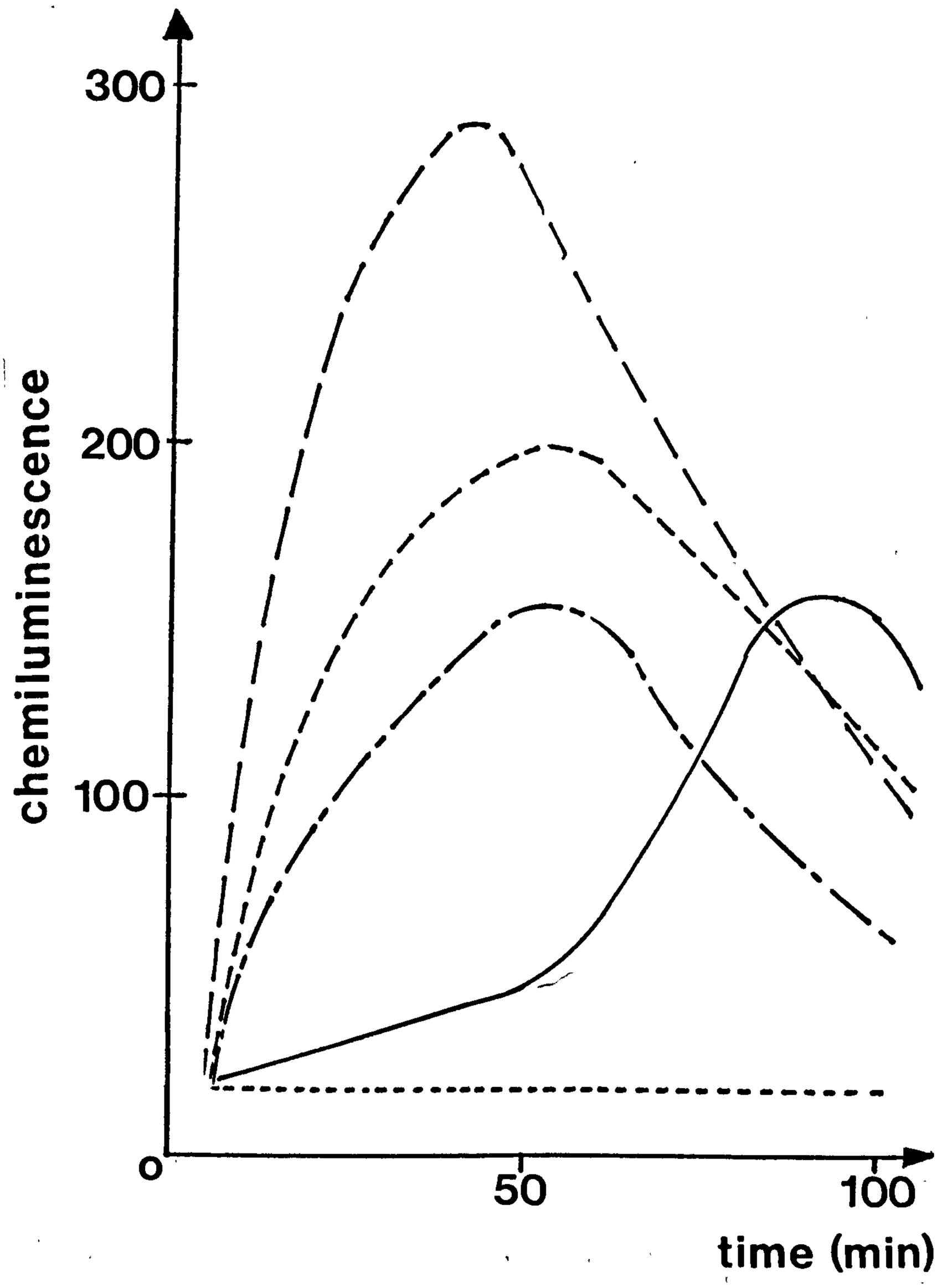
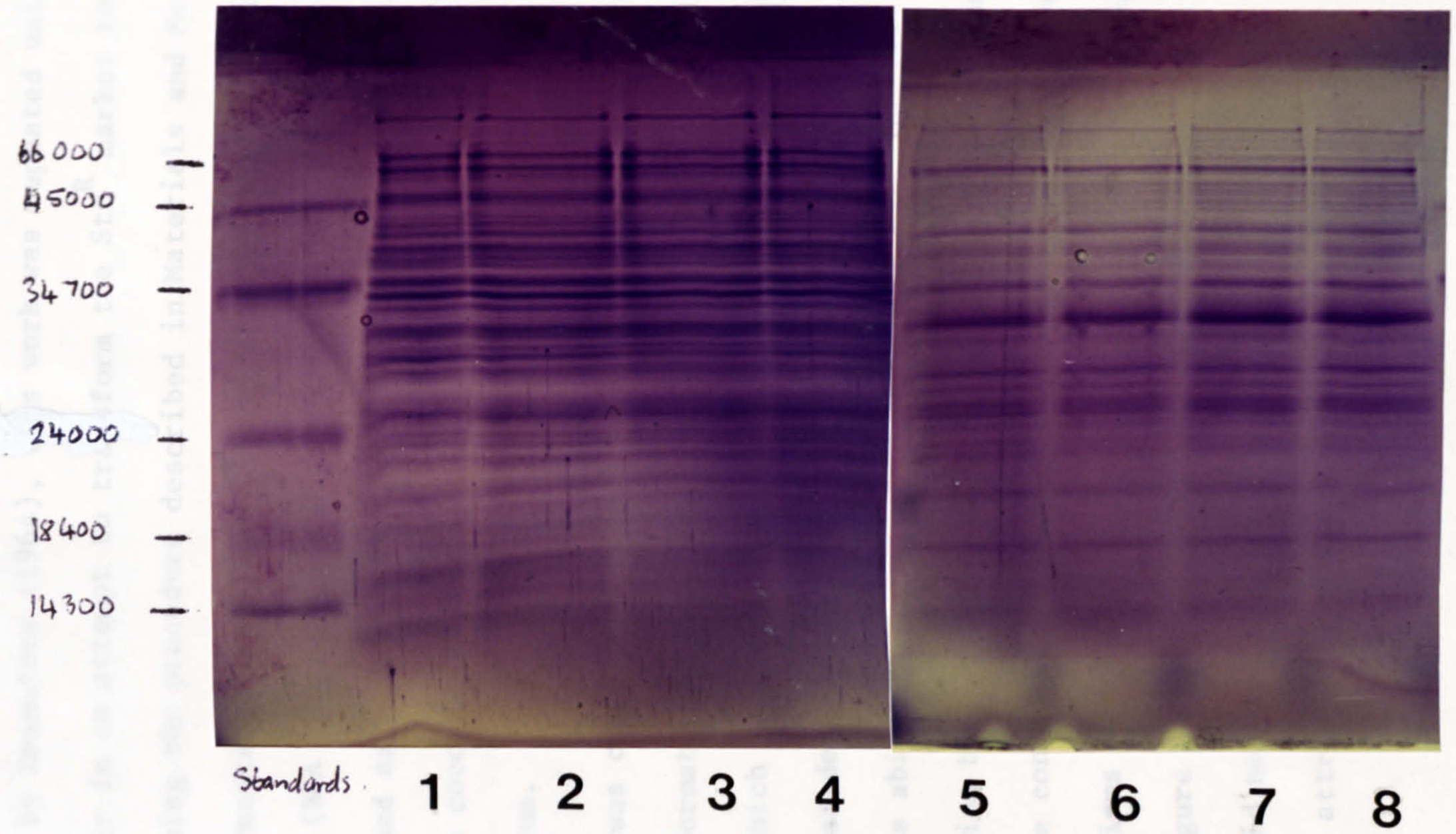

300
200
100
0
chemiluminescence
50
100
time (min)

Figure 9 : SDS-PAGE of whole cells of E. coli and B. pertussis transconjugants carrying plasmids.

