Molecular Characterization of the Plasmids in *Vibrio cholerae* strain V58.

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To my Mum and Dad,

for their love and

understanding

CONTENTS

ABSTRACT

V58 is one of the prototype strains used in the genetic analysis of *Vibrio cholerae* and has been found to contain three plasmids. The largest of these corresponds to the P sex factor which except for its ability to promote low level transfer of chromosomal markers is cryptic like the other two plasmids.

The aim of this work was to characterize these three plasmids on a molecular and physical basis as well as to investigate their possible functions. The three plasmids have been identified in whole genomic DNA extracts of V58 and sized by agarose gel electrophoresis and electron microscopy. Restriction endonuclease cleavage maps of the three plasmids have been constructed and the sum of the fragment sizes agrees with other measurements: P, 68 kb; large cryptic plasmid (lcp), 34 kb; small cryptic plasmid (scp), 4.7 kb.

Most of the *Eco*RI and *Xba*I fragments of P have been cloned and the proteins encoded within these fragments analysed in both whole cells and minicells. Concerted effort has failed to clone the remaining fragments suggesting they may contain lethal functions in the absence of other regions of P. The number of proteins detected in the subclones does not account for the potential coding capacity suggesting that important regulatory regions/genes have not been cloned on the fragments. Similarly the lcp and scp have been subcloned and the proteins analysed.

Possible properties of P have been examined including resistance to serum and metal ions, but no effect could be seen comparing P^+ and P^- strains. The ability of P to transfer to different hosts and its relationship to the incompatibility type strains has been examined. P is unique in its properties and also could be shown to encode a surface

exclusion system. It has been previously reported that P plays a role in the suppression of virulence of hypertoxinogenic strains. Studies here have demonstrated that the suppression of virulence by P is due to poor colonization of the small intestine. Transposon mutagenesis of P has enabled the regions associated with transfer and surface exclusion to be mapped on the plasmid, however the region(s) responsible for suppression of virulence could not be localized.

The lcp has no phenotype except that it can also be shown to be transferable and is identical to the V plasmid described in non-01 *V. cholerae* and a 31.5 kb (21 MDal) plasmid identified in Classical strains isolated during the sixth pandemic.

A role for the scp has not been identified. It appears to be the same as the 4.5 kb (3 MDal) plasmid detected in Classical strains isolated during the sixth pandemic.

Using subclones of these various plasmids, it has been possible to examine their distribution in other *Vibrio* species by DNA hybridization. None of the plasmids were found in *Vibrio* species other than in *V. cholerae* 01 even though numerous plasmids were detected in non-01 isolates.

STATEMENT

I state that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

Eveline Bartowsky

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ABBREVIATIONS

Ap	ampicillin
ATP	adenosine 5'-triphosphate
bp	base or nucleotide pairs
Bla	gene for β-lactamase encoding Ap^R
BSA	bovine serum albumin
cpm	counts per minute
cAMP	cyclic adenosine 3', 5'-monophosphate
CAT	chloramphenicol acetyltransferase
Cm	chloramphenicol
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
eop	efficiency of plating
EtBr	ethidium bromide
Ig	immunoglobulin
kDal	kilodaltons(s)
kb	kilobase pair(s) or 1000bp
Km	kanamycin
LPS	lipopolysaccharide
MDal	megadaltons
min	minute(s)
MM	minimal medium
NA	Nutrient Agar

NB	Nutrient Broth
pfu	plaque-forming units
PAGE	polyacrylamide-gel electrophoresis
^{R}	resistance
Rif	rifampicin
RNA	ribonucleic acid
rpm	revolutions pre minute
^{S}	(superscript) sensitive
SDS	sodium dodecyl sulphate
sec	second(s)
Sm	streptomycin
Spc	spectinomycin
TB	Tryptone Broth
Tc	tetracycline
Tn	transposon
Tris	Tris (hyroxymethyl)aminomethane
UV	ultraviolet
wt	wild type
[]	designates plasmid-carrier state

Chapter 1

Introduction

1.1 Introduction

Cholera is an acute diarrhoeal disease of man and was demonstrated by Robert Koch in 1883 to be due to the bacterium *Vibrio cholerae*. *V. cholerae* is a Gram negative non-invasive pathogen with a distinctive comma shape.

The existence of Asiatic Cholera can only be traced back to 1769, prior to this India was unknown to European physicians. During the period 1769 to 1790, cholera was very prevalent in Madras, India, afflicting many thousands of people. From 1814 the history of this disease can be traced quite accurately throughout the world, even though it may have existed in many parts of Asia prior to this time without coming under notice of the Europeans.

John Snow (1854) first described the association of cholera with contaminated water (Snow, 1965). Through meticulous examination of deaths from the disease in several districts of London, Snow was able to conclude "that cholera invariably commenced with the affection of the alimentary canal" and was a result of contaminated water supplies. Snow also believed that the propogation of the material which caused cholera was similar to that of the plaque, typhoid fever and yellow fever. The first description of the organism, which is directly responsible for cholera was in 1854 by Pacini, an Italian scholar of art and professor of anatomy. This organism was later named after him as *Vibrio cholerae* (synonym *Vibrio comma*) Pacini 1854.

V. cholerae of the 01 serotype can be subdivided into two biotypes, Classical and El Tor (Feeley, 1965; Sen, 1969), both of which are capable of causing human cholera. There have been seven pandemics of cholera in recent history. The first six, up until 1961, are thought to have been a result of the Classical biotype and the seventh due to the El Tor biotype (Barua and Cvjetunovic, 1970; Kamal, 1974).

Cholera is very often a fatal disease due to severe dehydration and loss of electrolytes. Waldemar Haffkine in the late nineteenth century was the first of many to attempt to produce a vaccine against cholera. Various forms of vaccines have been tried including parentally and orally administered forms, however, parental whole-cell and toxoid vaccines have been shown to be largely ineffective at producing lasting immunity in an unprimed population (Cash *et al.*, 1974; Finkelstein, 1984). However, parenteral cholera vaccines consisting of killed organisms are somewhat protective and appear to boost an existing immunity but do not protect for any substantial period of time when examined for efficacy in Bangladesh, Calcutta and the Phillipines (Feeley and Gangarosa, 1980; Levine *et al.*, 1983).

Recent efforts have concentrated on oral vaccines which would produce local immune responses with a longer lasting immunity. Oral vaccination is considered to produce the best immunity and was classically demonstrated by Levine and coworkers (1983) in a human volunteer trial at the Center for Vaccine Development at the University of Maryland. Volunteers were fed a virulent cholera strain (in the presence of bicarbonate to reduce gastric acidity and so permit the survival of the bacterium to the stomach) and after recovery were found to be immune to rechallenge with virulent *V. cholerae*. Immunity was shown to last at least three years. An effective attenuated live vaccine is yet to be discovered. Various attempts have been made

over the years, however, all potential candidates still elicit a mild diarrhoeal response which is considered an unacceptable side effect. The mutant, Texas Star SR which is A^-B^+ (cholera toxin) due to mutagenesis with nitrosoguanadine (Honda and Finkelstein, 1979) appeared a promising candidate, however, this attenuated strain still caused diarrhoea as manifested by one to several loose movements in about 24% of the recipients (Levine *et al.*, 1984).

Black *et al.* (1987) have examined the protective efficacy of a killed whole-vibrio oral vaccine with and without the B subunit of CT in adult volunteers and shown that the combination provided 64% protection whereas the whole vibrio vaccine administered alone provided 56% protection. This killed oral vaccine has a major advantage over current live vaccines in that illnesses in vaccinees were much milder than in controls and it has been suggested that the vaccine might provide a significant level of protection if given to a population in an endemic area. Initial field trials in Bangladesh indicate a protective efficacy of 85% for the combination and 58% for the whole vibrio vaccine alone (Black *et al.*, 1987).

An alternative approach for developing an effective oral cholera vaccine is through genetic engineering to introduce genes for cholera-protective antigens into an already successful live oral vaccine such as the Ty21a mutant typhoid vaccine developed by Germanier (1984). However, the bacterial factors responsible for the efficient induction of enteric mucosal immunity and the mechanisms by which such protection is mediated are only partly understood.

1.2 Pathogenesis of cholera.

Cholera is a diarrhoeal disease caused by organisms of the 01 serotype. The induction of disease by this organism occurs through a

combination of virulence determinants. Once the *V. cholerae* bacterium has successfully passed through the gastric acid barrier of the stomach and entered the small intestine, it utilizes an array of virulence properties to overcome the hosts nonspecific defence mechanisms of the small intestine peristalsis. The final diarrhoeal response is thought to be a consequence of a series of events: motility and chemotaxis are employed to penetrate the mucus gel, proteases, nuraminidase and DNases to degrade this mucus gel, pili (such as *tcp*) and haemagglutinins facilitate adherence to the intestinal epitheleal cells and allow colonization by the bacteria to permit efficient delivery of cholera toxin (CT) and possibly other toxins.

Non-motile *V. cholerae* exhibit a notable reduction in virulence inferring that motility via the single polar flagellum is an important virulence attribute (Attridge and Rowley 1983a, b). Freter and coworkers (1981a, b, c, d) have demonstrated that motile *V. cholerae* direct themselves to the mucosal surface in response to chemotaxis. Adherence to the enterocytes of the crypts and villi and colonization by the organism are more than likely due to components of the bacterial cell such as outer membrane proteins, the flagellum, pili and the lipopolysaccharide (LPS) (Manning, 1987). Colonization of the gut by the organism is an important factor in immunizing capacity (Levine *et al.*, 1977; Cray *et al.*, 1983; Pierce *et al.*, 1988). The OmpV protein is the major outer membrane protein (Stevenson *et al.*, 1985; Pohlner *et al.*, 1986), however, it does not appear to be a colonization factor even though it is strongly immunogenic (Taylor *et al.*, 1987; Manning and Haynes, 1984; Pohlner *et al.*, 1987). Several cell-associated haemagglutins (HA) have been identified (Boothe, 1985), however, their roles are uncertain. The *tcp* pilus (toxin co-regulated pilus) has been implicated as an important colonization factor (Taylor *et al.*, 1987) and

the minor protein, TcpG, rather than the major pilin protein, TcpA, is probably the adhesin (Taylor *et al.*, 1988a). A component of the flagellum, a flagellum-associated adhesin exists which cannot be expressed in the absence of the flagellum (Attridge and Rowley, 1983c) and appears to be involved in the process of adhesion to mucosa. LPS seems to play a role in adhesion to the gut epithelium (Chitnis *et al.*, 1982) in that purified Inaba LPS significantly inhibited attachment of *V. cholerae* Inaba to rabbit mucosa and antibodies against Ogawa or Inaba LPS prevented adhesion.

1.2.1 Cholera toxin.

Cholera toxin is one of many secreted proteins of *V. cholerae* and is a member of a family of related toxins found in a number of Enterobacteriaceae (Mekalanos, 1985). This toxin is the major virulence determinants for manifestation of the disease. It is an extracellular multimeric toxin which consists of two subunits A and B in the ratio of one to five, respectively. The A subunit is 27 kDal, synthesized as a single polypeptide chain which is usually proteolytically nicked to form two disulphide-linked polypeptides, A1 (22 kDal) and A2 (5 kDal) (Gill, 1976; Mekalanos *et al.*, 1979a). The A1 subunit is the enzymatically active moiety of the toxin and is known to promote the activation of adenylate cyclase in eukaryotic cells by catalyzing the ADP-ribosylation of a GTPase regulatory component of the cyclase complex (Cassel and Pfluffer, 1978). The 5 B subunits are 11,600 daltons and display a high affinity for the cell surface receptor, ganglioside GM_1 (Gill, 1976, Cuatrecases, 1973). The genes encoding and regulating CT biosynthesis are chromosomally encoded (Mekalanos *et al.*, 1979b, Sporecke *et al.*, 1984). The structural genes for the two subunits A and B of CT are present as the *ctxAB* operon and have been mapped on the chromosome in both

Classical and El Tor biotypes (Moseley and Falkow, 1980, Kaper *et al.*, 1981). Gerdes and Romig (1975) have demonstrated that the CT genes are not phage encoded and Kaper *et al.* (1981) demonstrated that these CT genes were not plasmid encoded.

The heat-labile enterotoxin (LT) of *E. coli* K-12 and CT cross-react antigenically and have similar structural and biochemical properties (Dallas and Falkow, 1979; Finkelstein, 1976). Southern DNA hybridizations using the cloned LT gene as a probe demonstrates that toxigenic strains of *V. cholerae* possess DNA sequences homologous to the LT genes of *E. coli*.but non-toxigenic strains do not (Gerdes and Romig, 1975; Moseley and Falkow, 1980).

Classical strains invariably possess two widely separated copies of the *ctxAB* operon (Pearson and Mekalanos, 1982) whereas many El Tor strains only contain a single copy (Moseley and Falkow, 1980). However, several El Tor strains have been identified that have duplications of *ctxAB* (Mekalanos, 1983). The multiple copies of the *ctxAB* operon in El Tor strains are arranged on large tandem repeats which are either 7 or 9.7 kb in length. This variation in size reflects the different number of copies of a 2.7 kb sequence (RS1) that is located at the junction of the duplication, as well as upstream and downstream from *ctxAB*. This RS1 element also appears to be responsible for the *ctxAB* amplification phenomenon which occurs during intestinal passage in animals (Holmes *et al.*, 1975; Mekalanos, 1983). Observed duplication and amplification properties of this sequence, has led to the suggestion that *ctxAB* is contained within a genetically mobile element, resembling a transposon (Mekalanos, 1983). The fact that amplification of the *ctxAB* did not occur in *recA* mutants (Goldberg and Mekalanos, 1986) supports the model which proposes that

amplification occurs via intramolecular recombination involving unequal crossover between the RS1 sequences flanking the *ctxAB* operon.

1.2.2 Regulation of cholera toxin.

Two types of regulatory mutations that alter the level of cholera toxin production have been identified. One class of mutants, *tox* mutants, which result in hypoproduction of CT, have been identified by Finkelstein and workers (1974). This locus has been mapped in *V. cholerae* Classical strain 569B to be closely linked to *his*-1 (Baine *et al.*, 1978). The other class of mutants, *htx*, results in hyperproduction of CT (Mekalanos *et al.*, 1978a, 1978b). This locus has been linked to *rif* on the genetic map of *V. cholerae* (Mekalanos *et al.*, 1979b). One of the *ctxAB* operons of Classical 569B strains has been cloned (Pearson and Mekalanos, 1979). The El Tor *ctxAB* operon has been mapped between *nal* and *his* on the *V. cholerae* chromosome (Sporecke *et al.*, 1984). One of the Classical *ctxAB* operons maps in a similar position. The *ctxAB* locus in Classical 569B and El Tor RV79 appears to be in the same relative position on the map, but it is not known where the second copy maps in the Classical strains.

The *toxR* gene, which positively controls the expression of CT, has been cloned from Classical strain 569B (Miller and Mekalanos, 1984). It has been identified as a transmembrane DNA-binding protein. *tox* mutants may have a defective *toxR* gene, since the cloned *toxR* gene is able to suppress the hypotoxinogenic *tox* mutation. All Classical strains have *toxR*. El Tor and non-toxinogenic strains have the *toxR* gene even though the non-toxinogenic strains do not have the *ctxAB* operon.

ToxS has recently been identified as a second regulatory gene system of CT (as well as other virulence determinants) and encodes a periplasmic protein which possibly interacts with the C-terminal end of

the ToxR trans-cytoplasmic membrane protein (Manning, 1988). It is postulated that ToxR and ToxS comprise a sensory transducer that converts environmental signals present *in vivo* into a change in virulence expression. A positive control system by ToxR and ToxS is exerted on at least 12 different gene products including CT (*ctxAB*), a pilus colonization factor (*tcpA*), an outer membrane protein (*ompU*) (Miller *et al.*, 1987) and the Acf colonization factor (Manning, 1988).

1.3 Plasmids.

Plasmids are circular double stranded DNA molecules which can be stably inherited without being linked to the chromosome and have been identified right across the genera of bacteria in both Gram positive and negative species.

Plasmids fall into two groups: either conjugative (capable of self transfer from one cell to another) or nonconjugative (incapable of self-transfer, but some may be comobilized by a conjugative plasmid). Conjugative plasmids capable of transferring chromosomal DNA between bacteria are often referred to as sex factors.