Tracks 1-4 E. coli JC3272 wild type, JC3272 (R68.45), JC3272 (RP1::Tn501) and JC3272 (RP1).

Tracks 5-8 B. pertussis 44122/7R wild type, 44122/7RP (R68.45), 44122/7R (RP1::Tn501) and 44122/7R (RP1).


66000
45000
34700
24000
18400
14300
Standards
1
2
3
4
5
6
7
8

3.20 Transformation in B. pertussis

Originally observed by Branefors (1964), this work was repeated using DNA from 44122/7R as donor in an attempt to transform the Str^R marker to Str^S 44122/7S recipients. Using the procedure described in Materials and Methods, Section 2.2.4 no transformants were obtained, although a few small colonies grew on selection plates (BGA containing streptomycin). However, these colonies were not confirmed as B. pertussis since they grew on nutrient agar, appeared as Gram-positive cocci in microscopy and failed to agglutinate in anti-B. pertussis antiserum.

When transformation was carried out in the presence of $CaCl_2$, at various concentrations, no transformation was observed, although as before, several small colonies appeared which could not be confirmed as being B. pertussis. Since no transformation was detected, the B. pertussis 44122/7S recipient strain was studied for its ability to produce deoxyribonuclease (DNase), which could prevent transformation by digestion of incoming DNA. A plate assay was used and as a positive control, Staphylococcus aureus Wood 46 was tested in parallel. Both organisms were examined for DNase activity at pH 6.8, 7.4 and 7.8 (Table 41, Figure 10). Staph. aureus produced DNase at all pH values but B. pertussis did not. Therefore the non-transformability of B. pertussis could not be attributed to extracellular DNase production.

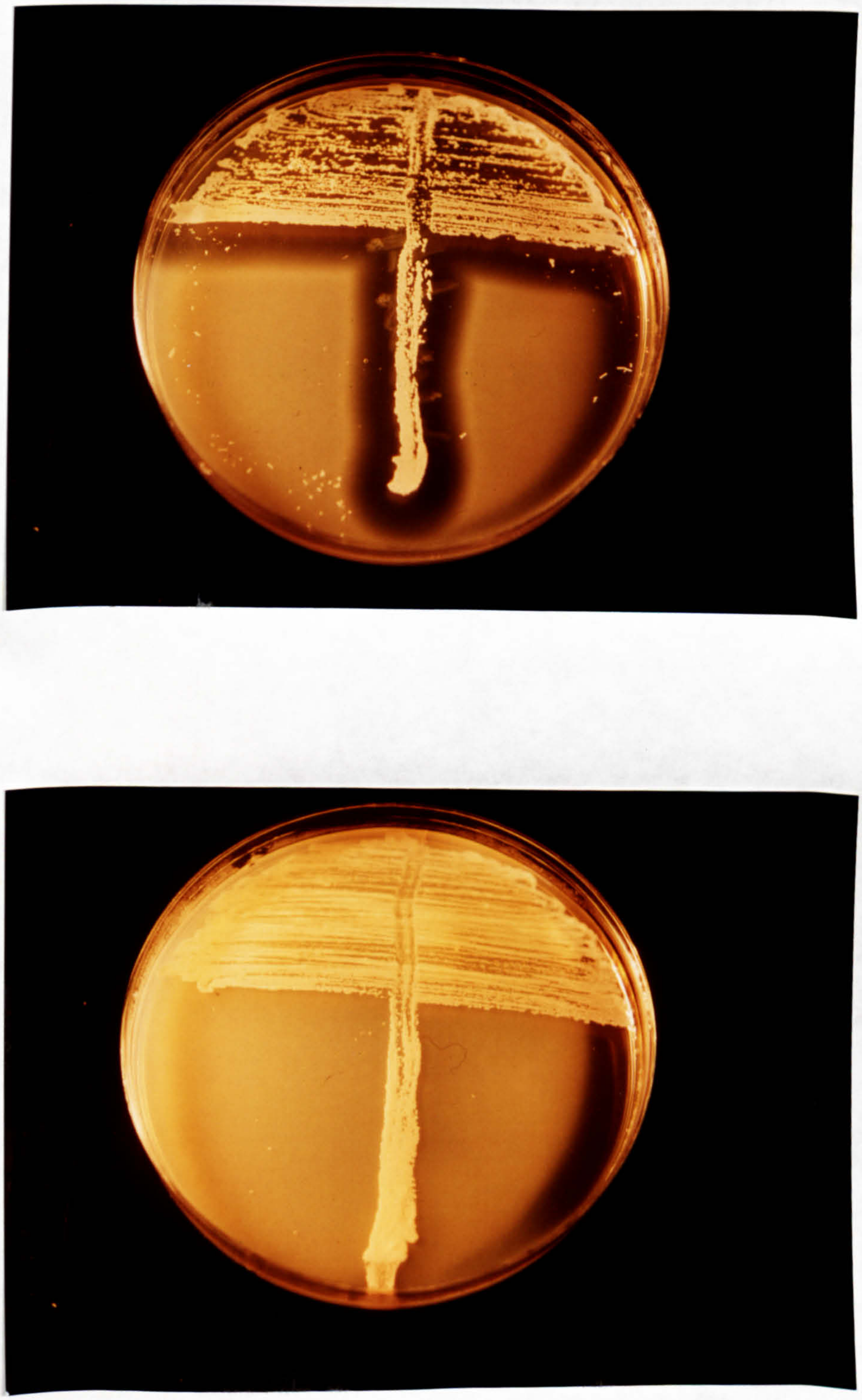
Table 41 DNase production at various pH values by Staph aureus and B. pertussis. Organisms were grown on a suitable medium containing DNA. The plates were flooded with 1N HCl giving rise to precipitation of DNA with zones of clearing in areas of enzyme activity.

Strain	Diameter (mm) of zone of clearing		
	pH 6.8	7.4	7.8
Staph aureus Wood 46	5	7	7
B. pertussis 44122/7S	0	0	0

Figure 10 : Determination of the ability of B. pertussis 44122/7S to produce extracellular DNase using a plate assay.

Top - Staph. aureus Wood 46.

Bottom - B. pertussis 44122/7S.



4. DISCUSSION

4.1 Development of a minimal medium.

Imaizumi et al. (1983) investigated the effect of the addition of various types of cyclodextrins to SSA and found methylated β-clyclodextrin to be the most effective in allowing B. pertussis growth from small inocula. The medium was called Cyclodextrin Solid Medium (CSM) and contained Casamino Acids but no BSA. In comparison with BGA, CSM was found to support growth of approximately the same number of colonies as BGA and omission of Casamino Acids led to less rapid growth of the colonies. In the current study, CSM containing Casamino Acids was found to give a viable count from small inocula comparable to that on BGA, as found by Imaizumi et al. (1983). Omission of Casamino acids, in addition to reducing colony growth, led to about a 100-fold reduction in plating efficiency, in contradiction to the findings of Imaizumi et al. (1983). Inclusion of BSA in the medium restored the plating efficiency to a high level, with an attendant increase in growth rate while inclusion of both BSA and casamino acids further increased plating efficiency, indicating that, singly, the components fail to remove all the inhibitors present. Although the effectiveness of MeβCD in overcoming inhibition may not have been as marked as claimed by Imaizumi, addition of BSA and casamino acids makes CSM a useful medium for use in genetic studies. However, for isolation of auxotrophic mutants and for their use as recipients in transfer of nutritional markers, the BSA concentration may have been sufficiently high to provide the mutants with the required amino acids. Although lower concentrations of BSA were suitable for B. pertussis growth, absence of BSA failed to support growth. Auxotrophic mutants, which were subsequently isolated, could not grow on CSM containing BSA unless the required amino acid was provided. This was also found to be the case for a Trp^- mutant which failed to grow in the presence of 0.15% BSA (Weiss and Falkow, 1983). BSA and MeβCD, in the medium, appear to have a synergistic effect on growth from small inocula. This can be explained if the effect of the MeβCD

is the same as that of BSA. Both components may neutralise growth inhibitors by interacting with fatty acids in the medium. Cyclodextrins, in common with albumin, are capable of operating as carriers of fatty acids in tissue cultures (Yamane et al., 1981). Albumin has been shown to be beneficial in the culture of the genus Leptospira (Johnson and Gary, 1962). Alternatively, MeβCD may stabilise and enhance uptake of an essential component such as glutathione by incorporation of reduced glutathione in the cavity of the MeβCD molecule. Having stabilised the component, MeβCD may enhance uptake into the bacterial cell.

Growth of B. parapertussis was found to be supported by SSA but only a small proportion of the total cell population was capable of growth on MacConkey Agar. However a single colony from MacConkey Agar gave a plating efficiency of 74%, following growth in liquid media. The growth of B. parapertussis on this medium may have selected a variant which may represent a phase-shift type of variation. Selection on SSA could be used to isolate variants of B. pertussis (Peppler, 1982) at low frequency. Variants capable of growth on nutrient agar were isolated in a similar manner (Peppler and Schrumpf, 1984a) as were variants of B. bronchiseptica by selection on Brucella agar (Peppler and Schrumpf, 1984b). It may be, therefore, that growth of B. parapertussis on MacConkey Agar selects for variants, in the cell population, which are analagous to Phase III B. bronchiseptica (Nakase, 1957) or Phase IV B. pertussis (Leslie and Gardner, 1931). These variants produced, after 4-5 days incubation, outgrowths from the colony giving rise to a star-like appearance. Since clonal proliferation at the periphery of a colony begins with a subset of all cells in the colony (Shapiro, 1984) it may be that there was a further population of variant cells within the original variant population. Further study of these properties would confirm if B. parapertussis undergoes phase-variation typical of the other members of the genus.

4.2 Mutagenesis.

Mutant isolation is an essential step in the development of a system of experimental genetics of a hitherto neglected organism in which a stock of mutants is lacking. The use of NTG in mutagenesis induces a high frequency of mutation at doses which result in little killing. Although NTG has been used in isolation of mutants of various bacterial species, the optimal conditions for mutagenesis have been mostly elucidated using E. coli K12 (Adelberg et al., 1965). However, NTG mutagenesis can result in the creation of multiple mutations and as a general rule, individual mutations should, if a suitable genetic system exists, be transferred into an isogenic background by transfer to a non-mutagenised recipient (Sparling, 1983). The method of isolation of B. pertussis antibiotic resistant mutants used NTG at an initial concentration of 200µg ml^{-1}. Mutants of B. pertussis strain Taberman and Tohama which were nalidixic acid resistant arose about 1·5 cm from the centre of the paper disk, suggesting that there was an optimal concentration of NTG at which such mutants are induced. NTG activity has been shown to be optimal at about 100µg ml^{-1} at pH 6.0, although a pH value of 7.0 reduced mutagenic activity by only 30% (Adelberg et al., 1965). It may be that an optimum NTG concentration exists at which Nal^R mutants of B. pertussis arise since Nal^R colonies were about equidistant from the centre of the disk. Treatment of Phase I B. pertussis with NTG has been found to induce a high level of phase change (C. Duggleby, Personal Communication). Similarly, NTG treatment to obtain antibiotic resistant mutants has given rise to organisms which have properties consistent with those of B. parapertussis (Kumazawa and Yoshikawa, 1978) leading to speculation that B. parapertussis is a mutant of B. pertussis which arises in nature due to the selective pressue of antibiotics. This is unlikely, however, since only erythromycin is used in antibiotic therapy, and is usually administered in the paroxysmal stage of the disease, when the

aetiological agent has all but disappeared from patients.

In this study, phase change was not induced by NTG treatment. Mutants and wild type strains all exhibited phase I properties : they agglutinated in Phase I antiserum and failed to grow on nutrient agar. However, it may be that mutant and wild type Tohama isolates lacked the 28 and 30 KD protein bands as revealed by SDS-PAGE, although subsequent work has cast doubt on this. Loss of these proteins has been associated with phase shift and loss of biological activities (Parton and Wardlaw, 1975; Wardlaw et al., 1976; Dobrogosz et al., 1979).

Isolation of B. pertussis auxotrophic mutants was carried out by a method similar to that of Miller (1972). Some strains (L84 Phase I and Phase IV strains) showed resistance to penicillin G in the auxotroph enrichment procedure. The natural resistance of some B. pertussis strains to penicillin G has been previously observed (Field and Parker, 1980), although the antibiotic was bacteriostatic at 5µg ml^{-1}. In this study, the susceptibility of each strain to penicillin G may have contributed to the differences observed in the number of presumptive auxotrophs obtained. e.g. Strain CSP1 gave rise to auxotrophs more readily than BPN6, but the parent of BPN6 (Tohama) was found to be resistant to low levels of ampicillin, reflecting reduced effectiveness of the penicillin G procedure.

4.3 Intrinsic antibiotic resistance in B. pertussis.

Relatively few studies on the susceptibility of B. pertussis to various antibiotics have been performed in vitro. The only antibiotic to which the organism is uniformly sensitive in vitro is erythromycin (Field and Parker, 1980; Bannatyne and Cheung, 1982; Zackrisson et al., 1983). Low level resistance to trimethoprim has been reported (Zackrisson et al., 1983) and

the organism has been found to exhibit resistance to some cephalosporins (Shishido et al., 1980). Mutants of B. pertussis, which would be suitable for use in genetic studies were not available at the outset of this study. The only antibiotic resistant strain available locally was the Str^R 44122/7R of Branefors (1964), although Weiss and Falkow (1982) used spontaneous Str^R and Rif^R mutants of Tohama as recipients in plasmid transfer studies. Screening of available strains for naturally-occurring antibiotic resistance yielded no suitable antibiotic resistance markers for use in genetic systems which involved transfer of plasmids encoding β-lactam resistance. However, B. parapertussis 10520 was found to be resistant to chloramphenicol, a property which was utilised in transfer of plasmids and chromosomal markers from Cm^S B. pertussis donors. Although little evidence of antibiotic resistance in Phase I strains was apparent, Phase IV strains tested showed resistance to antibiotics where the corresponding Phase I strain did not. This is in keeping with other findings which show tolerance to erythromycin and resistance to tetracycline, oleic acid, rifampicin and penicillin G to be characteristic of degraded strains (Weiss and Falkow, 1984; Peppler and Schrumpf, 1984).