Plasmids are usually not essential for cell survival but may encode many accessory functions which are important in medical, agricultural, industrial and environmental terms. Their functions include virulence properties such as exotoxins, enterotoxins, serum resistance and adhesiveness, production of colicins, haemolysins, surface antigens, antibiotic resistances, metabolism of organic compounds, plant tumour formation and biological nitrogen fixation (Timmis and Pühler, 1979).

Other plasmid related properties such as replication and recombination have yielded much information about these fundamental biological processes. Plasmids have played an important part in the discovery and development of recombinant DNA technology as vectors

for the propagation and expression of a wide variety of foreign genes. This has great potential in industrial, biological and medical disciplines. The wide interest in the biology of bacterial plasmids and DNA cloning techniques has led to a more detailed molecular understanding of the plasmids themselves including their structure, replication, conjugal transfer and inheritance.

Bacterial plasmids also play an important role in bacterial genetics since they are able to act as "movable genetic elements" which can acquire bacterial genes by integation into and imprecise excision from the chromosome and transfer them to another bacterium.

Plasmids are classified according to their characteristics: R plasmids confer resistance to one or more antibacterial drugs, Col plasmids code for antibacterial proteins referred to as colicins, degradative plasmids encode for a variety of catabolic enzymes and virulence plasmids increase the pathogenicity of bacteria in a variety of ways. Each of these types of plasmids can be found in a wide range of bacterial genera.

1.3.1 Sex factors.

Conjugative plasmids capable of transferring chromosomal DNA are often referred to as sex factors and have been identified in a variety of species. These plasmids are particularly useful in the mapping of genes on the bacterial chromosome. The F factor of *E. coli* K-12 is the best characterized sex factor and is capable of integrating into the chromosome and so promote chromosome transfer in other members of the Enterobacteriaceae including several *Salmonella* species as well as *Citrobacter freundii* and *Erwinia chrysanthemi* (Holloway and Low, 1987).

Sex factors have also been identified in the genus *Pseudomonas*. IncP plasmids originally found in *Pseudomonas aeruginosa* are able to

transfer genes between pseudomonads as well as between many other genera of Gram-negative bacteria. The K plasmid (or sex factor K) originally from *P. putida* is in fact part of an OCT plasmid aggregate which dissociates into a nontransmissible OCT plasmid and the transfer plasmid, factor K (Chakrabarty and Friello, 1974) and is able to initiate chromosomal gene transfer at a high frequency. This K factor has been used to map chromosomal genes of *P. putida* (Mylroie *et al.*, 1977). Orientated chromosome transfer promoted by the FP2 sex factor and other plasmids in *P. aeruginosa* has been used to map numerous genes and to demonstrate that the chromosome is circular (Holloway *et al.*, 1979).

Genetic manipulations to artificially introduce regions of homology between the chromosome and a plasmid in order to create an Hfr situation has been done using the bacteriophage Mu (demonstrated in *Klebsiella pneumoniae*). Introduction of a transposon into both the chromosome and the sex factor is another means of providing regions of homology and has been used in *V. cholerae* and the P sex factor (discussed in section 1.7).

1.3.2 Conjugative plasmids.

Conjugative plasmids have been found in a large variety of genera with many of these plasmids having a broad host range (Datta and Hedges, 1972). There are three common features shared by all conjugative plasmids. They encode: (1) the production of an extracellular filamentous organelle, a sex-pilus, which is essential for cell-recognition and mating aggregate formation, (2) a transfer (*tra*) region(s) specifying proteins for pilus biosynthesis, conjugal replication and the transfer of plasmid DNA, and (3) surface exclusion proteins in the cell envelope which prevent the cell being a recipient to a donor carrying the same or closely related plasmid.

Conjugative plasmids have been classified into different incompatibility (Inc) groups based on the ability of plasmids to coexist in the same cell. This property is intimately associated with plasmid replication, such that two plasmids which have the same or very similar replication systems will be incompatible because they will be competing for the same replication functions, and so are assigned to the same Inc group (reviewed by Novick, 1987). Plasmids with different replication systems will be able to coexist in the same cell and so are assigned to the different Inc groups. Plasmids in the same Inc group usually have similar conjugational systems and the pili which they specify are morphologically and serologically similar (Bradley, 1980) and may well have derived from a common ancestral source. Different Inc groups usually have conjugal systems which are phenotypically and genetically distinct.

Conjugative plasmids are invariably greater than 30 kb, because of the genetic complexity of the conjugal process. Sizes can range from about 40 kb, *Erwinia stewartii* has a 52 kb conjugative plasmid (Coplin *et al.*, 1985) and an IncN plasmid from *Klebsiella pneumoniae* is 39 kb with a 19 kb *tra* region (Thattle *et al.*, 1985) whereas many *Rhizobia* and *Agrobacteria* have conjugative plasmids larger than 200 kb.

1.3.3 Nonconjugative plasmids.

Nonconjugative plasmids are incapable of selftransfer, however some such as ColE1 and RSF1010 may be comobilized by a conjugative plasmid (Warren *et al.*, 1978; (Willetts and Wilkins, 1984, respectively). For comobilization of these plasmids one requirement is a DNA sequence referred to as *bom* (basis of mobilization). This sequence is specifically recognized and nicked by a plasmid-encoded mobilization protein, referred to as *mob*, to prime the plasmid for mobilization by a

single-strand transfer mechanism (Warren *et al.*, 1978). Also involved are plasmid encoded proteins, of which little is known about their properties (Brash and Meyer, 1986). Only conjugative plasmids will be discussed here, however for reviews on the mechanisms of comobilization of nonconjugative plasmids refer to Clark and Warren (1979) and Willetts and Wilkins (1984).

1.4 Plasmids in *Vibrio cholerae*

Plasmids have not been widely identified in the *Vibrio* genus. Few isolations of R factors have been reported, mainly in marine-borne species of *Vibrio* (Davidson and Oliver, 1986). The incidence of R factors in *V. cholerae* is low, however a sex factor, P (Bhaskaran, 1958), has been identified as well as several other cryptic plasmids (Cook *et al.*, 1984).

1.4.1 P factor.

The P factor of *V. cholerae* was first described by Bhaskaran in 1958. He found that fertile cells (P^+) were able to transfer chromosomal genes to P^- cells. An unusual characteristic of P^+ cells, is their ability to produce plaque-like clearings, referred to as lacunae. These lacunae can be demonstrated when P^+ cells are plated onto a lawn of P^- cells (Takeya and Shimodori, 1969) and were originally thought to be due to a bacteriophage (Bhaskaran, 1958). However, live P^+ cells are necessary to reproduce this phenomenon; neither killed P^+ cells, culture filtrates or extracts were able to induce the production of lacunae (Bhaskaran, 1958). These lacunae probably represent zones of inhibited growth caused by lethal zygosis (Parker and Romig, 1972).

The P factor is capable of derepressed self transfer to P^- recipients at a high frequency. Thus, the P factor is similar to the

fertility factor F of *E. coli* K-12. Like F, P is able to transfer chromosomal markers from one cell to another, however, this P mediated transfer of chromosomal markers only occurs at a low frequency. Unlike F, P appears to be unable to stably integrate into the host chromosome to form strains capable of high frequency recombination (Hfr) and this probably accounts for the low frequency of chromosomal marker transfer (Parker, *et al.*, 1979). Lack of homology between the P factor and the host chromosome, as indicated by the significant difference in the overall guanine plus cytosine (G+C) content (42% and 48%, respectively) has been suggested to be responsible for this inability to integrate into the host chromosome. However, perhaps a more plausible explanation for the inability of P to form Hfr strains is the lack of common insertions sequences between the P factor and the *V. cholerae* chromosome. In contrast, the F factor of *E. coli* has a base composition close to that of its host (Marmur *et al.*, 1961) and the presence of common insertion sequences, such as IS2, IS3 and Γ-δ (Tn1000) accounts for its ability to form Hfr's (Deonier and Hadley, 1980).

Johnson and coworkers (1979) constructed artificial Hfr strains in *V. cholerae* by introducing common sequences (transposons) into the P factor and the *V. cholerae* chromosome. The uses of these Tfr strains (Transposon-facilitated recombination) will be discussed later.

The P factor is present at approximately one copy per chromosome (Parker and Romig, 1972). It is not a resistance factor (Parker *et al.*, 1971; Parker and Romig, 1972) and no simple phenotypic markers have been identified . Sex pili have been demonstrated on the surface of about 60% of cells in a P^+ culture (Bhaskaran *et al.*, 1969) and are referred to as P pili however these P pili have not been purified or characterised in any great detail. From electron microscopic

measurements, the P pili do not resemble sex pili or common pili as described by Meynell *et al.* (1968). The P pili are 0.2 µm in length and have a diameter of 80 - 100 µm with one to four present per cell and seem to resemble pili described by Tweedy *et al.* (1968) which were 72 Ain diameter, 1.0 µm in length and were present in up to nine per organism. The only other function proposed for P besides the mobilization of chromosomal markers is a possible role in the suppresion of pathogenicity of *V. cholerae* (Sinha and Srivastava, 1978). This possible role is discussed in section 1.3.

1.4.2 R plasmids.

Over the last twenty years there has been a marked increase in the occurrence of resistance plasmids (R plasmids) in enteric bacteria mainly due to the increased use of antibiotics to treat bacterial infections. However, there have been few reports on outbreaks of chemotherapy tolerant *V. cholerae* bearing R plasmids even though there has been a marked increase in conjugative R plasmids in various Gram negative organisms (Kuwahara *et al.*, 1979). The fourth cholera epidemic in Tanzania saw the emergence of a R-plasmid, six months after the heavy use of tetracycline to combat the disease (Towner *et al.*, 1979). The isolated R plasmid exhibited multiple drug resistance, was transferable to *E. coli* K-12 and belonged to the IncC incompatibility group (Mhalu *et al.*, 1979).

Few R plasmids have been isolated from *V. cholerae* and nearly all of these belong to the incompatibility group IncC (Hedges and Jacob, 1975, Hedges *et al.*, 1977), with the exception of one plasmid, pJYI, which belongs to IncJ (Yokota and Kuwahara, 1977). Multiple drug-resistant strains of *V. cholerae* have been isolated but the presence of a R plasmid has not always been demonstrated (Kuwahara *et al.*, 1979). The risk of emergence of multiple drug-resistant *V. cholerae* is

negligible since few R plasmids are reportedly maintained in *V. cholerae* (Yokota *et al.*, 1972). Incompatibility group IncC are the most stable R plasmids in *V. cholerae* and have been found in isolates from geographically widely separated areas: Africa (Ouellette *et al.*, 1988), Algeria (Rahal *et al.*, 1973), Southern USSR (Hedges and Jacob 1975), Indonesia (Hedges *et al.*, 1977) and the Philipines (Yokota *et al.*, 1972). The presence of IncC R plasmids in *V. cholerae* is not unusual since these plasmids are found in an wide range of genera including *Pseudomonas*, *Proteus*, *Providencia* and *Klebsiella* (Witchitz and Gerbaud, 1972). IncP R plasmids also appear to be stable in *V. cholerae* (Hedges *et al.*, 1977), however, none have been found in clinical isolates (of *V. cholerae*). It has been suggested that the limited number of incompatibility groups of R plasmids in *V. cholerae* is due to the G + C content (Hedges *et al.*, 1977). Coliform bacteria have G + C ~50% and these readily form a large range of R plasmid groups whereas other bacteria which have a G + C % of approximately 40% (eg: *Providencia*) do not readily form R plasmid groups. Several workers have shown using *in vitro* transfer experiments that *V. cholerae* are poor recipients for R plasmids (Kuwahara *et al.*, 1963; Rahal *et al.*, 1973; Davey and Pittard, 1975), which could be a possible reason for the limited number of R plasmids isolated from *V. cholerae*.

The presence of plasmids in other *Vibrio* species has been observed. R plasmids have been isolated from *V. anguillarum*, a fish pathogen (Hayashi *et al.*, 1982) and *V. parahaemolyticus* (Arai *et al.*, 1985). Both these R plasmids belonged to incompatibility group IncC, were large in size (average 110 MDal) and could be transferred and stably maintained in *E. coli* K-12. Approximately 12% of clinical and environmental isolates of *V. vulnificus* (Davidson and Oliver, 1986) were shown to contain plasmids of varying size but these had no

demonstrable trait. Other lactose-fermenting *Vibrios* examined had a much higher incidence of plasmids, 62%, of varying size (2.1 - 150 MDal) (Davidson and Oliver, 1986).

Clinical and environmental isolates from *V. mimicus* were examined for resistance to a variety of antibiotics (Chowdhury *et al.*, 1986) and all clinical isolates were found to be susceptible to Km, Tc, Tm, Ap, Cm and Gm whereas the environmental isolates were resistant to all antibiotics except Cm and Gm. It has yet to be demonstrated whether this antibiotic resistance is contained on a R plasmid and is transferable.

1.4.3 Cryptic plasmids.

Few other plasmids have been identified in *V. cholerae.* Two apparently cryptic plasmids have been identified in a large number of Classical strains from the 6th cholera pandemic but were not found in El Tor isolates (Cook *et al.*, 1984). Newland *et al.* (1984b) examined 298 isolates of *V. cholerae* from both clinical and environmental sources with a wide geographical distribution and found that only 16% contained a plasmid.

The two plasmids identified in Classical strains by Cook *et al.* (1984) are 21 and 3 MDal in size. Johnson *et al.* (1979) reported the identification of two plasmids in strain V58 (Classical) and found them to be 3.2 and 24.7 MDal in size. These plasmids may well be similar or identical, since they were common to Classical strains, however, no characterization has not been reported to date. Datta *et al.* (1973) identified a plasmid present in both P^+ and P^- strains which has a molecular weight of 37 MDal. Further examination of these plasmids has not been reported.

1.4.4 V factor.

Another transmissible factor designated the V factor has been identified in a non-cholera vibrio strain (NCV 165) by its ability to produce lacunae on *V. cholerae* P$^-$ lawns (Bhaskaran and Sinha, 1971). This factor is capable of conjugative transfer but at a lower frequency than P and has demonstrated a very low level of chromosomal gene transfer and only when V$^+$ strains are incubated at 44.5° C prior to mating (Bhaskaran *et al.*, 1973). Bhaskaran and Sinha (1971) have reported that the P is repressed for transfer in the presence of V. When P is introduced into a V$^+$ cell, there is a brief lag period, during which the P factor is still capable of autonomous transfer as well as chromosomal mobilization (Bhaskaran and Sinha, 1971).

1.4.5 L factors.

Transmissible factors have been identified in non-01 *V. cholerae* and other *Vibrio spp.* and called L factors (Smigocki and Voll, 1986). These L factors (for production of lacunae) are able to produce lacunae on lawns of non-01 *V. cholerae* under the same conditions as the P factor. The production of lacunae by V and L factors was not due to a vibriocin or phage activity but rather probably corresponds to sites of active mating. Lacunae production could be transferred, presumably by conjugation, however, no plasmids could be identified in L$^+$ or V$^+$ strains (Smigocki and Voll, 1986). Smigocki and Voll (1986) suggest that a possible reason for the inability to isolate plasmid DNA from L$^+$ or V$^+$ strains may be that they belong to the incompatibility group IncJ, since it has been found that IncJ plasmids in *Pseudomonas* are difficult to extract (Hedges *et al.*, 1975).

1.4.6 *V. cholerae* non-01 plasmids.

A 33 kb plasmid from a *V. cholerae* non-01 (strain 91) has been shown to contain homologous sequences to the *tdh* gene (thermostable direct haemolysin) of *V. parahaemolyticus* (Honda *et al.*, 1986).

V. parahaemolyticus is a natural inhabitant of estuarine and marine environments and is associated with seafood-borne gastroenteritis (Fujino *et al.*, 1974; Blake *et al.*, 1980). A major virulence determinant of this *Vibrio* species is the thermostable direct haemolysin (TDH) (Sakurai *et al.*, 1973) or Kanagawa phenomenon (Miyamoto *et al.*, 1980) resulting in β haemolysis in blood agar.

Nishibuchi and coworkers (1985) have cloned the *tdh* gene from the chromosome of *V. parahaemolyticus*, localized the gene on a 1.3 kb *Hind*III fragment, sequenced and characterized the gene (Nishibuchi and Kaper, 1985).