4.4 Determination of optimal conditions for plasmid transfer and chromosome mobilisation in E. coli.

All matings in these experiments were initially done as filter matings which yielded high plasmid transfer frequencies. This method has been extensively used in studies on plasmid-mediated chromosome mobilisation, using R68.45 and RP1, in other species (Jacob et al., 1976; Megias et al., 1982). In this study frequency of mobilisation of the donor Nal^R marker was about 10^{-9} to 10^{-8}. Although R68.45 is a varient of R68 which has enhanced cma ability (Haas and Hollaway, 1976), mobilisation frequencies were still relatively low. Subsequently, the use of transposons to generate Hfr like donors was

studied (see below). The stability of cma ability in R68.45 has been shown to be increased by maintenance of the donors on kanamycin (Haas and Holloway, 1976), thus indicating that resistance to kanamycin can be used as a selective marker for strains which harbour a cma-proficient plasmid (Currier and Morgan, 1982). The presence of kanamycin, in this study, was found to enhance cma in E. coli and in cases where E. coli or B. pertussis transconjugants were used as genetic donors, kanamycin was included in the medium.

In order to produce a system of mating which would yield high plasmid transfer and chromosome mobilisation frequencies, and be suitable for use with B. pertussis, several types of mating systems were investigated. Filter matings produced highest transfer frequencies between E. coli strains but were prone to atmospheric contamination when used with B. pertussis. Mating of donor and recipient E. coli cells in broth containing 0.1% agar has been shown to result in a very large increase in the number of transconjugants obtained, when compared to mating in broth in the absence of agar (Groot Obbink and Ackerman, 1980). Mobilisation of the Ap^R marker increased 2000 times in the presence of 0.1% agar and it was postulated that agar facilitates formation of mating aggregates. However, the present study indicated that R-plasmid - borne antibiotic resistance was transferred at a slightly lower rate in the presence of agar in broth and the massive increases described by Groot Obbink and Ackerman (1980) were not apparent.

Plasmids such as those of the N and P groups were suspected as being better able to transfer on solid media rather than in liquids (Dennison and Baumberg, 1975). This was confirmed by Bradley et al. (1980) who demonstrated a 2000-fold increase in transfer frequency with plasmids of the N and P groups compared to broth matings, the P group plasmid studied being RP1. It appears as a general rule that matings performed on solid surfaces are much more efficient than those in liquid media (Beringer and Hopwood, 1976; Haas and Hollaway,

1976; Chatterjee, 1980). Mobilisation of chromosomal markers by R68.45 is mediated at comparable efficiency in both plate and filter matings, which are much more efficient than those in broth (Hamada et al., 1979).

In the present study, filter matings on solid media were found to mediate approximately a 50-fold increase in plasmid transfer frequency between E. coli strains compared to broth mating. Plate matings were marginally more efficient at allowing plasmid transfer than broth matings but were preferred, for use with B. pertussis, to filter matings due to their greater freedom from contamination. The great differences between transfer frequencies on solid and liquid media observed by other workers may have been due to the gentle shaking which was used in their broth matings but not in this study. This may have had the effect of disrupting mating aggregates and preventing plasmid transfer.

Another variable which may have an effect on plasmid transfer was donor: recipient ratio. Although most workers carry out plasmid transfers with donor: recipient ratios of 1:1, Chatterjee (1980), in transferring R68.45 to Erwinia chrysanthemi used donor:recipient ratios of 1:5 or 1:10, but did not comment on any differences which may have been observed. In the present study, using ratios of 1:1, 1:5 and 1:10, no effect on plasmid transfer between E. coli strains was evident but at 1:10 mobilisation of the Nal^R marker was reduced 10-fold. In another report, a 500-fold increase in mobilisation was reported as donor:recipient ratio changed from 5:1 to 1:30 (Hamada et al., 1979).

4.5 Plasmid transfer from E. coli to B. pertussis.

A problem in developing the plasmid transfer system between E. coli and B. pertussis was to allow sufficient time for mating and plasmid transfer to occur while prohibiting overgrowth of E. coli to such an extent to ensure that

mutants resistant to the selective antibiotic did not arise. This was overcome by addition of cephalexin to the mating medium (Regan and Lowe, 1977; Stauffer et al., 1983) which, while allowing B. pertussis to grow, prevented overgrowth of E. coli in overnight matings. Addition of cephalexin had no effect on plasmid transfer. Alternatively, B. pertussis cells were grown alone, before mating with E. coli for a short period of time between 4 and 6 hours. This procedure reduced the amount of E. coli growth and still allowed sufficient time for plasmid transfer to occur.

Plasmid R68.45 transferred from E. coli to B. pertussis at frequencies about 10-fold less than the lowest frequency observed for transfer between E. coli strains. This compared favourably with other inter-species transfers. For example, R68.45 transfrred from Ps. aeruginosa to Paracoccus denitrificans at frequencies of 10^{-8} to 10^{-7} per recipient (Paraskeva, 1979) and, in a situation more analagous to the present study, R68.45 transferred at about 5×10^{-3} per recipient from E. coli to Legionella pneumophila an organism in which similar problems of fastidious growth are encountered (Dreyfus and Iglewski, 1985).

Plasmid RP4 has been found to transfer at about 10^{-5} per recipient to B. pertussis from E. coli (Weiss and Falkow, 1982). In the light of these observations, the plasmid transfer system developed here compares favourably.

If the B. pertussis transconjugants obtained were to be of use in studying chromosome mobilisation, they must be capable of acting as genetic donors. This was shown to be the case, as R68.45 was found to transfer into a suitable E. coli recipient at a frequency about 100-fold lower than the transfer frequency from E. coli to B. pertussis. No attempt was made to screen for transfer of a B. pertussis chromosomal marker since it was unlikely that any transferred gene would undergo recombination in E. coli. Transfer of R68.45

between strains of B. pertussis, using Taberman R1 as recipient, was as high as the observed frequency between E. coli strains, indicating that the inter-species barrier has a major effect on plasmid transfer and expression. This may reflect the evolutionary diversity of donor and recipient restriction and modification systems. It may be that the more closely related the donor and recipient are, in evolutionary terms, the less the plasmid is susceptible to restriction and modification by the new host systems, and the species barrier becomes a less formidable obstacle.

In a similar situation to that of B. pertussis, R68.45 transferred at a frequency of 10^{-5} from L. pneumophila to E. coli while transfer to another L. pneumophila strain occurred at a 100-fold greater frequency (Dreyfus and Iglewski, 1985). A more extreme example of this was in studies on transfer of RP4 from E. coli to the gliding bacterium Myxococcus xanthus (Breton et al., 1985). RP4 transferred to M. xanthus at a frequency of 10^{-8}, but could not replicate in this organism and integrated into the host chromosome. However, RP4 transferred to another strain of M. xanthus at a frequency of 10^{-3} but again failed to replicate. This strain was known to possess a lower level or absence of restriction enzyme activity suggesting that the expression and replication of the plasmid, in distantly related species, is governed by the host restriction systems. It seems, therefore, that the ability of these plasmids to replicate in B. pertussis, E. coli and L. pneumophila, but not in M. xanthus may reflect the evolutionary divergence of restriction enzyme systems. The possibility that transfer of plasmid-borne antibiotic resistance was due to transformation was excluded due to continued transfer on medium which contained DNase. During cell to cell contact, the transferring DNA would not be susceptible to the action of DNase.

Using transconjugants of B. pertussis 44122/7R as genetic donors and Taberman R1 (Rif^R) as recipient, it was not possible to obtain mobilisation

of the donor Str^R marker. This was due to a high level of spontaneous mutation of donor and recipient to the selective antibiotics. In other Gram-negative species, Str^R and Rif^R have been shown to be linked (Bachmann, 1983; Mekalanos et al., 1979), and linkage of these markers was also reported to occur in B. pertussis (Weiss and Falkow, 1983).

4.6 Transfer of other plasmids to B. pertussis.

Plasmids RP1 and RP1::Tn501 transferred at high frequencies between strains of E. coli and transposon Tn501 was expressed in the recipient. R68.45supdnaG315 however, transferred at extremely low frequencies. This plasmid has been shown to possess enhanced cma ability in Rhizobium spp. (Ludwig and Johansen, 1980) but data on its transferability was not given. Although the plasmid transferred at low frequency to B. pertussis 44122/7R, it was not included in the range of plasmids which were used to mobilise chromosomal markers in this organism. Low transferability would create an even lower rate of mobilisation. All plasmids, including RP4::Tn7, were transferable from E. coli to B. pertussis 44122/7R using streptomycin to select for recipients. Although Tn7 encodes resistance to streptomycin in E. coli (Barth et al., 1976) selection with high streptomycin concentrations (200µg ml^{-1}) was found to prevent donor growth, although this concentration of antibiotic has been used to select for transconjugants of Caulobacter which carry Tn7 (Ely and Croft, 1982). For use of RP4::Tn7 in mobilising Str^R donor markers and employing the plasmid to mediate mobilisation to Str^R recipients, it was essential that Tn7 did not encode Str^R in B. pertussis. This was found to be the case. Expression of transposon-encoded resistance genes has been shown to be host dependent. For example, Tn5 encodes for Str^R in addition to Km^R in Rhizobium meliloti, but does not encode Str^R in E. coli (De Vos et al., 1984) since the necessary streptomycin phosphotransferase is not expressed in

E.coli (Putnoky et al., 1983). Tn5-encoded Str^R is also expressed in C. crescentus, Ps. putida and Acinetobacter calcoaceticus, but not in Klebsiella aerogenes (O'Neill et al., 1984). Recently it has been shown that Tn5 encodes Str^R in B. pertussis but not in E. coli (Weiss and Falkow, 1984). Although no similar studies on host dependent expression have been carried out with Tn7, it is possible that, as with Tn5, there may be differences in gene expression in non-enteric bacteria.

Transconjugants which carried plasmids RP1 and RP1::Tn501 were compared for their ability to mobilise chromosomal markers between strains of B.pertussis. The presence of transposons and insertion sequences has been shown to affect the ability of the plasmid to form Hfr-like donors and subsequently to promote chromosome mobilisation (Kleckner et al., 1977). The cma ability of R68.45 in A. tumefaciens could be improved by insertion of homologous Tn5 sequencies on the chromosome and in the plasmid, leading to Hfr formation and enhanced chromosome mobilising ability (Pischl and Farrand, 1983). Hfr formation was more likely to be the reason for enhanced cma, rather than R-prime formation since gene transfer occurred in a polarised manner, and R-prime plasmids would have contained chromosomal material from both sides of the Tn5 insertion site, which they did not. The insertion of Tn501 into plasmid RP1 greatly enhanced the ability of this plasmid to promote chromosome transfer in Rh. sphaeroides (Pemberton and Bowen, 1981), promoting polarised transfer from possibly two origins on the chromosome. Tn1 and Tn5 failed to enhance mobilisation and the cma enhancing ability of Tn501 may have been due to the high frequency with which Tn501 transposes, allowing formation of Hfr like donors to occur more readily (Stanisich et al., 1977). In cases where transconjugants of B. pertussis carrying RP1::Tn501 were selected on $HgCl_2$, the transposon was maintained, but plasmid-borne antibiotic resistance was lost i.e. the plasmid behaved as a suicide vehicle for delivery of Tn501 to the B. pertussis chromo-

some. A possible explanation for this is that transposition of Tn501 to the bacterial chromosome occurred but the absence of selection for plasmid maintenance caused the plasmid to disappear from the population, leaving Tn501 in the chromosome. It is therefore feasible to generate transposon insertion mutants using RP1::Tn501 as a delivery plasmid. Another explanation is that, upon selection of transconjugants on kanamycin and carbenicillin, the entire plasmid, via cointegrate formation, inserted into the chromosome. Tn501 cointegrates are efficiently resolved to final transposition products only after growth in the presence of Hg salts (Sherratt et al., 1981). If selection was carried out in kanamycin and carbenicillin, then resolution of cointegrates would not occur and the entire plasmid may have integrated into the chromosome. Selection on Hg salts alone will induce cointegrate resolution, which may leave the plasmid unable to replicate.

4.7 Effect of antigenic modulation on plasmid transfer.

Weiss et al. (1983) found that the frequency of plasmid transfer from E. coli to B. pertussis was increased by addition of 10mM $MgSO_4$ to the mating medium. This procedure may have induced antigenic modulation in the B. pertussis recipients, changing them from X-mode to C-mode (Lacey, 1960). Antigenic modulation is known to be induced at $MgSO_4$ concentrations of 10-11mM (Brownlie, et al., 1983). Antigenic modulation involves changes in cell-envelope composition (Wardlaw et al., 1976; Wardlaw and Parton, 1979) which may have had an effect on plasmid transferability if C-mode cells form conjugative aggregates more readily than X-mode cells. An alternative explanation is that, in mating of donor and recipient, fatty acids excreted by the E.coli donor had a bactericidal or inhibitory effect on X-mode B. pertussis, while C-mode cells, being resistant to fatty acid inhibition (Pollock,1949) were unaffected. Results in this study showed that mating in medium containing

$MgSO_4$ (20mM) had no effect on plasmid transferability and that no killing of B. pertussis cells by E. coli occurred.

4.8 Agarose gel electrophoresis of plasmid DNA.

Initial attempts to visualise plasmid DNA prepared from B. pertussis used the methods of Portnoy et al. (1981), Holmes and Quigley (1981) and the SCFSB (Materials and Methods, Section 2.2.2) as used in the laboratory of D. Sherratt, University of Glasgow. Although the Portnoy and SCFSB procedures were suitable for the preparation of plasmid DNA from E. coli, they were not as suitable for B. pertussis. The boiling procedure of Holmes and Quigley was highly effective when used for E. coli and plasmids prepared by this method were susceptible to digestion by restriction enzymes. However, the procedure was less suitable for use with B. pertussis. A problem was apparent at the stage of cell lysis. E. coli cells resuspended evenly in STET lysis buffer, thus giving rise to more efficient lysis by lysozyme, as indicated by the 'fluffy' pellet recovered after centrifugation. With B. pertussis, cells tended to clump in STET, despite the inclusion of Triton X-100, and were not as susceptible to lysozyme activity. The pellet following centrifugation, contained some whole cells. Thus, recovery of plasmid DNA would have been inefficient, which explains the difficulty in obtaining clearly visible bands in agarose gels.