Using a 415 bp probe to the *tdh*, Nishibuchi has demonstrated that a haemolysin produced by some *V. cholerae* non-01 strains, NAG-rTDH (related to the TDH (Yoh *et al.*, 1986)) has the gene encoding NAG-rTDH on a plasmid (Honda *et al.*, 1986). Homologous sequences to the *tdh* gene of *V. parahaemolyticus* were detected on a 33 kb plasmid from the non-01 *V. cholerae* strain 91.

1.5 Role of plasmids in pathogenicity.

1.5.1 Virulence plasmids.

Many bacterial species harbour plasmids which play a role in the virulence of the host bacterium.

Salmonella species are often associated with gastrointestinal infections. The wild type *S. typhimurium* LT2 strain harbours a 100 kb (60 MDal) plasmid essential for the virulence of the plasmid (Jones *et al.*, 1982; Gulig and Curtiss, 1987). Cured strains are avirulent,

however, the inability to reintroduce the plasmid into a cured strain has not permitted confirmation of its role. This 100 kb plasmid has been shown to encode serum resistance (Helmuth *et al.*, 1985). Helmuth *et al.* (1985) have examined 337 isolates of *Salmonella* for their plasmid content and found that of the *S. typhimurium, S. enteritidis, S. dublin* and *S. choleraesuis* isolates 90% or more carried a serotype-specific plasmid. The sizes of these plasmids were 60 MDal, 37 MDal, 56 MDal and 30 MDal respectively and these plasmids were necessary for virulence. Strains of *S. infantis. S. panama* and *S. heidelberg* harboured plasmids, however, these plasmids could not be correlated with virulence.

Pathogenic *E. coli* (ETEC) harbour plasmids which encode the virulence determinants (Parry and Rooke, 1985). Adherence-factor antigens K88 and K99 are plasmid encoded (reviewed by Elwell and Shipley, 1980; Gaastra and deGraaf, 1982). The heat labile enterotoxin LT of *E. coli* K-12 is plasmid encoded (Elwell and Shipley, 1980). Enteropathogenic *E. coli* (EPEC) are diarrheogenic *E. coli* which do not have the known heat-labile and heat-stable enterotoxins nor do they invade the intestinal mucosa (Edelman and Levine, 1983). A 60 MDal plasmid encoding an adherence factor (EAF) is required for full virulence by human EPEC, (Nataro *et al.*, 1987). Nataro and workers have demonstrated homology between the 60 MDal plasmid and other plasmids in EPEC strains ranging from 55-65 MDal and that this plasmid belongs to the IncFII group. It has also been suggested that EAF plasmids are in fact closely related yet do not carry other phenotypes characteristic of *E. coli* virulence plasmids.

Two other *E. coli* groups which are diarrheogenic are the EHEC (enterohemorrhagic *E. coli*) and EIEC (enteroinvasive *E. coli*) (Levine, 1987). EHEC have only been recently isolated, 1982, and cause an

unusual clinical syndrome of diarrheal disease, haemorrhagic colitis and are caused by *E. coli* 0157:H7, a new bacterial enteric pathogen (Riley *et al.*, 1982). Strains of this serotype 0157:H7 possess a 60 MDal plasmid which plays a role in virulence (Karch *et al.*, 1988) which encodes a fimbrial antigen.

EIEC strains closely resemble *Shigella* like having the capacity to invade and proliferate within epithelial cells (DuPont *et al.*, 1971). The invasive capacity of a large 140 MDal plasmid (Harris *et al.*, 1982). These have been reviewed by Levine (1987).

Some colicin V plasmids of *E. coli* may increase the virulence of bacterial strains harbouring them (Smith, 1974b; Smith and Huggins, 1980). There are several determinants on these plasmids which may increase virulence, such as: encoding genes for a high affinity hydroxamate siderophore (Stuart *et al.*, 1980); serum resistance (Binns *et al.*, 1979); Nillus and Savage, 1984) or possibly the colicin V protein may inhibit phagocytosis of macrophages *in vitro* (Ozanne *et al.*, 1977), however, this latter phenomenon has been disputed (Evans *et al.*, 1978). Smith and coworkers (1983) have shown that strains repressed in conjugal genetic transfer were able to adhere to the tissue in greater numbers than those in which the plasmids were derepressed. This suggests that the conjugal transfer functions of certain colicin V plasmids influence a property that may be involved in the pathogenesis of invasive *E. coli* strains.

Shigellosis is an important disease throughout the world and virulence (through the invasion process) can be demonstrated to be in part due to a large plasmid harboured by the strains. *Shigella sonnei* harbours a non-conjugative 120 MDal plasmid which is necssary for virulence and encodes production of the formI antigen associated with the O-side chain of lipopolysaccharide (Kopecko *et al.*, 1980; Sansonetti

et al., 1981). *Sh. flexneri* is responsible for a significant proportion of bacillary dysentry and the involvement of a large nonconjugative (but mobilizable) 140 MDal plamsid has been demonstrated (Sansonetti *et al.*, 1982). This plasmid either encodes or regulates the expression of some function(s) required for epithelial cell penetration, however, unlike the formI plasmid of *Sh. sonnei* it does not encode synthesis of any lipopolysaccharide surface component.

A large proportion of the *Pseudomonas* species contain indigenous plasmids (Piwowarski and Shaw, 1982: Currier and Morgan, 1983), many of which are of a large molecular weight. Plasmids in strains of *Ps. syringae pv.tomato* were found to range between 29-103 kb (Bender and Cooksey, 1986), however, most were phenotypically cryptic and only two of 41 strains contained plasmids which were conjugative.

In soil *Pseudomonas* species, the ability to catabolize toluene via benzoate and substituted benzoates appears to be almost always plasmid encoded (Williams and Murray, 1974), but the plasmids themselves differ considerably in structure and properties (Williams and Worsey, 1976: Duggelby *et al.*, 1977). These TOL plasmids invariably have a high molecular mass, in the range of 16 to 135 kb (25 x 10^6 to 202 x 10^6 daltons) (Duggelby *et al.*, 1977). For example, the archetype TOL plasmid pWW0 isolated from *Ps. putida mt-2* is 117 kb in size (Nakazawa and Yokota, 1973).

The ability to induce the formation of tumours, referred to as crown gall, in a wide range of dicotyledenous plants is encoded on plasmids in *Agrobacterium tumefaciens* (reviewed by Gheysen *et al.*, 1985; Nester *et al.*, 1984). *A. tumefaciens* contain a diverse group of conjugative plasmids termed Ti plasmids (tumour inducing). As the result of the insertion of a segment of the Ti plasmid into the plant genome results in uncontrolled proliferation of the plant cells resulting

in tumour formation occurs. These conjugative plasmids can be greater than 200 kb in size.

The bacterium *Rhizobium* is capable of forming a nitrogen-fixing symbiositic relationship with legumes which is very specific: alfalfa/symbiont: *R. meliloti*, peas: *R. leguminosarum* and clover: *R. trifolii*. Plasmids found in *Rhizobia* species play an important role as many of the symbiotic genes (*nod*, (nodulation), *nif*,(nitrogen fixation)) are located on large plasmids (Sym plasmid) and some are self transmissible. Djordjevic and co-workers (1983) have reported a self-transmissible 140 MDal Sym plasmid from *R. leguminasarum* which encodes clover nodulation and nitrogen-fixation functions. This has been shown to be able to transfer to various *Rhizobium*.

The etilogical agent of the plague is *Yersinia pestis* (Braude, 1981) and *Y. enterocolitica* and *Y. pseudotuberculosis* are causative agents of gastrointestinal illnesses in humans. It has been demonstrated by several groups that plasmids carried by *Yersinia* are involved in virulence (Gemski *et al.*, 1980; Portnoy *et al.*, 1981). Also associated with virulence is Ca^{2+} dependence and the production of V and W antigens which have been shown to be encoded on a family of related plasmids in *Yersinia* (Portnoy and Martinez, 1985).

1.5.2 Plasmids and toxins.

Many bacterial toxins are associated with plasmids and bacteriophages. In *E. coli*, the heat labile enterotoxin (LT) is plasmid encoded (Elwell and Shipley, 1980) and the heat-stable enterotoxin (ST) is encoded on a transposon, Tn*1681* (So *et al.*, 1979) most often found inserted in plasmids. The toxin genes of *Corynebacterium diphtheriae* are found on a temperate bacteriophage (Groman, 1955). Shiga toxin, a cytotoxin produced by *Shigella dysenteriae* type 1 is chromosomally encoded but a related toxin has been identified on a

phage (Smith *et al.* 1983). Certain strains of *E. coli* and *V. cholerae* produce a protein toxin which is structurally and immunologically related to Shiga toxin (O'Brien *et al.*, 1984a). This group of related toxins are often referred to as Vero cell cytotoxins or Shiga-like toxins and have been implicated as a cause of diarrhoea and several of the Shiga-like toxins have been shown to be encoded by a family of related bacteriophages (O'Brien *et al.*, 1984b; Newland *et al.*, 1985a).

1.5.3 P factor and pathogenicity.

Plasmids may also play a role in pathogenicity besides encoding structural and regulatory genes. Plasmids may reduce the virulence of their bacterial host as seen with the plasmid Sa, originally from *Shigella flexneri*, it suppresses tumorogenicity in *Agrobacterium tumefaciens* (Farrand *et al.*, 1981).

Sinha and Srivastava (1978) have suggested that the P and V plasmids suppress the pathogenicity of *V. cholerae* due to a decreased level of toxin production. This effect was most noticeable in Classical strains. The observation that P is repressed for transfer in El Tor strains unless the bacteria are exposed to thermal shock at 44.5°C (Bhaskaran *et al.*, 1973), could explain why there is a lack of effect due to P in El Tor strains. Khan *et al.* (1985) suggested that toxin biosynthesis is regulated on plasmid-borne genes or that genes regulating toxin biosynthesis in *V. cholerae* are located on the P plasmid. This was based on the observation that strains cured of the P factor produced as much CT as the parent strain and reacquisition of the P factor by the cured strain again resulted in suppression.

The effect of plasmids on virulence in *V. cholerae* has also been examined by Hamood *et al.* (1986). They used a variety of plasmids including cloning vectors pRK290 and pBR322, three different incompatibility group plasmids RP4 (IncP), $R_{ts}1$ (IncT) and Sa (IncW),

two Tn derivatives of the P factor (pSJ5; P::Tn*1* and pSJ8; P::Tn*9*) and two R plasmids isolated in Bangladesh (pVH1 and pVH2).

Each plasmid was examined for its effects on ability to a induce fluid accumulation response in infant mice (FA); ability to produce cholera toxin in a YI adrenal cell assay and ability to colonize the upper part of the small intestine of infant mice, as evaluated by dual infection competition experiments. It was found that RP4, pRK290, Sa, pSJ5, pSJ8 and pBR322 had no effect on virulence, R factors $R_{ts}1$ and pVH1 reduced it but the other *V. cholerae* R factor enhanced the diarhoeal response. Hamood *et al.* (1986) suggested that the R factors isolated from Bangladesh may contain virulence-related genes or perhaps contain a regulatory gene which controls the expression of chromosomally encoded virulence factors however this unique enhancing affect on virulence was not related to CT, haemolysin or a colonization factor.

Thus, the role played by the P factor (and V factor) in affecting pathogenicity is unclear. Khan *et al.* (1985) reported the suppression of pathogenicity due to a decrease in toxin production as measured by fluid production in adult rabbit ileal loops, whereas Hamood *et al.*, (1986) could find no effect on virulence as measured by FA response on *in vitro* toxin production, and suggested that alterations in other virulence attributes are more likely (Hamood *et al.*, 1986).

1.6 Mechanisms of genetic exchange.

Transfer of genetic material between two bacterial cells can occur by three different mechanisms: transformation, or transfection which involves the uptake of DNA, from solution; conjugation which requires cell to cell contact for the unidirectional transfer of DNA between the

two cells and transduction in which a bacteriophage acts as the "shuttle vector" between two bacterial cells.

1.6.1 Transformation.

The uptake of genetic material from solution by a bacterium is referred to as transformation and was first observed in 1928 by Griffith who was studying with the epidemiology of pneumococcal pneumonia (Griffith, 1928). The observation that avirulent pneumococci (forming rough colonies (R)) could be "transformed" into virulent pneumococci (forming smooth colonies (S)) simply with the incubation of killed S cells with R cells led to the first reported case of transformation. This however, was not widely accepted until 1944 when Avery and fellow workers were able to identify the transforming factor as DNA (Avery *et al.*, 1944). Transformation has been accomplished with only a few bacterial species including *Haemophilus*, *Neisseria*, *Streptococcus*, *Staphylococcus* and *Bacillus*. The Enterobacteriaceae are not naturally transformable, however, an artificial state of competence can be established following the treatment of cells with cold $CaCl_2$. This method of transformation was first described by Mandel and Higa (1970), who observed that the incubation of a suspension of *E. coli* K-12 cells with bacteriophage lambda DNA in $CaCl_2$ at 0°C resulted in the subsequent appearance of plaques. Since this initial observation, the process of transformation has been employed extensively with plasmids, especially in the cloning of DNA fragments in plasmid vectors (Curtiss *et al.*, 1977).

Transformation of chromosomal markers in *E. coli* K-12 is not an efficient method of genetic mapping because of the *recBC* nuclease activity (Hoekstra *et al.*, 1980). Hoekstra and coworkers (1980) have observed cotransformation of linked markers in *E. coli* K-12 by using a

recB, *recC*, *sbcB* mutant strain, however, very little is known about this system and so it has a limited use in genetic mapping in *E. coli* K-12.

In contrast, much of the initial genetic mapping of the gram positive organism *Bacillus subtilis* has been done by transformation because it is naturally competent (Dubnau and Goldthwaite, 1967). This process of genetic mapping has its limitations in that it is restricted to portions of the genome and too large fragments may break, perhaps resulting in the interference of linkage (Kelly, 1967). The limited extent of genetic linkage by transformation has made the construction of an uninterupted transformation map impossible. Bacteriophage PBS1 transduction studies have allowed the linkage of large clusters of loci and all the linkage groups have been linked to one another to form a circular chromosomal map (Lepesant-Kejzlarova *et al.*, 1975).

1.6.2 Transduction.

The transfer of bacterial genetic information from one cell to another via a bacteriophage particle is referred to as transduction. The transduction process can be used in genetic mapping. Transduction by bacteriophage can be either generalized, where any bacterial gene may be transferred or specialized in which a particular bacteriophage can only transfer certain genes. The distance between two mutational sites that can cotransduce in the same particle can be estimated either form the frequency of joint transduction of distinct genes or from the frequency of recombination between sites within a gene. This approach has been widely used in genetic mapping in a variety of bacteria.

Two of these processes, namely conjugation and transduction have been utilized in genetic mapping of the *V. cholerae* chromosome and is discussed in more detail below.

1.6.3 Conjugation.

Bacterial conjugation is a complex process whereby DNA is transferred from a donor to a recipient cell via direct cell to cell contact and was first reported by Lederberg and Tatum (1946). Conjugation was subsequently shown to be due to the presence of the F factor of *E. coli* (Cavalli-Sforza, 1950; Hayes, 1953). Many conjugative plasmids have been identified in a wide variety of both Gram negative (Bukhari *et al.*, 1977) and Gram positive bacteria (Clewell, 1981). The process of conjugation in Gram negative organisms differ markedly from that of Gram positive organisms The genes for conjugation are usually plasmid encoded. The Gram negative systems all specify surface filaments for donor-recipient recognition whereas the Gram positive plasmid encoded systems do not have surface filaments but rather involve sex pheromone-induced aggregations. Only Gram negative systems will be discussed here, however, for an extensive review of Gram positive conjugation refer to Clewell (1981) and Clewell *et al.* (1985).

The conjugal transfer systems of IncF, IncI, IncN and IncP plasmids are different (Willetts, 1977b). Little DNA homology is observed between plasmids of these groups (Grindley *et al.*, 1972), however, plasmids of all four groups encode systems for DNA replication, conjugal transfer and surface exclusion.

Conjugation mediated by plasmids of the Inc groups N,P,W function more efficiently on solid surfaces than in liquid medium (Dennison and Baumberg, 1975; Bradley, 1981) or in a physical environment that provides an increased surface-to-volume ratio (Singleton, 1983) and this is due to the fragile nature of the pili.