4.9 Chromosome mobilisation in E. coli.

Although plasmids such as R68.45, RP1 and RP4 have been used widely as tools for studying the genetics of many bacterial species, the relative abilities of each plasmid, to promote chromosome mobilisation in E. coli has received little attention. In this study, the ability of plasmids RP1, RP1::Tn501 and R68.45 to mobilise Str^R in E.coli was compared. In addition, the ability of RP4::Tn7 to

mobilise Nal^R was compared with R68.45, to determine which of the plasmids was most efficient at promoting mobilisation before use in B. pertussis. The most efficient plasmids relative to R68.45, itself a mutant of R68.44 which mobilises markers at high frequencies (Haas and Holloway, 1976), were RP1 and RP4::Tn7. In other species, R68.45 has been more efficient than RP4 in promoting mobilisation e.g. in Rh. leguminosarum (Beringer et al., 1978). The presence of Tn501 on RP1, enabled mobilisation to occur in Rh. sphaeroides while RP1 itself was not fertile (Pemberton and Bowen, 1981). In this study, RP1::Tn501 failed to enhance mobilisation of the E. coli Str^R marker. Tn501 was found to insert into RP1 at many sites (Bennett et al., 1978) and displayed regional specificity. If this was true also of chromosomal insertion, then the mobilisation of Str^R may be low because none of the preferred insertion sites are close to this marker. Thus, formation of Hfr like donors may occur, but Str^R transfer occurs at low frequencies, or not at all, if the conjugation bridge is broken before transfer of the marker. A similar situation may explain the high frequency of mobilisation of the Nal^R marker which was observed using RP4::Tn7. In E. coli, Tn7 has a 'hot spot' in the chromosome into which it inserts at high frequency (Lichtenstein and Brenner, 1981). This site lies between the dnaA and ilv markers, at minute 82 on the E. coli linkage map (Barth et al., 1978). The Nal^R (gyrA) mutation lies at 48 minutes (Bachmann, 1983). Therefore, insertion of RP4::Tn7 at this position may allow a high frequency of transfer of the marker if conjugation lasts for a sufficient length of time - at least 34 minutes.

4.10 Chromosome mobilisation in B. pertussis.

Initial attempts to mobilise chromosomal antibiotic resistance markers in B. pertussis were thwarted by high spontaneous mutation rates to the selective antibiotics. However, using Nal^R recipients, mobilisation of the donor Str^R

marker occurred most efficiently with plasmids RP4::Tn7 and R68.45. In all cases, mobilisation of antibiotic resistance markers and, in subsequent experiments, prototrophic markers was mediated most efficiently by RP4::Tn7 and R68.45. The basis of high frequency mobilisation by RP4::Tn7 may be similar to that in E. coli i.e. there is a unique chromosomal 'hot spot' in the bacterial chromosome of B. pertussis, into which RP4::Tn7 forms an Hfr-like donor by a transposition-recombination event. The 'hot spot' may be close to the resistance markers under study. Such 'hot spots' have been shown to be present in other species such as C. crescentus (Ely, 1982), Vibrio spp. (Thomson et al., 1981), Xanthomonas campestris (Turner et al., 1984) and Ps. aeruginosa (Caruso and Shapiro, 1982). Transposition of Tn7, followed by plasmid integration via the homologous DNA sequences, may lead to polarised gene transfer. Differences in mobilisation frequencies for a selected marker from 100-fold to about equal by RP4::Tn7 and R68.45 may be due to mobilisation from a single site, by RP4::Tn7 while R68.45 mobilises from multiple origins (Haas and Holloway, 1978), and is less suitable for localisation of genes in linkage studies. In addition, R68.45 mobilises only short sequences of the chromosome (Hermann and Gunther, 1984). However, formation of an Hfr like donor via Tn7 transposition, similar to FP2 donors which behaved as an Hfr like donor and allowed mapping of the whole chromosome (Herrmann and Gunther, 1984), would allow a wide range of genetic studies on linkage in B. pertussis. Formation of such a donor via RP4::Tn7 transposition and mobilisation from a single origin would be a suitable system.

RP1::Tn501, although capable of transferring plasmid genes at high frequency, exhibited only low chromosome mobilisation ability, on occasions lower than that of the parent RP1. This was in contradiction to the findings of Pemberton and Bowen (1981). Tn501, in B.pertussis has been found to insert in virtually all cases tested, into a single site on the chromosome (Weiss and Falkow,1983). In Rh.sphaeroides, one or possibly two transfer origins were present upon RP1::Tn501

integration into the chromosome (Pemberton and Bowen, 1981). It may be that, in this study, Tn501 insertion is at a site distant from the selected marker, or, if it inserts at a single site, then that site may be within the selected gene, thus causing a polar mutation and non-expression of the chromosomal marker.

A satisfactory outcome of these studies was the use of CSM as a selective medium for mobilisation of donor prototrophic markers. It was possible to begin a comparison of co-transfer of prototrophic and antibiotic resistance markers and only the lack of suitable auxotrophic strains now prevents this system being used for studies on linkage of B. pertussis chromosomal genes. It was possible to compare linkage of two markers, Nal^R and Gly^+ by selection of recombinants on CSM containing nalidixic acid. In order to grow on CSM in the presence of the antibiotic, the auxotrophic (Gly^-) mutation must first be corrected, followed by transfer of the antibiotic resistance gene. Only those bacteria which have inherited both markers will grow on the selective medium. Therefore, it was possible to begin to compare the ability of cma-proficient plasmids to promote co-transfer of genes in B. pertussis. Only one other report of linkage in B. pertussis exists, in which Str^R and Rif^R were shown to be linked by co-transfer by plasmid pUW942 (Weiss and Falkow, 1983). A problem encountered in the current study was the high reversion rate of some of the auxotrophic mutants to prototrophy, which excluded their use in linkage studies. This may be overcome by generation of a series of transposon induced auxotrophic mutants which would remove the problems of multiple mutations and spontaneous reversion since only one copy of a transposon such as Tn5 would be present in the chromosome. Since transposons rarely excise spontaneously from the chromosome, auxotrophic mutations would be stable. Only one other study of mobilisation of prototrophic markers in B. pertussis has been carried out. In a less detailed report, Weiss and Falkow (1983)

mobilised a donor Trp^+ marker to an auxotrophic recipient at a frequency 1000 times the reversion frequency. Plasmid R68.45 has been used to mediate mobilisation of a Thy^+ marker in Legionella pneumophila at a frequency about 1000 times that of the spontaneous reversion rate. (Dreyfus and Iglewski, 1985).

Using this gene transfer system, correction of a Tn5-induced Hly^- (deficient in haemolysin production) mutation, in B. pertussis BP347, was attempted. Mobilisation of the donor Str^R marker occurred with RP4::Tn7 at about 100 times greater frequency than that observed with R68.45. However, no Hly^+ recombinants were observed in either case. An interesting observation was that the BP347 recipient, although resistant to kanamycin was not, as reported by Weiss and Falkow (1984), resistant to streptomycin. However, these workers reported that a mutant selected for chromosomal streptomycin resistance was resistant to much higher concentrations of antibiotic than was the Tn5 mutant. This may explain why it was possible to obtain mobilisation of a donor Str^R chromosomal marker in the presence of Tn5 in the recipient.

4.11 Chromosome mobilisation to B. parapertussis.

Inter-species transfer of chromosomal genes by plasmid R68.45 has been used to transfer Rif^R and Str^R genes from Rhizobium leguminosarum to R. meliloti (Johnston et al., 1978). Since a close phylogenetic relationship exists between B. pertussis and B. parapertussis as measured by DNA-DNA hybridisation (Kloos et al., 1978), it would be reasonable to expect that mobilisation of a B. pertussis chromosomal marker across the species barrier to B. parapertussis could occur. If this was the case, then it may be possible to obtain expression of genes involved in e.g. production of pertussis toxin in B. parapertussis.

Initial attempts to transfer plasmids and chromosomal genes from B. pertussis to B. parapertussis used as a selective mechanism, the ability of the recipient to grow on nutrient agar. However, this proved to be impractical due to the high spontaneous reversion rate of the recipient to the selective antibiotic and of the donor to Gna^+ (ability to grow on nutrient agar). This is a similar phenomenon to that observed by Peppler and Schrumpf (1984) who isolated variants of B. pertussis which were capable of growing on nutrient agar. Weiss and Falkow (1984) also observed a high rate of spontaneous phase shift in B. pertussis.

It was possible to use MacConkey Agar as a selective medium for B. parapertussis transconjugants. However, due to the low plating efficiency of B. parapertussis, and the possibility of selection of variants within the population, this system was not regarded as being satisfactory. However, using BGA containing nalidixic acid and chloramphenicol to select for B. parapertussis recombinants, it was possible to obtain expression of the B. pertussis BPT2 Nal^R marker. As in earlier experiments, plasmid RP4::Tn7 was the most efficient at promoting mobilisation of the donor marker. Using this system it may be possible to use B. parapertussis as a recipient in studies aimed at mapping the B. pertussis chromosome. Because of its less fastidious nature, isolation of B. parapertussis auxotrophs may be easier and more suitable for use as recipients in mobilisation of nutritional markers. An available selection mechanism may be to use SSA plates on which phase I B. pertussis will not grow. Such studies may allow the location of genes involved in the regulation or production of pertussis toxin.

4.12 Transposon mutagenesis in B. pertussis.

The advantage of transposon mutagenesis methods in preference to chemical mutagens have already been discussed. Although it was demonstrated that

plasmid RP1::Tn501 could be used to generate Tn501 insertion mutants of B. pertussis, two suicidal chimeric plasmids were studied for their abilities to deliver transposons. Using plasmid pUW964, small Km^R colonies were obtained which could not be further characterised. However, mutants carrying Tn501, from plasmid pUW942, failed to demonstrate plasmid borne antibiotic resistance. Weiss and Falkow (1983) found that, in some cases, it was possible to create genetic donors by insertion of the entire plasmid into the chromosome, and that Tn501 insertions generated a dominant class of thiamine - requiring auxotrophic mutants. In this study, no such genetic donors were found but generation of auxotrophic mutants may have occurred, although further characterisation of the mutants proved inconclusive.

Transposon insertion mutagenesis of B. parapertussis yielded large numbers of mutants, by selection on SSA. Although further characterisation was not possible, it would be of interest to determine, using such mutants, if there is a stepwise phase variation in this organism and to compare the properties of the mutants to those of the spontaneous variants which were capable of growth on MacConkey Agar.

4.13 Restriction modification in B. pertussis

The presence of a HindIII like restriction enzyme in B. pertussis was first reported by Greenaway (1980) and confirmed by Weiss and Falkow (1982) who demonstrated that modification of HindIII restriction sites on plasmid R388 occurred by B. pertussis. In the present study it was shown that chromosomal DNA from B. pertussis and B. parapertussis, while being susceptible to the action of EcoRI, could not be digested with HindIII. As a control for enzyme activity, HindIII failed to digest methylated λ DNA but digested non-methylated λ DNA. Thus, it appears that B. parapertussis, like B. pertussis probably contains an enzyme with HindIII-like activity and its own DNA is

protected by methylation. In accordance with the enzyme nomenclature rules of Smith and Nathans (1973), the restriction enzyme from B. pertussis has been named BpeI (Roberts, 1983).

4.14 Bacteriophage studies.

Various theories of bacteriophage induced conversion of B. parapertussis to B. pertussis have been postulated in recent years (see Introduction), although little experimental evidence exists in support of them. However, following the reports of isolation of lysogenic bacteriophage from B. pertussis by Lapaeva et al. (1980) and Mebel et al. (1981) experiments aimed at repeating their work were undertaken. None of the experiments in this study to observe formation of phage particles using Mitomycin C or to observe spontaneous lysis by viable phage particles yielded results compatible with those obtained by the previous workers. Since these studies, no further reports of phage conversion have been forthcoming.

However, B. pertussis was shown, in the present study, to be capable of supporting growth of a bacteriophage. The thermoinducible Mucts62 (Howe, 1973) inserted into plasmid RP4 (Boucher et al., 1977) was transferred by conjugation to B.pertussis and production of viable phage particles was induced by growing transconjugants in broth at 39°C or 42°C. This showed that when inserted into RP4, the Mu prophage can be introduced into B. pertussis, a species taxonomically distinct from the few enteric bacteria which form the normal host range of Mu; E. coli K12, Shigella dysenteriae (Taylor, 1963) and Citrobacter freundii (de Graaf et al., 1973). In addition, the Mu genome was expressed in B. pertussis and led to formation of plaque-forming particles on an E. coli lawn. The presence of Mu on plasmid RP4 was shown to reduce the transfer frequency of the plasmid from E. coli to R. meliloti (Boucher et al., 1977) and a similar effect occurred in transfer of RP1 (=RP4) and

RP4::Mu *cts* 62 to *B. pertussis*. This may be due, however, to different donor strains being used to transfer the two plasmids. Expression of the Mu genome and production of viable phage particles gave rise to 5×10^2 to 3×10^5 p.f.u. ml^{-1} in *Pseudomonas solanacearum* (Boucher *et al.*, 1977). This was comparable to the yield of particles produced from *B. pertussis* at 39°C or 42°C. Thus, by using a plasmid vector which has a broad host-range in Gram-negative bacteria (Datta and Hedges, 1972; Olsen and Shipley, 1973) it was possible to obtain expression of a narrow host range bacteriophage in a wide range of ecologically diverse species.

4.15 Effect of plasmids on stimulation of neutrophil chemiluminescence.

The presence of certain R-factors in *E. coli* conferred upon the organism the property of resistance to serum bactericidal activity (Fietta *et al.*, 1977). Further study with plasmid R6·5 indicated that the serum - resistance gene product of this plasmid was the *traT* protein (Moll *et al.*, 1980). The protein was subsequently found to be responsible for the enhancement of *E.coli* resistance to phagocytosis (Aguero *et al.*, 1984). Thus, it was shown that an R-plasmid gene was capable of modifying the bacterial cell surface to favour invasiveness. Since R-plasmids such as RP1 and R68.45 carry genes which allow for self-transmissibility (*tra* genes) the effect of the presence of such plasmids on phagocytosis of *E. coli* and *B. pertussis* was studied. Differences in resistance to phagocytosis, as indicated by stimulation of neutrophils, were apparent between strains. In *E. coli* JC3272, RP1 and R68.45 exhibited an ability to reduce the chemiluminescent response, an effect which was not as apparent in *B. pertussis* 44122/7R transconjugants as in BPT2 transconjugants. However, in BPT2, the presence of R68.45 may have delayed the neutrophil response. Unlike plasmid R6.5, RP4 has been shown to have no effect on *E. coli* resistance to serum (Fietta *et al.*, 1977), and appears

in correlation, to have little effect on stimulation of phagocytes. However RP1, R68 and R68.45 decrease virulence and serum resistance in P. aeruginosa PAD (Wretlind et al., 1985). These alterations do not appear to be due to a single factor such as tra gene-dependent cell surface alterations. The presence of traT on low copy number plasmids may not produce a significant reduction in phagocytosis since the traT gene cloned into low copy number vectors produces a much less well defined anti-phagocytic effect than in high copy number vectors (Aguero et al., 1984). The presence of transposons Tn501 and Tn7 caused a massive increase in chemiluminescence due to E. coli and B. pertussis BPT2. This may be due to an alteration of the cell surface by a transposon encoded product. Tn7 and Tn501 have not been characterised with respect to membrane proteins which they may produce, but Tn10 has been shown to produce a 36,000D membrane protein which was inducible in the presence of tetracycline (Levy and McMurray, 1974). Such a protein may appear in whole cell SDS-PAGE analysis, but no difference in profiles of E. coli or B. pertussis due to the presence of Tn7 or Tn501, or plasmids were observed, when compared to parent strains which did not carry plasmids.