Incompatibility group HI plasmids are found in the Enterobacteriaceae and are frequently associated with chloramphenicol

resistance in Salmonella typhi (Smith, 1974a). These plasmids are temperature sensitive for transfer (Taylor and Levine, 1980), plasmid R27 has been shown to have two widely separated transfer regions both of which are essential for conjugation (Taylor *et al.*, 1985). The transfer genes of the IncN plasmids are located within a single 22.5 kb *tra* region (Thatte and Iyer, 1983) whereas *tra* genes of IncP10 plasmid R91-5 from *P. aeruginosa* and IncP plasmid RP4 (IncP1 in *P. aeruginosa*) lie in two or three distinct regions (Barth, 1979; Moore and Krishnapillai, 1982). ColIb-P9 is a large 93 kb conjugative plasmid belonging to the $IncI_1$ group and is related to R64 and R144. Three distinctive *tra* regions have been identified on the ColIb-P9 plasmid (Rees *et al.*, 1987) The distribution of *tra* genes into more than one region has also been reported for a Ti plasmid in Agrobacterium tumefaciens (Holsters *et al.*, 1980).

1.6.3.1 Hfr formation.

Hfr strains of *E. coli* K-12 capable of transfer of chromosomal markers at high frequency arise from the integration of a conjugative plasmid into the bacterial chromosome by one of several possible types of recombination events. Cavalli-Sforza (1950) and Hayes (1953) were the first workers to report such conjugational donors of chromosomal markers at high frequency and since this initial observation the understanding of this mechanism has increased vastly. Hfr strains have been formed by various methods including spontaneous or UV induced integration, using *E. coli* K-12 mutants which are defective in initiation of DNA replication at high temperatures integrative suppression; (Iida, 1977). The repeated integration of the F plasmid at certain sites of the chromosome has been shown in at least some cases to be due recombination events between an insertion sequence (IS

element) on the factor and a homologous IS element on the chromosome (Davidson *et al.*, 1975).

It has been possible to construct Hfr strains with sites of integration distributed around the *E. coli* K-12 chromosome. These have been extremely useful for mapping of various genes as well as for strain construction (Low, 1972, review of most integration sites; Low, 1987)

1.6.3.2 F of *E. coli* K-12 as a model conjugation system.

The *tra* region of the F factor is the most defined transfer region. However, *tra* regions from other plasmids are beginning to be investigated further and major differences are being noted. The most prominent one is that not all systems involve a single region but rather two or three blocks, such as for RP4 (Lanka and Barth, 1981), ColIb-P9 (Rees *et al.*, 1987) and pKM101 (Winnans and Walker, 1985). Other differences noted, especially for IncI$_1$, B, K, and Z are that two distinctive pili are synthesized (Bradley, 1983, 1984). The two types are a thick rigid pilus thought to be essential for conjugation and a thin flexible pilus (immunologically distinct) which stabilizes mating aggregates in liquid.

The IncI$_1$ conjugation system also includes a DNA primase, which is involved in processing the transferred plasmid DNA (Merryweather *et al.*, 1986). Another feature of IncI$_1$ system is an EDTA-resistant nuclease activity co-ordinately expressed with the transfer genes (Rees *et al.*, 1987).

Similarities between these various systems are cell-to-cell interactions mediated by conjugative pili, transfer of an unique strand of plasmid DNA initiated at the origin of transfer site (*oriT*) and synthesis to regenerate double-stranded plasmids in both the donor and

recipient cell (Ippen-Ihler and Minkley, 1986). The expression of exclusion genes is also a common feature (Hartskeerl *et al.*, 1985).

Conjugal DNA tranfer consists of a number of highly specific DNA processing steps. The F factor of *E. coli* K-12 has been the model system for the study of these events and this provides us with a basis for the understanding of the conjugal process. Because of the complexity of the process of conjugation, only an overview of the mechanism will be presented here. A more detailed description of the different associated phenomena can be obtained from the reviews by Ippen-Ihler and Minkley (1986) and Willetts and Skurray (1987).

The transfer (*tra*) operon on the F plasmid comprises at least 25 genes within a region of approximately 33 kb which represents about one third of the plasmid. The *tra* genes can be divided into five groups. Most of the genes belong to the first group: *tra A,L,E,K,B,V,W,C,U,N,F,Q,H,G* are involved in the formation of F pilus which provides the initial contacts between donor and recipient cells. These DNA transfer events occur while the conjugating bacteria are in cell surface contact, a process dependent upon the function of the donor sex pilus.

traN and *traG* are in the second group and are involved in stabilization of the mating pairs (Achtman, 1975; Willetts and Skurray, 1980; Manning *et al.*, 1981).

Conjugal DNA metabolism is the function of the third set of *tra* genes, namely, *tra M,Y,D,I,Z* (Willetts and Skurray, 1980; Willetts and Wilkins, 1984). The *traJ* gene is involved in the regulation of transfer. The product of *traJ*, TraJp, is encoded on a separate but adjacent operon (Helmuth and Achtman,1978) and is a positive control element for the majority of the *tra* genes. The fifth functional region are the *traS* and *traT* genes which are involved in surface exclusion.

1.6.3.3 Regulation of the *tra* operon.

The majority of F-like plasmids isolated from nature are repressed for conjugal DNA transfer (Watanabe and Fukasawa, 1962; Meynell *et al.*, 1968) and at least six fertility inhibition systems have been identified: FinC, FinOP, FinQ, FinU, FinV and FinW. The FinOP system inhibits *tra* expression indirectly via *traJ*, the other systems are believed to repress *tra* expression directly (Willetts and Skurray, 1980).

The regulation of the *tra* operon of F like plasmids involves two stages: the first stage is fertility inhibition in which the products of the *finO* and *finP* genes repress transcription of *traJ* (Willetts, 1977a; Finnegan and Willetts, 1973) and in the second stage, the traJ protein is required for transcription of the *traYZ* operon. The *finO* gene of F is inactivated due to an IS*3* insertion (Clark, 1985; Cheah and Skurray, 1987), however, FinO can be provided *in trans* by many compatible finO$^+$ F-like plasmids, such as R1 and R100 (Egawa and Hirota, 1962; Meynell *et al.*, 1968). The *finP* gene is located close to *traJ* (Johnson *et al.*, 1981) and the product is a ca. 78 bp RNA product which may possibly act in conjunction with the *finO* protein to prevent translation or transcription or both of *traJ*. Control of conjugation by a *finP* RNA is at least superficially similar to control of plasmid ~~regulation~~ replication (Stougaard *et al.*, 1981) and of *ompF* (Mizuno *et al.*, 1984) by antisense RNA molecules.

The product of *traJ*, TraJp, is a cytoplasmic protein of 24,000 daltons which, positively controls the expression of the *tra* operon which is in turn positively controlled by certain chromosomally encoded products but is negatively regulated by the FinOP fertility inhibition system. There are at least five distinct chromosomally encoded products essential for *tra* gene expression by the F plasmid and its derivatives (for a review see Silverman, 1985).

TraJp has been reported to be the positive regulator for the expression of the *traM* and *traYZ* operons, via their promoters P_M and P_{YZ}, respectively (Achtman *et al.*, 1971,1972; Finnegan and Willetts, 1973; Willetts, 1977a; Sambucetti *et al.*, 1982; Gaffney *et al.*, 1983). It has also been proposed that TraJp functions as an anti-terminator for the *traY-Z* transcripts (Willetts and Skurray, 1980; Gaffney *et al.*, 1983) and/or is necessary to increase the stability of *tra* mRNA (Fowler *et al.*, 1983). Its precise role in *tra* gene expression still remains obscure.

1.6.3.4 Surface exclusion.

Surface exclusion is the phenomenon whereby cells carrying a plasmid are poor recipients with donors carrying the same or a closely related plasmid. Four surface exclusion specificity systems have been defined in the F-like plasmids (Manning and Achtman, 1979).

Two genes in the *tra* operon, *traS* and *traT* are essential for surface exclusion expression but not for conjugal DNA transfer. Each is responsible for a different aspect of surface exclusion but for the full effect both genes are required.

The *traS* product (TraSp) is a 16,861 dalton very hydrophobic cytoplasmic membrane protein with no apparent signal sequence (Jalajakumari *et al.*, 1987). TraSp has a minor effect on mating aggregate formation but effectively prevents DNA transfer by inhibiting the triggering of conjugal DNA metabolism.

The *traT* product (TraTp) is a 23,709 dalton peptidoglycan associated outer membrane lipoprotein produced with a 2,223 N-terminal precursor (Jalajakumari *et al.*, 1987). TraTp prevents cells from forming stable mating aggregates and has been recently proposed by Minkley and Willetts (1984) to inhibit conjugation by binding to the tip of the F pilus which binds at the zones of adhesion (Bayer, 1979).

Riede and Eschbach (1986) have demonstrated that TraTp interacts directly with OmpA to prevent OmpA-specific phages from binding suggesting that TraTp possibly mediates surface exclusion as a result of preventing the F pilus binding to OmpA as also proposed by Minkley and Willetts (1984).

TraTp has been shown to be responsible for serum resistance of *E. coli* K-12 cells harbouring F-like plasmids F, R6-5 and R100 (Moll *et al.*, 1980). It has also been implicated in reducing the susceptibility of the host bacterium to phagocytosis by macrophages (Nillus and Savage, 1984) and to hydrophobic antibiotics such as fusidic acid, erythromycin, vancomycin and novobiocin.

1.7 Genetic analysis in *V. cholerae*

Transfection of *V. cholerae* with phage DNA has been reported (Balganesh and Das, 1979), but it has not proven to be reproducible and transformation of *V. cholerae* cells has not been successfully demonstrated. The production of an extracellular DNase by *V. cholerae* cells may be one of the reasons for the inability to make *V. cholerae* cells competent for transformation or transfection (Focaretta and Manning, 1987). Transduction has been reported by several workers (Ogg *et al.*, 1981 and D.A.Relman M.D.thesis, Harvard-Massachusetts Institute of Technology, Cambridge, 1982). Generalised transducing phages have been identified but a cholera phage capable of specialised transduction has yet to be isolated. A conjugal gene transfer system exists in *V. cholerae* and is mediated by the naturally occurring conjugative plasmid, the P factor (Bhaskaran, 1960; Parker and Romig, 1972; Johnson and Romig, 1979b).

1.7.1 Vibriophages.

A small group of phages capable of lysogeny in both biotypes include VcA-1, Vc A-2 and VcA-3 and are serologically related to the Kappa type phage even though this phage is confined mainly to El Tor strains. This group of phages is useful in genetic experiments in *V. cholerae* since they are able to integrate randomly into the host chromosome (Goldberg and Murphy, 1983; Johnson and Romig, 1981) (Mekalanos *et al.*, 1982). The mutator property of these phages closely resembles that of phage Mu (Taylor,1963; Bukhari, 1976). Lysogeny of the host bacteria may result in the inactivation of a particular gene and this has been used to generate a large number of auxotrophic mutants (Goldberg and Murphy, 1983; Johnson *et al.*, 1981) as well as deletion strains lacking the cholera toxin gene (Mekalanos *et al.*, 1982). Homology between VcA-1 and a hybrid P factor, P::Tn*1* (pSJ15) which contains a defective VcA-1 genome (Johnson and Romig, 1981) has been used to form Tfr donor strains and so map the cholera toxin operon (Sporeke *et al.*, 1984).

Another contribution to the study of *V. cholerae* genetics is the generalized transducing phage, CP-T1 (Ogg *et al.*, 1981). CP-T1 was the first generalized transducing phage to be described and three further phages VP-M, VP-2 and VP-T have subsequently been isolated (Relman, M.D. thesis, 1982)). CP-T1 has been examined in considerable detail (Guidolin *et al.*, 1984) and its cell surface receptor has been identified as LPS (Guidolin and Manning, 1985). Bacteriophage VP-M appears to be identical to CP-T1 as seen by restriction analysis and VP-2 differs only slightly from CP-T1. The third generalized transducing phage identified by Relman (M.D. thesis, 1982), VP-T, appears to be totally unrelated to either of the other phages. CP-T1 is able to transduce widely separated markers and is capable of

propagating on both biotypes. Bacteriophages VP-M and VP-2 are very similar to CP-T1 and would also appear to mediate generalized transduction (Relman,M.D. thesis, 1982).

The generalized transducing bacteriophage CP-T1 packages its DNA into the phage capsid by a "headful packaging" mechanism (Guidolin *et al.*, 1984). In this method packaging of DNA commences at a *pac* site on a concatameric intermediate and continues until the phage capsid is full. The amount of DNA required to fill a head is more than the length of the genome resulting in terminal redundancy. An important feature of this "headful" packaging mechanism is the identification of the *pac* site, from which encapsulation commences (Guidolin and Manning, 1988). The *pac* site is utilized once only on each concatamer to initiate packaging, and subsequent packaging events show no site specificity.

The subcloning of the CP-T1 *pac* site (packaging site) into a transposon could lead to the possibility of high frequency generalized transducing lysates of *V. cholerae*. This would enable chromosomal DNA to be packaged at high frequency and markers could be mapped by a gradient of transduction. By using various *pac* sites in the chromosome as well as different orientations of *pac* it should be possible to accurately map any gene.

1.7.2 Genetic mapping.

Genetic mapping in *V. cholerae* has been limited to the use of the conjugative plasmid P or its derivatives. A linkage map of the Classical strain 162 has been constructed using P factor crosses (Parker *et al.*, 1979), however, this map is limited to seventeen ordered markers and five linked but unordered markers. Due to the low transfer frequencies of chromosomal markers (10^{-5} to 10^{-6}) and that

the nonselected markers are poorly linked to selected markers, this system of mapping chromosomal markers is inefficient.

A system based on Hfr-like donors has been devised (Johnson and Romig, 1979a) and has been the result of construction of Tfr donors (transposon-facilitated-recombination). Hfr donors in *E. coli* are formed by cointegration via a single crossover between homologous insertion sequences on both the F plasmid and bacterial chromosome. Improved *V. cholerae* donors have been constructed by the deliberate introduction of homology by means of insertion of transposon Tn*1* into both the chromosome and the P factor (Johnson and Romig, 1979b). These Tfr donors transfer genes at a high frequency from origins of transfer of P within the chromosome specified by the chromosomal Tn*1* copy. Transfer of the chromosome in both directions is possible by having Tn*1* in opposite orientations within the P factor. These donors have been useful for mapping genes in both biotypes of *V. cholerae* (Sublett and Romig, 1981; Newland *et al.*, 1984a).

The Tfr system has been used to confirm the genetic map of the Classical strain 162 (Sublett and Romig, 1981) which had been constructed by conventional P^+/P^- crosses, to map the position of several loci; *htx* (hyperproduction of cholera toxin) in Classical Ogawa strain 569B (Mekalanos *et al.*, 1979b), *cha* (chicken erythrocyte haemagglutinin), *pmx* (polymyxin B resistance) (Green *et al.*, 1983); the *ura*-201, *ile*-201 and *val*-201 alleles in an El Tor strain (Newland *et al.*, 1985b), the *hly* locus for the haemolysin between *arg* and *ilv* (Goldberg and Murphy, 1984) and the *xds* locus for the extracellular DNase between *pro*-1 and *ile*-201 in an El Tor Ogawa strain (Newland *et al.*, 1985b).

1.8 Ojectives of this study.

The Classical *V. cholerae*-01 strain V58 was first reported by Bhaskaran (1956) and was of interest because of the presence of the P sex factor which had a similar property to the F sex factor of *Escherichia coli* K-12, in that it could transfer chromosomal markers from one cell to another. It's characterization has been greatly neglected over the years with its only usefulness being in the mapping of genes on the *V. cholerae* chromosome in both Classical and El Tor strains. The examination of any plasmids from *V. cholerae* in any depth has not been carried out.

It is proposed in this study to examine the three plasmids at both a molecular level and a physical level. This type of study would enable comparisons to be made with other similar plasmids as well as providing a useful tool in probing plasmids within the *Vibrio* genus. A study of other possible properties endowed to these plasmids is proposed as well as examining their relationship with other cryptic plasmids isolated from *Vibrio* species. Another objective of this study is a detailed examination of the potential role played by the P factor in the virulence of *V. cholerae*.

Chapter 2

Materials and Methods

2.1 Growth of bacteria.

2.1.1 Media

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Difco), prepared at double strength (16 g/litre) with added sodium chloride (NaCl) (5 g/litre), or Oxoid, prepared as 10 g/litre peptone, 10 g/litre lab-lemco and 5 g/litre NaCl, were the general growth medium used for *E. coli*, *S. typhimurium* and *Aeromonas* strains. *V. cholerae* strains were grown in Brain Heart Infusion (BHI) (Difco), prepared as directed by the manufacturers. Luria broth (LB) and 1% tryptone broth (TB) were prepared as described by Miller (1972). Minimal A medium (M13 minimal media) was also prepared as described by Miller (1972), and was supplemented prior to use with $MgSO_4$, glucose and thiamine-HCl to 0.2 mg/ml, 2 mg/ml, 50 µg/ml final concentration, respectively.

NA is nutrient agar, which is blood base agar (Difco), prepared without the addition of blood, or Oxoid nutrient broth with the addition of 1.5% agar. BHI agar is BHI broth with the addition of 1.5% agar. Soft agar contains equal volumes of NB and NA.

2.1.2 Antibiotics

Antibiotics were purchased from Sigma (ampicillin, kanamycin sulphate, rifampicin, spectinomycin) and Calbiochem (chloramphenicol, streptomycin sulphate, tetracycline, HCl).

Antibiotics were added to broth and solid media at the following concentrations: ampicillin (Ap), 25 µg/ml; chloramphenicol (Cm), 25 µg/ml; kanamycin (Km), 50 µg/ml; rifampicin (Rif), 100 µg/ml; spectinomycin (Spc), 40 µg/ml; streptomycin (Sm), 100 µg/ml; tetracycline (Tc), 8 µg/ml for *E. coli* and 4 µg/ml for *V. cholerae* strains.

2.1.3 Incubations

Incubations were at 37°C unless otherwise specified. Normally liquid cultures were grown in 20 ml McCartney bottles, or 100 ml side-arm flasks. Optical densities (OD) were measured at 650 nm using a Unicam Instruments spectrophotometer.

2.1.4 Growth rate of bacteria

The growth rate of a bacterial strain during the exponential phase in a particular medium was followed spectrophotometrically and by determination of a viable count every thirty minutes over a 4 hour period.

2.2 Chemicals and Reagents

Chemicals were Analar grade. Phenol, sodium dodecyl sulphate (SDS) and sucrose were from BDH Chemicals. Tris was Trizma base from Sigma.

The following electrophoresis grade reagents were obtained from the sources indicated: HGT and Sea Plaque agarose (Seakem), acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N'-methylene-bis-acrylamide and urea from BBL.

The sodium salt of adenosine-5'-triphosphate (ATP) and dithiothreitol (DTT) were obtained from Sigma.

Radionucleotide α-[^{32}P]-dATP was from BRESA and [^{35}S]-methionine was from Amersham.

Phosphorylated linkers (1 unit = 40 - 50 µg) *Nde*I (8-mer) and *Eco*RI (8-mer) obtained from Biolabs in lyophilized form were resuspended in 0.1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA) pH8.0 and stored frozen at -20°C.

2.3 Enzymes

The following enzymes were purchased from Sigma: deoxyribonuclease I (DNase I), ribonuclease A (RNase A) and lysozyme. Pronase was from Boehringer-Mannheim.

2.3.1 Restriction endonucleases

The majority of restriction endonucleases were purchased from Boehringer-Mannheim or Pharmacia except for *Aha*III, *Ava*I, *Bcl*I, *Nde*I, *Sca*I, *Ssp*I and *Stu*I which were purchased from Biolabs.

2.3.2 DNA modifying enzymes

DNA modifying enzymes were purchase from Biolabs (T4 DNA ligase), Amersham (T4 DNA polymerase) and Boehringer-Mannheim (DNA polymerase I; alkaline phosphatase (calf intestinal), molecular biology grade; and Klenow fragment of DNA polymerase I).

2.4 Bacterial strains and Plasmids

Vibrio cholerae strains used are listed in Table 2.1. Strains of the El Tor biotype were distinguished from the Classical biotype by resistance to the antibiotic polymyxin B (50 units/ml) and sensitivity to biotype specific typing phages. Table 2.2 describes the *Escherichia coli*, *Salmonella typhimurium* and *Aeromonas* strains used in this study. Cultures were stored lyophilised in ampoules or frozen at -70°C after

Table 2.1

Vibrio cholerae STRAINS

Strain	Biotype/Serotype	Genotype/Phenotype	Source/Reference
V58	Classical Ogawa	*ilv arg his*	K. Bhaskaran
V58 P$^-$	Classical Ogawa	*ilv arg his*	K. Bhaskaran
V498	Classical Ogawa	V58 Spc^R	this study
V499	Classical Ogawa	V58 Rif^R	this study
V507	Classical Ogawa	V498[P::Tn*3*-1]	this study
V508	Classical Ogawa	V498[P::Tn*10*-1]	this study
V509	Classical Ogawa	V499[P::Tn*3*-1]	this study
V510	Classical Ogawa	V499[P::Tn*10*-1]	this study
KB9 V$^+$	Classical Ogawa	*ilv arg his str*	B.S. Srivastava
569B	Classical Inaba	*str*	K. Bhaskaran
V685	Classical Inaba	569B Rif^R	this study
V628	Classical Inaba	569B Spc^R	this study
569B P$^+$	Classical Inaba		R.K Holmes
569B P$^+$V$^+$	Classical Inaba		B.S. Srivastava
017	El Tor Ogawa	*str*	K. Bhaskaran
RV69	Classical Ogawa	P$^+$ *arg1 his1 ilv1*	C.Parker
1621	El Tor Ogawa	*met lys* Rif^R	J. Ogg
V135	El Tor Ogawa	P::Tn*3*-1 *met lys* Rif^R	this study
V136	El Tor Ogawa	P::Tn*10*-1 *met lys* Rif^R	this study
1621	El tor Ogawa		J. Ogg (1978)
V108	El Tor Ogawa	1621[P::Tn*3*-1]	this study
V109	ElTor Ogawa	1621[P::Tn*10*-1]	this study

Table 2.1 (continued)

Vibrio cholerae STRAINS

Strain	Biotype/Serotype	Genotype/Phenotype	Source/Reference
CD63	Classical Inaba	569B P::Tn*1*	B.S. Srivastava
CD17	El Tor Inaba	wild type prototype type phage IV^R	B.S. Srivastava
CD17 P$^+$	El Tor Inaba	wild type prototype type phage IV^R	B.S. Srivastava
CA401	Classical Inaba		C. Parker
V697	Classical Inaba	CA401 Rif^R	this study
CA411	Classical Ogawa		C. Parker
V697	Classical Ogawa	CA411 Rif^R	this study

Genetic markers are as described in Parker *et al.* (1979)

Table 2.2

OTHER BACTERIAL STRAINS

Strain	Genotype/Phenotype	Source/Reference
Escherichia coli K-12		
LE392	F^-, *supF, supE, hsdR, galK,*	
	trpR, metB, lacY, λ^-	Berman *et al.* (1981)
DH1	*gyrA*96, *recA*1, *relA*1, *endA*1,	
	thi-1, *hsdR*17, *supE*44, λ^-, F^-	D. Hanahan (1983) from B. Bachmann (CGSC)
DS410	F^-, *minA, minB, rpsL*	Dougan and Sherratt (1977)
S17-1	RP4 2-Tc::Mu-Km::Tn 7	
	pro, res^-, mod^+	R. Simon (1984)
CSH45	*c*I857*S*am7	Miller (1972)
Salmonella typhimurium		
#4419	*metA*22, *metE*551, *trpD*2,	
	*ilv*2, *ilv*452, *hsdLT*6, *hsdSA*29,	
	*strA*120	Bullas and Ryu (1983)
LB 5010	*galE* derivative of #4419	
	leu, P1 sensitive	Bullas and Ryu (1983)

Table 2.2 (continued)

OTHER BACTERIAL STRAINS

Strain	Genotype/Phenotype	Source/Reference
Aeromonas		
hydrophila	A006	M. Atkinson
sobria	A187	M. Atkinson
caviae	A321	M. Atkinson
caviae	V14	M. Atkinson
unidentified	AB 1	T. Chakraborty
unidentified	AB 3	T. Chakraborty
unidentified	Pappu	T. Chakraborty

Table 2.3

PLASMID CLONING VECTORS

Plasmid	Antibiotic marker	Source/Reference
pACYC184	Cm, Tc	Chang and Cohen, (1978)
pBR322	Ap, Tc	Bolivar *et al.*, (1977)
pJRD158b	Ap, Tc	Davison *et al.*, (1984)
pSC101	Tc	Cohen *et al.*, (1973)
pRK290	Tc	Ditta *et al.* (1980)
pLG339	Km, Tc	Stoker *et al.*, (1982)
pSUP202-1	Ap, Cm	Simon *et al.*, (1984)
pSUP301	Ap, Km	Simon *et al.*, (1984)
pSUP401	Cm, Km	Simon *et al.*, (1984)

Table 2.4

TRANSPOSONS

Transposons	Antibiotic marker	Source of Transposon	Source/Reference
Tn*3*	Ap	R1-19	P. Reeves
Tn*5*	Km	F'$_{ts}$ *lac*::Tn*5*	Sansonetti *et al.*, (1981)
Tn*10*	Tc	R100-1	P. Reeves
Tn*1725*	Cm, Km	pRU669	Ubben and Schmitt (1986)
Tn*1736Tc*	[Cm^R]^a, Tc, Ap	pRU885	Ubben and Schmitt (1987)
Tn*1732*	Km	pRU664	Ubben and Schmitt (1987)

a expressed only from an external promoter

resuspending bacterial growth from plates in a mixture of 0.4 ml 80% (v/v) glycerol plus 0.6 ml 1% peptone (Difco, Bacto-peptone). Working cultures were stored in glycerol at −25°C. The plasmids and transposons used are listed in Tables 2.3 and 2.4 respectively.

2.5 Electron Microscopy

DNA was spread in 0.5 M ammonium acetate at neutral pH following the method of Kleinschmidt and Zahn (1959). Electron micrographs were taken in a Philips EM301 transmission electron microscope.

2.6 Transformation procedure

This is basically a modification of the procedure of Brown *et al* (1979). *E. coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaking culture (in NB) was diluted 1:20 into NB and incubated with shaking until the culture reached an OD_{650} of 0.6. The cells were chilled on ice for 20 min, pelleted at 4°C in a bench centrifuge, resuspended in half volume of cold 100 mM $MgCl_2$, centrifuged again and resuspended in a tenth volume of cold 100 mM $CaCl_2$. The cells were allowed to stand for 60 min on ice, after which time they were considered as competent. Competent cells (0.2 ml) were mixed with DNA (volume made to 0.1 ml with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and left on ice for a further 30 min. The cell/DNA mixture was heated at 42°C for 2 min, chilled on ice for 15 min, 3 ml NB was added and this was then incubated with shaking at 37°C for 1-2 hr. The culture was plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were run as a control.

2.7 DNA isolations

2.7.1 P Factor DNA isolation

P factor DNA was isolated from *V. cholerae* cells using the technique of Meyers *et al.* (1976). Cells from a 1 litre culture were harvested (4,500 rpm, 15 min, 4°C, in a MSE Coolspin) and resuspended in 50 ml of 25% sucrose in 10 mM Tris-HCl 1 mM EDTA pH 8.0 and transferred to a 250 ml Ehrlemeyer flask on ice. Freshly prepared lysozyme (10 ml of 5 mg/ml in 0.25 M Tris-HCl pH 8.0) was mixed in gently and incubated for 5 min. 20 ml of 0.25 M EDTA pH 8.0 was then added and incubated for a further 5 min after which the cells were lysed with 8 ml of 10% SDS. The SDS was mixed in gently by swirling until the solution became viscous. Chromosomal DNA was precipitated with the addition of 17.5 ml of 5 M NaCl, swirling thoroughly until the entire solution had a whitish appearance. This mixture was left at 4°C overnight after which the precipitated chromosomal DNA was pelleted at 15,000 rpm, 20 min, (SS34, Sorvall). The supernatant was retained and CsCl was added to a density of 0.93 gm/ml. Ethidium bromide (10 mg/ml) was added at 0.5 ml/10 ml. The DNA was banded by centrifugation at 50 K in a 60 Ti Beckman rotor, at 15°C for 20 hr. The DNA band was removed by side puncture of the tube with a 19G needle attached to a 10 ml syringe. This DNA was then rebanded at 50 K in a 80 Ti tube at 15°C for 20 hr. The DNA band was removed as above, the ethidium bromide was extracted using CsCl saturated isopropanol and the CsCl removed by dialysis overnight against 2 litres of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C (with two changes of buffer). DNA was stored at 4°C.

A small scale version of this extraction procedure was used for rapid screening of the presence of P factor in *V. cholerae* strains. 3 ml of an overnight culture was pelleted in an Eppendorf tube and lysed,

appropriately scaled down as described above. The chromosomal DNA was precipitated by incubation on ice for 2-4 hr followed by centrifugation. The supernatant containing the P factor DNA was extracted with phenol once and precipitated by the addition of one tenth volume 3 M sodium acetate and two volumes of absolute ethanol and standing at -70°C for 30 min. The precipitate was collected by centrifugation for 15 min (Eppendorf 5414), washed once with 1 ml of 70% ethanol, dried under vacuum and resuspended in 20 µl TE buffer.

2.7.2 Purified plasmid vector DNA

Plasmid DNA was purified by a three step alkali lysis method (Garger *et al.*, 1983). Cells from a 1 litre culture were harvested (6,000 rpm, 15 min, 4°C, Sorvall GS-3 rotor) and resuspended in 24 ml of solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). Freshly prepared lysozyme (4 ml of 20 mg/ml in solution 1) was mixed with the cell suspension and incubated at room temperature for 10 min. Addition of 55 ml of solution 2 (0.2 N NaOH, 1% SDS) followed by 5 min incubation on ice resulted in total lysis of the cells. After the addition of 28 ml of solution 3 (5 M potassium acetate pH 4.8) and incubation on ice for 15 min, protein, chromosomal DNA and high molecular weight RNA were removed by centrifugation (8,000 rpm, 20 min, 4°C in a Sorvall GSA rotor). The supernatant was then extracted with an equal volume of a TE saturated phenol, chloroform, isoamyl alcohol mixture (25:24:1). Plasmid DNA from the top aqueous phase was precipitated with 0.6 volume of iso propanol at room temperature for 10 min, and collected by centrifugation (10,000 rpm at 4°C for 35 min in a Sorvall GSA rotor). After washing in 70% ethanol, the pellet was dried under vacuum and resuspended in 4.6 ml TE. Plasmid DNA was purified from contaminating protein and RNA by centrifugation on a two step CsCl ethidium bromide gradient according to Garger *et al.*

(1983). The DNA band was removed by side puncture of the tube with a 19G needle attached to a 1 ml syringe. The ethidium bromide was extracted using CsCl saturated isopropanol. CsCl was then removed by dialysis overnight against 2 litres TE at 4°C with two changes of the buffer. DNA was stored at 4°C.

2.7.3 Plasmid DNA for screening

Plasmid DNA for the screening of inserts was extracted from *E. coli* cells by either of two methods.

Method 1.

Rapid plasmid preparation by the boiling method of Holmes and Quigley (1981) was performed as follows: Cells from 1 ml of a 10 ml shaken overnight culture were pelleted in an Eppendorf tube in an Eppendorf 5414 centrifuge for 30 sec, resuspended in 50 µl STET buffer (5% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0). Lysozyme (5 mg/ml) (5 µl) was added and the samples were placed in boiling water for 35 sec and immediately spun for 10 min in an Eppendorf centrifuge. The chromosomal pellet was removed and plasmid in the supernatant precipitated with 0.6 volumes of propan-2-ol at -20°C for 10 min. DNA was pelleted by centrifugation in an Eppendorf for 10 min, washed once with 1 ml of 70% ethanol, dried under vacuum and dissolved in 20 µl TE buffer.

Method 2.

Triton X-100 cleared lysates were prepared by a modification of the procedure of Clewell and Helinski (1969, 1970) from 10 ml overnight cultures following sedimentation of cells in a bench centrifuge. Cells were frozen, thawed and resuspended in 0.4 ml 25% sucrose, 50 mM Tris-HCl pH 8.0. Lysozyme (50 µl, 10 mg/ml freshly prepared in H_2O) and 0.25 M EDTA pH 8.0 (50 µl) were added to cells

in microfuge tubes and left to stand on ice for 15 min. 0.5 ml of TET buffer (50 mM Tris-HCl, 66 mM EDTA pH 8.0, 0.4% Triton X-100) was added followed by a brief mixing by inversion of the tubes. The chromosomal DNA was then pelleted by centrifugation (15,000 rpm, 20 min, 4°C, in a Sorvall SS34 rotor). The supernatant was extracted twice with TE saturated phenol and twice with diethyl ether. Plasmid DNA was precipitated by the addition of an equal weight of iso propanol and standing at -70°C for 30 min. The precipitate was collected (10 min, Eppendorf 5414), washed once with 1 ml 70% ethanol, dried under vacuum and resuspended in 50 µl TE buffer.