4.16 Transformation.

Although several reports of transformation of Str^R (Branefors, 1964; Al Sallami, 1981) and prototrophic markers (Kloos et al., 1978) exist, no transformation of Str^R was obtained in the present study. Due to the nature of the experimental method, there are several stages at which contaminating organisms may be introduced. Thus, the only colonies which grew on selective media were contaminants, which casts suspicion on the previous reports. Neither the Branefors nor the Kloos studies were ever subject to independent verification by other workers and it may be that the transformation method was not suitable for use in developing a gene transfer system. This is not because B. pertussis cannot be transformed. Weiss and Falkow (1982) demonstrated transformation by plasmid DNA which had been modified at the HindIII sites. B. pertussis does not produce DNase, as demonstrated in this study.

Transformation in B. pertussis therefore remains obscure and requires further study.

4.17 Conclusions.

The components of a system which will allow transfer of B. pertussis chromosomal genes have been developed and used to demonstrate conclusively that it is possible to begin a detailed genetic study of this organism. It is now possible to isolate strains which carry selectable properties and to use them in the production of a genetic map of the B. pertussis chromosome. In addition, the use of B. parapertussis as a recipient in gene transfer may allow location of virulence-associated genes. Evidence has been found that B. parapertussis may undergo a form of phase variation, which would be worthy of further study. Very few bacteria of medical or agricultural importance have been characterised genetically, and the use of plasmids and transposons to study properties of these bacteria, such as nodulation in Rhizobium and the mechanisms of virulence of Vibrio cholerae and L. pneumophila is at an early stage. Use of such techniques in the study of B. pertussis will provide a powerful new tool in the elucidation of the mechanisms of pathogenesis of whooping cough.

APPENDICES

APPENDIX 1 - Media Preparation

Bordet-Gengou Agar

40g of dehydrated Bordet-Gengou Agar Base (Gibco Europe Ltd.) was added to 1L of distilled water containing 10% (v/v) glycerol. The medium was steamed to dissolve and sterilised by autoclaving at 15 p.s.i. for 15 min (121°C). The medium was then cooled to 50°C in a water bath, and 20% sterile defibrinated horse blood was added and well mixed. Antibiotics were added as required. The medium was poured into sterile single or triple vent 9cm plastic petri-dishes (Sterilin, Feltham, Middlesex). Bubbles were removed by brief flaming. To remove excess surface water, plates were left at room temperature overnight. Plates were stored at 4°C and used within 3 weeks of preparation.

Stainer and Scholte Medium (Stainer and Scholte, 1971).

The following are dissolved in 800ml of distilled water.

	g/l
L-glutamate (monosodium salt)	10.72
L-proline	0.24
NaCl	2.5
KH_2PO_4	0.5
KCl	0.2
$MgCl_2.6H_2O$	0.1
$CaCl_2$	0.02
Tris (hydroxymethyl-methylamine)	6.075

The pH was adjusted to 7.4 with 2.5N HCl. The volume was made up to 990ml with distilled water and autoclaved at 15 p.s.i. for 15 mins. Before use the following were dissolved in 10ml of distilled water and filter sterilized with a Millipore filter of pore size 0.45µm (Millipore S.A. Molsheim, France).

The total volume was then added to the bulk medium.

	g/l
L-cysteine	0.04
$FeSO_4$-$7H_2O$	0.1
Ascorbic acid	0.02
Nicotinic acid	0.004g
Glutathione	0.1g

Cyclodextrin Solid Medium (CSM)

This was prepared according to Imaizumi et al. (1983) using Stainer and Scholte medium as the basal medium. CSM contained 0.5g per litre of Casamino Acids (Casein Hydrolysate, Peptone No. 5, Gibco Ltd.), 1.0g per litre of Heptakis (2,6-O-Dimethyl) β-Cyclodextrin (MeβCD) obtained from Dr. Imaizumi, Teijin Institute for Biomedical Research, Tokyo, Japan and 15.0g per litre of agar (Oxoid Agar, Technical No. 3). Bovine serum albumin (Sigma) 7.5% w/v was added to final concentration of 0.05% (w/v). For use as a minimal medium, Casamino Acids were omitted and amino acid solutions (0.2% w/v) added to give a final concentration of 20µg/ml.

E. coli Minimal Agar (ECMA) (Clowes and Hayes, 1968).

	g/l
NH_4Cl	5.0
NH_4NO_3	1.0
Na_2SO_4	2.0
K_2HPO_4	3.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.1
Oxoid Agar Technical No. 3	15.0

Ingredients were added to 1L of distilled water and autoclaved at 15 p.s.i. for 15 min. 20ml of glucose 10% w/v was added before pouring. Thiamine (0.02% w/v) was added to a final concentration of 2µg ml^{-1}.

1% Casamino Acids Solution

10g Casamino Acids (Casein Hydrolysate acid, Peptone No. 5. Gibco Ltd.)

0.1g $MgCl_2.6H_2O$

0.015g $CaCl_2$

5g NaCl

The ingredients were dissolved in 980ml of distilled water and the pH adjusted to 7.0 with 2.5N NaOH. For culture storage at -70°C 20% glycerol (v/v) was added and 1ml amounts dispensed in bijoux bottles. The medium was autoclaved at 15 p.s.i. for 15 min.

Cohen and Wheeler Medium (Cohen and Wheeler, 1946)

Casamino Acids (Casein Hydrolysate acid, Peptone No. 5) (Gibco)	10g
Yeast extract (Gibco)	2.5g
Soluble starch	1.5g
$MgCl_2.6H_2O$ 25% solution	1.6ml
$CaCl_2$ 10% solution	0.1ml
$FeSO_4.7H_2O$ 1% solution	1.0ml
$CuSO_4.5H_2O$ 1% solution	0.05ml
L-arginine 0.01% solution	1.0ml
L-histidine 0.005% solution	1.0ml
KH_2PO_4	0.5g

Starch was dissolved by steaming for 30 min. The remaining ingredients were added and the total volume made up to 1L with distilled water. The pH was adjusted to 7.8 with 1N NaOH.

APPENDIX 2 - Antibiotic and amino acid screening

Oxoid multodisks

Oxoid S3 Multodisks have 8 arms which are impregnated with antibiotics at the following concentrations :

Antibiotic	Code	Concentration
Chloramphenicol	C	10µg
Erythromycin	E	10µg
Sulphafurazole	SF	100µg
Methicillin	CB	10µg
Penicillin G	P	1.5 Units
Ampicillin	PN	2µg
Streptomycin	S	10µg
Tetracycline	TE	10µg

Amino acid solutions

For selection of prototrophic recombinants and isolation of auxotrophic mutants, the following mixtures of amino acids, purines and pyrimidines (pools) were incorporated in CSM at concentrations of 20µg ml^{-1}.

Pool Number

1	2	3	4	5
6	Adenine	phenylalanine	alanine	arginine	leucine
7	Cytosine	serine		ornithine	glycine
8	Guanine	tryptophan	threonine	aspartate	isoleucine
9	Thymidine	tyrosine	methionine		histidine
10	Uracil		lysine		valine

APPENDIX 3 - Buffers for chemiluminescence studies

Hepes - buffered saline (HBS)

NaCl	8g
KCl	0.4g
$MgCl_2.6H_2O$	0.2g
$CaCl_2$	0.14g
glucose	1g
HEPES	2.388g
phenol red	10ml of 0.5% w/v solution
distilled water	1L

HEPES is N-2-hydroxyethyl piperazine N'-2-ethane sulphuric acid.

Divalent cation-free HBS-EDTA was prepared by omitting the calcium and magnesium salts and adding 0.292g of EDTA (ethylenediaminetetra-acetic acid) per litre.

HBS and HBS-EDTA were buffered to pH 7.6 with 1N NaOH.

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