2.7.4 Genomic DNA from *V. cholerae*

Genomic DNA was prepared essentially as described by Manning *et al.*, (1985). Cells from a 20ml shaken overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl). The pellet was then resuspended in 2 ml 25% sucrose, 50 mM Tris-HCl pH 8.0, and 1 ml lysozyme (10 mg/ml in 0.25 M EDTA pH 8.0) was added and the mixture incubated on ice for 15 min. TE buffer (0.75 ml) and 0.25 ml lysis solution (5% sarkosyl, 50mM Tris-HCl, 0.25 M EDTA pH 8.0) was added together with 10 mg solid pronase. The mixture was gently mixed, transferred to a 50 ml Ehrlenmeyer flask and incubated at 56°C for 60 min. This was followed by two extractions each with TE saturated phenol and diethyl ether. The genomic DNA was then precipitated from the solution by the addition of two volumes of cold 95% ethanol. The precipitate was washed twice with 70% ethanol, dried under vacuum for 60 min and allowed to resuspend in 1 ml TE buffer. This was achieved by heating to 56°C for several minutes. This generally yielded high molecular weight DNA at concentrations ranging from 0.5-1 mg/ml.

2.7.5 Preparation of lambda DNA

(i) Preparation of lambda phage

Lambda phage were prepared essentially as described by Miller (1972). An overnight culture of temperature sensitive lysogenic strain (*c*I857*Sam*7) of lambda was diluted 1:20 in 500ml of NB in a 2 litre Ehrlenmeyer flask and grown to early exponential phase by shaking at 30°C. The culture was heat induced at 42°C for 20 minutes standing in a water bath with occasional swirling followed by a further incubation with shaking at 37°C for 3 hours. The cells were tested for induction before proceeding further by removing 1 ml and adding 50 µl of chloroform which should result in lysis. If lysis occurred the cells were harvested by centrifugation and resuspended in 50 ml TM buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$) and transferred to a metal Sorvall GSA bucket. 7.5 ml of chloroform was added to the cell suspension which was incubated with shaking at 37°C for 30 minutes. Cellular debris was collected by centrifugation (8,000 rpm, 15 min at 4°C) and the phage in the supernatant were precipitated with PEG6000 (10% (w/v) and 0.5 M NaCl) overnight at 4°C. Precipitated phage were pelleted and allowed to resuspend by leaving 5 ml of TM buffer on the pellet overnight at 4°C. This phage suspension was banded on a 3 step CsCl gradient (56%, 45% and 31% CsCl w/w in TM buffer) in a SW41 rotor at 34 K for 1 hour. The phage band was removed by side puncture of the tube with a 19G needle attached to a 1 ml syringe. CsCl was removed by consecutive dialysis against NaCl (4 M, 2 M and 1 M in TM buffer), 1 hour each at 4°C with a final dialysis against TM buffer overnight at 4°C.

(ii) Extraction of lambda DNA

Extraction of bacteriophage lambda DNA was essentially as describe by Maniatis *et al.* (1982). Bacteriophage were lysed by the addition of EDTA pH 8.0 to a final concentration of 20 mM, pronase to a final concentration of 50 µg/ml and SDS to a final concentration of 0.5%. This was mixed by inverting several times and incubated for 1 hour at 37°C. The mixture was extracted once with TE saturated phenol, the aqueous phase was then extracted once with a 50:50 mixture of phenol and chloroform and once with chloroform. The aqueous phase containing the lambda DNA was dialysed against three 1000-fold volumes of TE buffer.

2.8 DNA manipulations

2.8.1 DNA quantitation

The concentration of DNA was determined by measurement of absorption at 260 nm and assuming an A_{260} of 1.0 is equal to 50 µg DNA/ml (Miller, 1972).

2.8.2 Digestion of DNA by restriction endonucleases

Cleavage reactions with restriction enzymes were performed using SPK buffer (10X stock is 200 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 5 mM dithioerythritol, 1 mM EDTA, 500 mM KCl and 50% glycerol). 0.1-0.5 µg of DNA or purified restriction fragments were incubated with 1 unit of each restriction enzyme in a final volume of 20 µl, at 37°C for 1-2 hours. The reactions were terminated by heating at 65°C for 10 min. Prior to loading onto a gel a one tenth volume of tracking dye (15% Ficoll, 1 mg/ml bromophenol blue) was added.

2.8.3 Analytical and preparative separation of restriction fragments

Electrophoresis of digested DNA was carried out at room temperature on horizontal, 0.6%, 0.8% or 1.0% agarose gels (Seakem HGT), 13 cm long, 13 cm wide and 0.7 cm thick. Gels were run at 100V for 3-4 hr in TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8). After electrophoresis the gels were stained in distilled water containing 2 µg/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light (354 nm) and photographed on either Polaroid 667 positive film or 665 negative film.

For preparative gels Sea Plaque (Seakem) low gelling temperature agarose at a concentration of 0.6% was used for separation of restriction fragments. DNA bands were excised and the agarose melted at 65°C. TE saturated phenol was added, mixed in thoroughly and left at room temperature for 15 min and then spun in an Eppendorf centrifuge to separate the phases. The aqueous phase containing the DNA was transferred to a new tube and re-extracted with phenol until no precipitate at the interphase remained. The DNA was precipitated with one tenth volume 3M sodium acetate and two volumes of ethanol at -70°C for 2 hours. The DNA was pelleted, washed in 70% ethanol, dried under vacuum and resuspended in 20 µl of TE buffer.

2.8.4 Size determination of restriction fragments

The sizes of restriction fragments were determined by comparing their relative mobilities on the gels with those of DNA molecules of known size. Fragments generated by the action of each restriction endonuclease are numbered sequentially from the largest to the smallest fragment according to their relative molecular mobilities on agarose gels (eg. *Eco*RI-1, *Eco*RI-2 etc.). The standards used were lambda DNA cleaved with *Eco*RI, *Kpn*I, *Hin*dIII, *Bam*HI, *Sac*I, *Nhe*I and *Xba*I (Philippsen *et al.*, 1978) and *Bacillus subtilis* phage SPP1 cut with

*Eco*RI (Ratcliff *et al.*, 1979; Franzon and Manning, 1986). Sizes of large fragments were calculated from the sum of the sizes of their sub-fragments obtained after digestion with a second enzyme.

Lambda DNA was prepared as described above and bacteriophage SPP1 DNA was from laboratory stocks and prepared similarly.

2.8.5 Alkaline phosphatase treatment of vector DNA.

3 µg digested vector DNA was incubated with 1 unit of alkaline phosphatase for 20 min at 37°C. The reaction was stopped with 5 mM EDTA pH 8.0 and heated to 65°C for 10 min. The DNA was extracted once with phenol followed by one ether extraction and precipitated with two volumes of ethanol and one tenth volume of 3 M sodium acetate.

2.8.6 *In vitro* cloning of P factor DNA

Purified P factor DNA (3 µg) was cleaved with either *Eco*RI or *Xba*I restriction enzymes. This was combined with 1 µg of similarly cleaved pACYC184 or pJRD158b plasmid DNA (alkaline phosphatase treated), respectively, then ligated using 1 unit of T4 ligase in a volume of 50 µl in a final buffer concentration of 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.6 mM ATP for 16 hours at 4°C. The ligated DNA was then used directly for transformation of strain LE392. Transformants were screened for insertional inactivation of the CAT gene with pACYC184 prior to plasmid isolation. Clones were reintroduced into strain LE392.

2.8.7 Nick translation method

Nick translation reactions with DNA polymerase I were modified from Maniatis *et al.* (1982) and carried out as follows: 25 µCi α-[^{32}P]-dCTP (1700 µCi/mmole in ethanol) was dried in vacuo in a microfuge tube, resuspended with 80 µl water, 10 µl 10X nick

translation buffer (500 mM Tris-HCl pH 7.2, 100 mM $MgCl_2$, 1 mM DTT, 500 µg/ml BSA) 1 µl each of 2 mM dATP, dGTP and dTTP. DNA (1 µg) to be nick translated was added. To this mixture 1 µl DNase (0.1 µg/ml) was added and incubated at 37°C for 10 min. DNA polymerase I (5 units) was added into the reaction mix, and allowed to incubate at 16°C for 2 hr. DNA was separated from unincorporated label by centrifugation through a small column of Sepharose CL-6B (Pharmacia).

2.8.8 Southern transfer and hybridization

Unidirectional and bidirectional transfer of DNA from agarose gels to nitrocellulose paper (Schleicher and Schüll) was essentially as described by Maniatis *et al.* (1982).

Prior to hybridization with radiolabelled probe, filters were incubated for 4 hr at 44°C in a prehybridization solution containing 50% formamide, 50 mM sodium phosphate buffer, pH 6.4, 5X SSC (0.34 M NaCl, 75 mM sodium citrate, pH 7.0), 5X Denhardt's reagent and 83 µg/ml single stranded Herring sperm DNA (Sigma) (Maniatis *et al.*, 1982). Prehybridization fluid was discarded and replaced with a fresh hybridization buffer (as for prehybridization solution, with the exclusion of Herring sperm DNA). Denatured probe (approximately 10^6 cpm) was added and hybridization allowed to occur for 16-24 hr at 44°C.

Filters were washed twice with shaking at 37°C for 30 min in 2X SSC containing 0.1% SDS. This was followed by two further washes in 0.2X SSC. After drying in air (15 min, room temperature) filters were covered in plastic wrap and placed on film for autoradiography at -70°C with intensifying screens.

2.8.9 End-filling or end-labelling with Klenow fragment

Protruding ends created by cleavage with *Hin*dIII and *Eco*RI were filled in using the Klenow fragment of *E. coli* DNA polymerase I. Typically, 1 µg of digested DNA, 2 µl 10X nick-translation buffer (Maniatis *et al.*, 1982), 1 µl of each dNTP at 2 mM and 1 unit Klenow fragment were mixed and incubated at 37°C for 30 min. The reaction was stopped by heating at 60°C for 10 min, followed by removal of unincorporated dNTP's and enzyme by centrifugation through a Sepharose CL-6B column (Pharmacia). End-labelling of such protruding ends was carried out as above using α-[^{32}P]-dCTP.

2.8.10 End-filling or end-labelling with T4 DNA polymerase

3' protruding ends created by cleavage with *Pst*I and 5' ends were converted to blunt ends with T4 DNA polymerase in a final volume of 25 µl containing 2 µg DNA, 2 units T4 DNA polymerase, 1 µl of each dNTP at 2 mM and 2.5 µl 10X T4 DNA polymerase buffer (Maniatis *et al.*, 1982). After a 5 min incubation at 37°C the reaction was stopped by heating at 70°C for 10 min. Salt, unincorporated nucleotides and enzyme were removed by passage through a Sepharose CL-6B (Pharmacia) column. End-labelling of such ends was carried out as above using α-[^{32}P]-dCTP.

2.8.11 Ligation of linkers to blunt DNA ends

Phosphorylated *Eco*RI and *Nde*I linkers were ligated to blunt ends generated by T4 polymerase or Klenow fragment by overnight incubation of 1 µg plasmid DNA with approximately 0.05 µg linkers and 400 units T4 DNA ligase in a final volume of 10 µl of 1x linker-kinase buffer (Maniatis *et al.*, 1982).

2.8.12 Manipulations with the cholera toxin operon.

The *ctxAB* operon encoding the structural genes for CT (A and B subunits) including the promoter and regulatory region for these genes has been subcloned on a 5.1 kb *Pst*I–*Eco*RI fragment from Classical strain 569B into pBR322 resulting in pJM17 (Mekalanos *et al.*, 1983).

In order to examine the effect of the P and V factors on CT production, the chloramphenicol acetyltransferase gene (CAT) has been introduced downstream of the *ctxAB* promoter. Two approaches were used in this construction: insertion of a promoter detecting transposon, Tn*1736Tc* and the direct insertion of the CAT gene after the *ctxAB* promoter.

2.9 pPM854 construction

The 5.1 kb fragment of the *ctxAB* operon was subcloned into the mobilizable vector pSUP301 (Simon, 1984). The *Eco*RI site of pJM17 was cleaved, end-filled with Klenow and the addition of *Pst*I linkers enabled the fragment to be subcloned into pSUP301 which results in the inactivation of the Bla gene. Transformants were screened for Km^R and Ap^S, plasmid DNA extracted and examined for the presence of the *ctxAB* fragment. One such isolate was pPM854.

2.10 Promoter detection using Tn*1736Tc*

Insertion of the promoter detecting transposon, Tn*1736Tc* (Ubben and Schmitt, 1987) downstream from the *ctx* promoter in plasmid pPM854 was essentially as described by Ubben and Schmitt (1987). The temperature sensitive plasmid pME305::Tn*1736Tc* (pRU885) was introduced into LE392[pPM854] via conjugation and transconjugants were selected on NA + Km, Tc at 30°C. Plasmid DNA was extracted from 6 individual transconjugants cultured at 42°C, transformed into competent

LE392 cells and fusion-positive transformants were selected on plates containing a low concentration of Cm (10 µg/ml) and Km (50 µg/ml). Single transformants were cultivated for determination of minimal inhibitory concentration (MIC) of chloramphenicol and restriction mapping of the pPM854::Tn*1736Tc* insertions.

2.11 Construction of pPM860, a *ctxAB* deletion

A second approach to measure the effects on the *ctxAB* promoter was by the deletion of part of the *ctxA* and *ctxB* genes and the insertion of the CAT cartridge. Plasmid pPM1603 is a derivative of pUC19 with the CAT cartridge inserted into the *Bam*HI site (courtesy of A. Barker, this laboratory). Plasmid pPM854 was subjected to digestion with *Xba*I and *Sac*I which removes a 1.9 kb internal fragment of the *ctxAB* operon which was replaced with the 0.9 kb *Xba*I-*Sac*I fragment from pPM1603 which includes the CAT cartridge. The resultant ligation was transformed into competent LE392 cells and transformant colonies screened for the plasmid with the correct insert. One such isolate is pPM860 which has the CAT cartridge inserted in the correct orientation for it to be transcribed from the *ctxAB* promoter.

2.12 Protein procedures

2.12.1 Whole cell extracts

Cells from 1 ml of a 10 ml shaken overnight culture were pelleted in an Eppendorf tube and resuspended in 100 µl of 1x sample buffer (Lugtenberg *et al.*, 1975). A sample of 10 µl was run on a SDS polyacylamide gel.

2.12.2 Small scale cell envelope isolation

Whole membrane material was isolated from 10 ml mid-exponential phase cultures by the method in Manning *et al.* (1982). The cultures were centrifuged for 10 min in a bench centrifuge and the cells washed with 10 ml 30 mM Tris-HCl pH 8.1. The pellet was resuspended in 0.2 ml 20% sucrose, 30 mM Tris-HCl pH 8.1, and the suspension transferred to SM24 tubes (Sorvall) on ice. After the addition of 20 µl lysozyme (1 mg/ml in 0.1 M EDTA pH 7.3) incubation was continued on ice for 30 min. The cells were then placed in an ethanol-dry ice bath for 30 min. After thawing, 3 ml of 3 mM EDTA pH 7.3 was added and the cells sonicated in a Branson sonicator (four, 15 sec pulses on 50% cycle). Membrane material was pelleted by centrifugation (20,000 rpm, 60 min, 4°C, Sorvall) and resuspended in 100 µl 1x sample buffer (Lugtenberg *et al.*, 1975). Samples were stored at -20°C. 10-15 µl amounts were loaded onto SDS-polyacrylamide gels.

2.12.3 Small scale cell fractionation

Whole cells were divided into constituent fractions of periplasmic, cytoplasmic and membrane fractions from 10 ml of cells. Cells from a 10 ml overnight culture were pelleted in a bench centrifuge, washed in 10 ml of 30 mM Tris-HCl pH 8.1 and resuspended in 0.2 ml of 20% sucrose 30 mM Tris-HCl pH 8.1. They were then transferred to a SM24 tube (Sorvall) and kept on ice. Whole cells were converted to sphaeroplasts with 0.02 ml 1 mg/ml lysozyme in 0.1 M EDTA pH 7.3 and incubated on ice for 30 min. Centrifugation at 10 K for 10 min pelleted the sphaeroplasts and the supernatant was retained as the periplasmic fraction. The pellet was frozen in an ethanol-dry ice bath for 30 min and after thawing 3 ml of 3 mM EDTA pH 7.3 was added and vortexed to disperse the pellet. Cells were sonicated in a Branson sonicator (four, 15 sec pulses on 50% cycle) and the mixture was spun

at 35 K in a Beckman 50 Ti rotor for 60 min in a L8-80 ultracentrifuge. The supernatant was retained as the cytoplasmic fraction and the pellet represents the membrane fraction.

A sample of the whole cells (1 ml) was treated as for whole cell extracts. Periplasmic and cytoplasmic fractions are concentrated by precipitating the proteins with 5% TCA and incubating on ice for 30 min. Proteins were pelleted in a microfuge and resuspended in 100 µl of 1x sample buffer (Lugtenberg *et al.*, 1975). All samples were stored at -20°C.

2.12.4 Outer and inner membrane separation

This is a modification of the method by Achtman *et al.* (1979). Cells were grown to mid-exoponential phase in 100 ml of BHI, pelleted and resuspended in one tenth volume of minimal medium. These cells were labelled with 25 µCi of [^{35}S]-methionine, pelleted and resuspended in 5 ml 0.75 M sucrose in 10 mM Tris-HCl pH 7.8. An equal volume of sphaeroplasting solution was added (100 µg/ml lysozyme in 40 mM EDTA pH 8.0) and stirred slowly at 4°C for approximately 15 min. Formation of sphaeroplasts was checked microscopically by phase contrast. Sphaeroplasts were frozen and thawed resulting in a viscous solution. If the solution was too viscous a very small amount of DNase was added and incubated at 37°C for 5-10 min. This mixture was diluted with 10 mM Tris-HCl, 10 mM EDTA, pH 7.8 and centrifuged at 50 K in a 50 Ti rotor for 90 min in a L8-80 ultracentrifuge. The supernatant was retained as the periplasmic/cytoplasmic fraction. Proteins in this sample were precipitated in 5% TCA. The pellet was resuspended in 400 µl 25% sucrose (wt/wt) 10 mM Tris-HCl pH 7.8 10 mM EDTA (a 50 µl sample was retained) and loaded onto a 30-55% (wt/wt) sucrose step gradient (in 10 mM Tris-HCl pH 7.8). The gradient was centrifuged in a SW41 rotor at 32 K for 18 hours in a L8-80 ultracentrifuge.

Fractions (0.5 ml = 15 drops) were collected dropwise from the bottom of the tube.

The refractive index of the fractions was determined to give an indication of the gradient and measurement of the number of counts in each fraction gives an indication of the distribution of protein.

Fractions were diluted 1:4 with water and proteins precipitated with 5% TCA. A 10 µl sample of each fraction was run on a polyacrylamide gel to determine the distribution of proteins. This polyacrylamide gel can be autoradiographed after being dried down onto Whatman 3MM paper.

2.12.5 Minicell procedures

Minicells were purified and the plasmid-encoded proteins labelled with [^{35}S]-methionine as described by Kennedy *et al.* (1977) and Achtman *et al.* (1979). This involved the separation, from a 500 ml overnight culture in LB medium, of minicells from whole cells by centrifugation through two successive sucrose gradients prepared by freezing and thawing 20% sucrose in BSG (0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, 100 µg/ml gelatine). After preincubation to allow for degradation of long lived mRNAs the minicells were then pulse-labelled for 60 min with 25 µCi [^{35}S]-methionine. Minicells were subsequently solubilized by heating at 100°C in 100 µl of 1x sample buffer (Lugtenberg *et al.*, 1975).

2.12.6 SDS polyacrylamide gel electrophoresis (PAGE)

SDS-polyacrylamide gel electrophoresis was performed on either 11-20% linear gradients or 15% polyacrylamide gels using a modified procedure of Lugtenberg *et al.* (1975) as described by Achtman *et al.* (1978). Samples were heated to 100°C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 4 hours. Staining of

proteins was achieved by incubation in Coomassie Brilliant blue G250 (0.06%) in 5% periodic acid overnight with gentle agitation. Destaining was with several changes of 5% acetic acid and gentle agitation for 24 hours.

Molecular weight markers were either from Pharmacia: phosphorylase B (94 kDal), bovine serum albumin (67 kDal), ovalbumin (43 kDal), carbonic anhydrase (30 kDal), soybean trypsin inhibitor (20.1 kDal) and lysozyme (14.4 kDal) or from Sigma: myosin (205 kDal), β-galactosidase (116 kDal), phosphorylase B (97.4 kDal), bovine albumin (66 kDal), egg albumin (45 kDal), pepsin (34.7 kDal), carbonic anhydrase (29 kDal), trypsinogen (24 kDal), β-lactoglobulin (18.4 kDal) and lysozyme (14.3 kDal).

2.12.7 Autoradiography

SDS-PAGE gels were dried down on Whatman 3MM chromatography paper at 60°C for 2 hr on a Bio-Rad gel drier. For [^{35}S]-methionine, autoradiography was performed at room temperature for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [^{32}P], the gels were exposed to film for 6-72 hr at -20°C, using intensifying screens.

2.13 Transposon mutagenesis

2.13.1 Transposition with Tn*1725*

Tn*1725* (Cm^R) transposition to plasmid DNA was performed in the following manner: pRU669 (R*ts*1::Tn*1725*) (Ubben and Schmitt, 1986) was transferred into an *E. coli* K-12 derivative harbouring the target plasmid by mating for 3 hr at 30°C in standing culture consisting of 0.1 ml of an overnight culture of MM294[pRU669], 0.9 ml LE392[pPM804] and 1 ml NB broth. Following plating of 0.1 ml of mating mix on NA

containing Cm and Tc, independent exconjugants were purified, and used for growing up an overnight culture at 37°C selecting for both the transposon (Cm^R) and the plasmid (Tc^R). Triton X-100 lysates (10 ml) prepared from these cultures were used to transform LE392 again selecting for both the plasmid and the transposon. Transformants were incubated at 37°C and independent transformants were taken for analysis.

2.13.2 Transposition with Tn*5* and Tn*10*.

Transposition with these transposons was carried out in a similar manner as for Tn*1725*. R plasmid R100-1 was used as a source of Tn*10* (Tc^R) and F_{ts}*lac*::Tn*5* (Sansonetti *et al.*, 1981) was used as a source of Tn*5* (Km^R).

2.14 Bacteriophage methods

2.14.1 Plaque assay

Indicator bacteria (0.1 ml), from a freshly grown culture were pre-incubated with bacteriophage (0.1 ml of neat or 100-fold dilutions) at 37°C for 10 min. 4 ml soft agar (44°C) was then added and poured as an overlay onto a NA plate. The plates were incubated inverted at 37°C.

2.14.2 Plate propagation of bacteriophage

Bacteriophage (0.1 ml of an appropriate dilution to give almost confluent lysis) and indicator bacteria (0.1 ml, from a freshly grown culture) were preincubated at 37°C for 10 min, mixed with 5 ml of soft agar (44°C) and poured as an overlay onto a NA plate. After incubation at 37°C the overlay was removed from the NA plate, 2-3 ml of NB added, thoroughly mixed and centrifuged to pellet the agar. The supernatant was filtered through a 0.45 μm Millex filter (Millipore

Corp.) to remove any contaminating bacteria. This was then titrated and used as a phage stock.

2.14.3 Bacteriophage efficiency of plating

Relative efficiencies of plating (e.o.p.) were determined by titrating each phage in soft agar overlays on both the parent and the strains of interest. These titres were compared to the titre of the phage on the appropriate propagating strain, which was assigned an e.o.p. of 1.

2.15 Serum resistance

Serum resistance was tested using the microassay method as described by Moll *et al.*, (1979). Human serum was used at 2 fold dilutions.

2.16 Metal resistance

Resistance to metals was tested using concentrations as suggested by Trevors *et al.*, (1985). Each bacterial strain was barstreaked across a gradient plate, prepared by pouring a NA plate of which half is removed and replaced by NA containing the appropriate metal salt.

2.17 Ileal loops.

Enteropathogenicity in ileal loops of adult rabbits was carried out essentially as described by Sinha and Srivastava (1978). Bacterial strains were grown to early exponential phase in BHI broth and 1 ml samples were injected into closed ileal loops of adult rabbits. Autopsies were performed after 18 hours. Accumulation of fluid was usually observed with a virulent strain and ileal loops with accumulated fluid were scored as positive and loops without fluid as negative. Fluid accumulation ratio was the volume of fluid (ml) per cm length of ligated loop. Viable counts were performed to determine the

inoculum injected into each loop. Rabbits were deprived of food for 24 hours and of water 12 hours before the experiments.

2.18 Motility test for *V. cholerae*

2.18.1 Soft agar overlay assay

Motility was tested by swarming of the bacteria in soft agar and is based on a modification by S.Attridge (PhD thesis, Universtity of Adelaide, 1979) of the sloppy-agar overlay method devised by Stocker (1949). A fresh culture of the test organism was diluted and plated onto NA such that 100-200 colonies would develop per plate. Following overnight incubation, each plate was overlaid with 5 ml of 0.3% soft agar, allowed to set at room temperature and incubated at 37°C for 2-3 hours. Colonies which comprise motile bacteria develop a halo as the bacteria swim in the soft agar overlay.

2.18.2 Capillary test for bacterial chemotaxis.

The chemotactic ability (movement towards an attractant source) and motility of a bacterium are directly related. The capillary test for chemotaxis of Freter and O'Brien (1981a) was employed using glucose and L-methionine as attractants.

2.19 Baby mouse protection assay.

Virulence of strains was determined by the infant mouse model as described by Attridge and Rowley (1983a) adapted from Ujiiye *et al.* (1968).

The baby mice (6-8 days old) were removed from their parents about 6 hours before use, to permit emptying of stomach contents. The challenge strain was grown in BHI at 37°C to a concentration of ~2 x 10^9 bacteria/ml. The organisms were spun out of the growth medium

and resuspended in PS (peptone saline, 0.1% w/v solution of proteose-peptone in saline (0.9% NaCl)). Serial ten-fold dilutions were prepared in PS and each was used to feed one group of 8 mice. Each mouse received 0.1 ml of bacterial suspension. The mice were not returned to their mothers but were kept in tissue-lined plastic containers in the laboratory. 48 hours after challenge, the survival of mice within each group was noted and these data used to construct a plot of accumulative percentage mortality versus $\log_{10}$ challenge dose (using the method of Reed and Muench, 1938). By interpolation it was then possible to determine the (48 hour) LD_{50} dose for a given strain - that is, the number of organisms capable of killing (within 48 hours) 50% of the mice to which it is administered.

2.20 *In vivo* colonization test

The *in vivo* colonization capacity of various 569B strains in the presence, absence or combination of the P and V factors was examined in infant mice. Infant mice were fed 5 x 10^6 bacteria and were sacrificed 24 hours after challenge when their small intestine was excised. These were then homogenized, diluted and plated onto NA + Sm (100 µg/ml). The recovery of organisms was determined and the colonies overlain with 0.3% soft agar in order to examine the percentage of motile bacteria (as described above).

2.21 *In vitro* assay of adherence

Adherence of *V. cholerae* to the gut was examined in 5 cm gut segments of baby mice, following the method of Attridge and Rowley (1983a).

The small intestines from baby mice were excised from just below the duodenum in 5 cm lengths and washed in saline (0.9% NaCl) to

remove faecal material. The segments were suspended in phosphate buffered saline (PBS pH7.3) at 37°C. Bacteria which had been grown to late exponential phase were added to the intestinal segments to a final concentration of 2 x 10^7/ml. Following a 15 min incubation at 37°C the intestinal segments were washed thoroughly in 3 saline baths to remove any bacteria which had not attached firmly and homogenized in 5 ml volumes of saline. Samples of the homogenate were plated onto NA + Sm (100 µg/ml) to inhibit the growth of intestinal commensals. The percentage of adherence was calculated by expressing the total number of bacteria bound to the tissue as a percentage of the number of organisms added initially.

2.22 Cholera toxin assay.

The amount of CT produced in the presence and absence of the P and V factors in *V. cholerae* 569B was measured by the GM_1 ganglioside technique of Holmgren (1973). Ganglioside GM_1, the receptor for CT was obtained from SIGMA. Strains were grown in TB + 66 mM NaCl pH6.5 for 18 hours at 30°C (Miller *et al.*, 1987) and the culture supernatant following filtration through a 22 µm Milex filter (Millipore Corp.) was used to measure CT. Anti-cholera toxin raised in rabbits was a generous gift from G. Mayrhofer.

2.23 Measurement of chloramphenicol acetyltransferase (CAT)

2.23.1 Minimal inhibitory concentration (MIC) of chloramphenicol

The level of resistance due to the presence of the CAT gene was determined by growth in increasing concentrations of Cm. 50 µl of an overnight culture was added to 1 ml of TB broth + 0.066mM NaCl pH6.5 containing 1, 5, 10, 25, 50, 100 or 200 µg/ml chloramphenicol for *V.*

cholerae and 10, 25, 50, 100, 250, 500 or 1000 µg/ml chloramphenicol for *E. coli* K-12. The tubes were incubated standing overnight at 37°C. A positive result was readily determined by an increase in turbidity of the fluid.

2.23.2 Spectrophotometric assay for CAT

Cellular extracts were prepared essentially as described by Close and Rodriguez (1982) and spectrophotometrically assayed according to Shaw (1975). Shaken overnight cultures grown in TB + 66 mM NaCl at 37°C with shaking (Miller *et al.*, 1987) were diluted 1 in 20 into the same medium and grown to mid-exponential phase at 37°C with shaking. 1.5 ml cells were harvested and resuspended in 0.5 ml extraction buffer (50 mM Tris-HCl, pH 7.8, 30 µM dithiothreitol). The cells were disrupted using a Branson sonicator and the extract was cleared of cell debris by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was assayed for CAT activity in a 1 ml reaction volume. The rate of increase in absorbance at 412 nm was measured to determine enzyme units in nmol of dithiobisnitrobenzoic acid reduced per minute per mg of total protein. Protein was assayed by the method of Bradford (1976) using a dye reagent concentrate (Bio-Rad) and bovine serum albumin (Sigma) as a standard.

2.24 Conjugations

2.24.1 Conjugation with *V. cholerae*.

Overnight broth cultures grown in BHI were diluted 1 in 20 and grown to early exponential phase with slow agitation. Donor and recipient bacteria were mixed at a ratio of 1:10 and left standing at 37°C for 1 hour. Dilutions of the mixture were then plated out onto selective media and incubated overnight at 37°C.

Transfer frequencies in liquid medium were measured by performing a conjugation for 60 min and the frequency calculated as the number of transconjugants per donor cell, since there was an excess of recipients.

2.24.2 Filter matings

For filter matings, donor and recipient bacteria were mixed at a ratio of 1:1, the cells pelleted and gently resuspended in 200 µl of NB and spread onto a membrane filter (0.45 µm, type HA, Millipore) on a NA plate. This was incubated overnight at 37°C. The cells were then resuspended in 10 ml NB and samples plated onto appropriate selective plates.

2.24.3 Surface exclusion index.

Conjugation conditions were as described. The donor strain, 1621, containing the P::Tn derivative under test was mixed with recipient cells, V628, harbouring P::Tn3. Dilutions of the mixture were plated onto media containing Spc and selection for the incoming plasmid. The surface exclusion index was calculated as the factor by which the number of transconjugants decreased when the recipient contained the P factor as compared to the P^- recipient.

2.25 Incompatibility testing

Table 2.5 lists the incompatibility test plasmids used to evaluate the incompatibility group to which the P factor belongs. These strains were kindly provided by D. Stevens (Royal North Shore Hospital, Sydney).

R factors were introduced into *V. cholerae* from *E. coli* by either liquid or filter matings. Incompatibility of the R factor and P derivatives was tested by using the R factors as donors and P^- or

Table 2.5

STANDARD INCOMPATIBILITY TEST PLASMIDS

Plasmid	Inc Group	Marker
R724	B	Cm, Sm, Su, Tc
R40a	C	Ap, Km, Su
R711b	D	Km
R455-2	FI	Ap, Cm, Sm, Su
R1-16	FII	Km
R124	FIV	Tc
R726	H	Cm, Sm, Su, Tc
R144-3	I	Km
TP114	I2	Km
R391	J	Km
R387	K	Cm, Sm
R446b	M	Sm, Tc
R447b	N	Ap,Km
R702	P	Km, Sm, Su, Tc
R401	T	Ap, Sm
R1460	U	Km, Sm, Su, Tc
R905	V	Km, Sm, Su
Sa	W	Cm, Km, Sm, Su
R6K	X	Ap, Sm

P::Tn*3* as recipients. *V. cholerae* transconjugants containing both plasmids were tested for plasmid incompatibility by their ability to retain the plasmid after two successive subcultures (1 in 100) after overnight incubation in the absence of selection and plating onto NA, following replica plating onto selective media for one or the other plasmid. Stability of the plasmids was scored as a percentage of cells containing the test plasmid after plating.

2.26 Lacunae assay.

An overnight culture of the donor strain was diluted 1 in 20 into fresh BHI and grown to early exponential phase (OD_{600nm} 0.4) with slow agitation. This was then diluted to 10^{-5}. The recipient strain was diluted 1 in 10 into fresh BHI. A 0.1 ml sample of both, the donor and the recipient were mixed with 5 ml of BHI soft agar and poured onto a NA plate. These plates were incubated upright for 6 hours at 37°C followed by overnight incubation at room temperature. Colonies with lacunae were scored the following morning.

2.27 Thermostable direct haemolysin (TDH) detection

The detection of the thermostable direct haemolysin (TDH) in *E. coli* K-12 harbouring subclones of the lcp in pACYC184 was carried out as described by Nishibuchi and Kaper (1985).

Analysis for homologous DNA to the *tdh* gene on the P and V plasmids and the large cryptic plasmid was performed by Southern hybridization using a 415 bp probe to the *tdh* gene, kindly provided by M. Nishibuchi.

2.28 Computer generated DNA sequence analysis.

The potential for constructing promoters as a result of cloning the CAT cartridge or inserting Tn*1736Tc* into the *ctxAB* operon was examined utilizing the Pustell sequence analysis programs (International Biotechnologies, Inc.)

Chapter 3

Identification of plasmids in *V. cholerae* strain V 58 and examination for possible functions.

3.1 Introduction

Plasmids are non-essential self-replicating extrachromosomal deoxyribonucleic acid molecules (DNA) which may be cryptic or play a role in the survival of their bacterial host cell. Plasmid-encoded functions may include: resistance to heavy metals (Trevors *et al.*, 1985); serum resistance (Moll *et al.*, 1980); restriction/modification systems (Arber, 1974) and determinants of bacterial pathogenicity, such as the production of enterotoxins, exotoxins and haemolysins (Elwell and Shipley, 1980), adhesion and invasion factors as seen with *Escherichia*, *Shigella* and *Yersinia* (Levine, 1987; Sansonetti *et al.*, 1982; Zink *et al.*, 1980).

Plasmids isolated from *V. cholerae* include R factors which nearly all belong to the incompatibility group IncC (Davey and Pittard,1975; Hedges and Jacob,1975; Hedges *et al.*, 1977) with the exception of two R factors from the IncJ group (Yokota and Kuwahara, 1977; Kuwahara *et al.*, 1979) and two cryptic plasmids detected in a number of Classical *V. cholerae* strains from the sixth pandemic (Cook *et al.*, 1984). Several plasmids have been isolated in other *Vibrio* species, including *V. parahaemolyticus* (Guerry and Colwell, 1977; Arai *et al.*, 1985), *V. mimicus* (Chowdhury *et al.*, 1986), *V. fluvialis* and *V. anguillarium* (Nakajima *et al.*, 1983). These have often been large

plasmids of the order of 90 - 150kbp (60 - 100 Md). The P factor is one of the few plasmids stably maintained in *V. cholerae*, but is poorly characterized in terms of function.

This chapter describes the characterization of three plasmids in the *V. cholerae* 01 Classical strain V58 and examines the possible functions of these three plasmids.

3.2 Identification of three plasmids in *V. cholerae* strain V58

The *V. cholerae* Classical Ogawa strain V58, originally isolated by Bhaskaran (1960), was used as a source of the P factor. Crude plasmid DNA extracts were prepared from V58 and V58 P^- and subjected to agarose gel electrophoresis. This revealed the presence of a plasmid which correlated with the P^+ phenotype and two additional plasmids, which had not been previously described (Figure 3.1).

These three plasmids in strain V58 have also been identified by electron microscopy (EM) (Figure 3.2). By measurement of the contour lengths of molecules, and by use of pBR322 DNA as a length standard, sizes for these three plasmids were calculated: the P factor, 68 ± 1.6 kb; large cryptic plasmid, 34.1 ± 0.13 kb and the small cryptic plasmid, 4.7 ± 0.13 kb. These sizes correlated with those determined by restriction analysis (Chapters 4 and 5).

3.3 Possible properties of the P factor

Conjugal transfer of chromosomal markers and a possible role in the suppression of virulence (Sinha and Srivastava, 1978) are the only known functions of P. Consequently analyses of the P factor for characteristics conferred by plasmids in other bacterial/plasmid systems

Figure 3.1.

Identification of plasmids in *V. cholerae* strain V58. Whole genomic DNA extracts from *V.cholerae* 01 strain V58 (Classical Ogawa) and its P^- derivative, were separated by electrophoresis through an 0.6% agarose gel. This revealed the presence of three plasmids: P (68 kb); lcp (34 kb) and scp (4.7 kb).

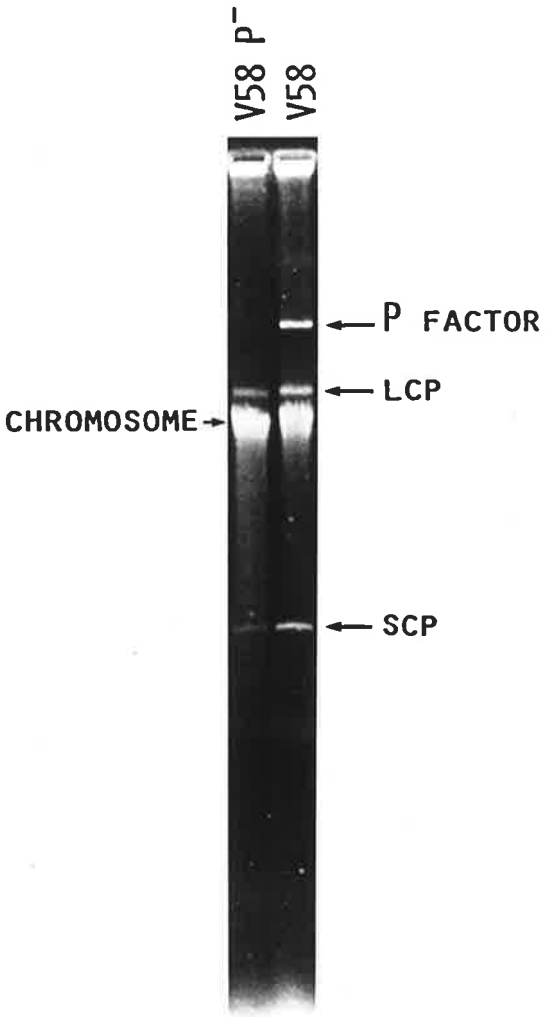

V58 P⁻
V58
P FACTOR
LCP
CHROMOSOME
SCP

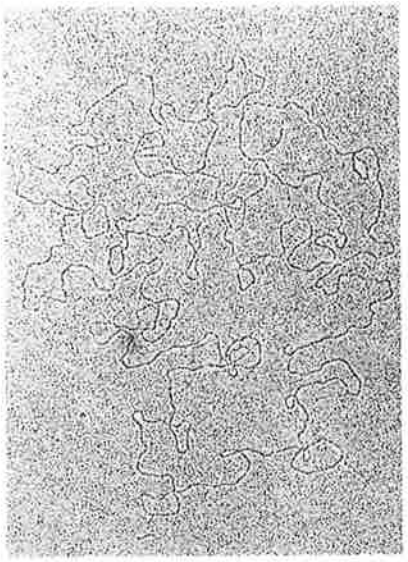
Figure 3.2.

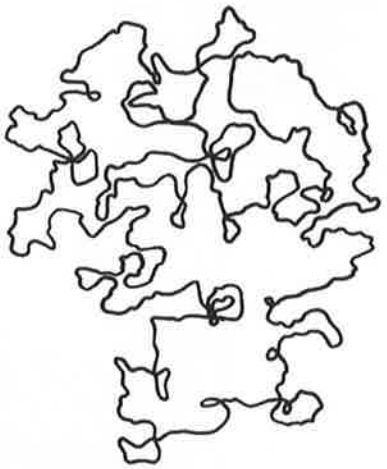
Electron micrographs of the plasmids present in strain V58. Plasmid pBR322 was used as a standard to calculate the length of the plasmids. Diagrammatic representations of the electronmicrographs are also shown.

A: P factor. Size was determined to be 68.2 ± 1.6 kb.

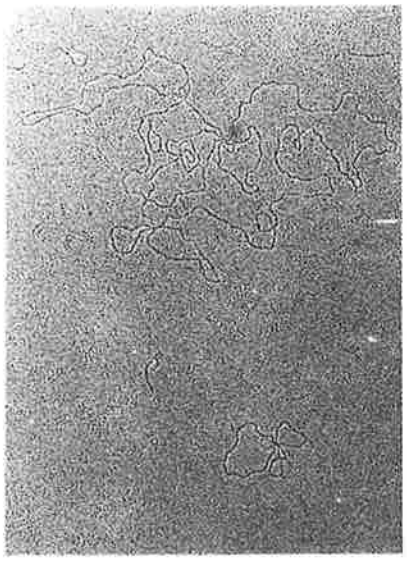
B: The two cryptic plasmids. The larger plasmid lcp (top) (34.2 ± 0.13 kb) and the smaller plasmid scp (bottom) (4.7 ± 0.13 kb).

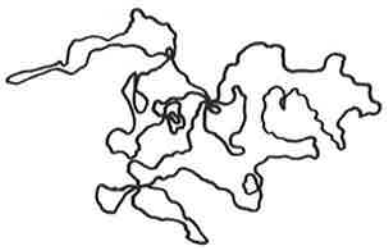
A





B





were undertaken. The role of P in virulence will be examined in Chapter 7.

3.3.1 Serum Resistance.

The F factor of *E. coli* K-12 and a number of other F-like plasmids can confer the ability upon the strains harbouring them (Moll *et al.*, 1980) to resist the bactericidal action of serum. This effect is due to the product of the *traT* gene which is also involved in surface exclusion. To examine whether the P factor was able to confer serum resistance to *V. cholerae* a colorimetric microassay method was employed (Moll *et al.*, 1979) with normal human serum.

Classical *V. cholerae* strain V58 and its P^- derivative were mixed with serum at various dilutions (0-16% v/v) and examined for serum resistance. *E. coli* K-12 strain JC3272, and JC3272[pRS31] (carrying the *traT* region of the F factor) (Skurray *et al.*, 1976), were included as controls (Figure 3.3). Whereas JC3272[pRS31] had an endpoint of 8%, JC3272 was susceptible to 4% serum. No difference was observed in the serum resistance exhibited by P^+ or P^- *V. cholerae* strains. Even though P is capable of surface exclusion (as will be demonstrated in Chapter 4), it does not appear to mediate serum resistance in *V. cholerae*.

3.3.2 Metal Ion Resistance.

Large plasmids commonly endow strains carrying them with resistance to metal ions (Summers *et al.*, 1978; Trevors *et al.*, 1985). P^+ and P^- strains of *V. cholerae* have been compared for their capacity to grow in the presence of eleven different metals. The metals examined were: arsenic, boron, cadmium, chromium, copper, cobalt, lead, mercury, molybdenum, tungsten and zinc. Test strains were bar streaked across a Nutrient Agar plate with the ion under test

Figure 3.3.

Analysis of the ability of various strains to resist the bactericidal action of human serum. *V. cholerae* strain V58 and its P$^-$ derivative, with *E. coli* K-12 strain JC3272 and JC3272[pRS31], were exposed to human serum at the dilutions shown. The colourmetric microassay showed that the minimal serum concentrations required for growth inhibition were: 4% (v/v) for V58, V58 P$^-$ and JC3272, whereas JC3272 [pRS31] required 8% (v/v). The arrows indicate the endpoints of serum resistance measurements.

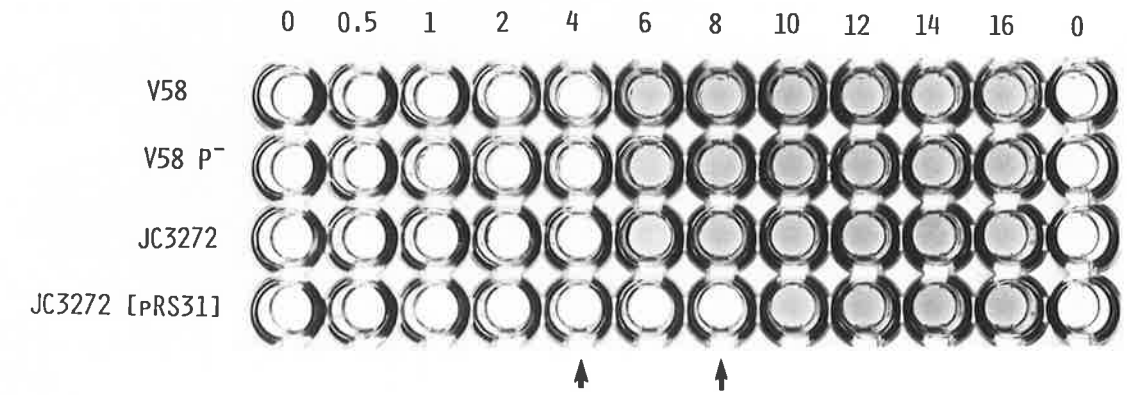
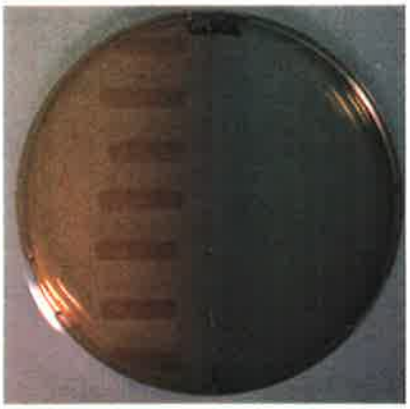

0 0.5 1 2 4 6 8 10 12 14 16 0
V58
V58 P⁻
JC3272
JC3272 [pRS31]

Figure 3.4.

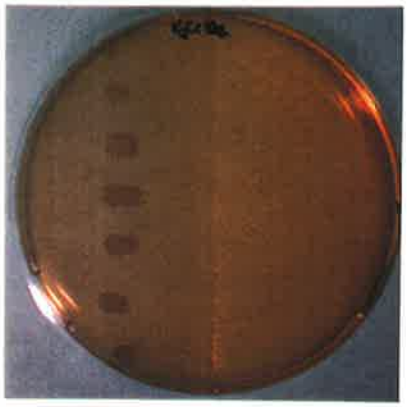
Ability of *V. cholerae* strains with or without the P and/or V factors to grow in the presence of metal ions. The strains were bar streaked on NA plates with inorganic salts present in only half of the plates, as indicated. Classical strain 569B and El Tor strains CD17 and 017 harbouring either the P or V factors were examined.

569B
569B P^+
569B V^+
CD17
CD17 P^+
O17
O17 P^+



$CuSO_4$

569B
569B P^+
569B V^+
CD17
CD17 P^+
O17
O17 P^+



K_2CrO_4

569B
569B P^+
569B V^+
CD17
CD17 P^+
O17
O17 P^+

$CrCl_3$

569B
569B P^+
569B V^+
CD17
CD17 P^+
O17
O17 P^+

$PbNO_3$

569B
569B P^+
569B V^+
CD17
CD17 P^+
O17
O17 P^+

$NaBH_4$

present in only half of the plate. Classical and El Tor strains with or without the P and/or V factors were tested. Metal resistance tests for Classical strain 569B and El Tor strains 017 and CD17 are shown in Figure 3.4. No differences in resistance were observed in the presence or absence of either the P or V factors with any of the metals examined.

3.3.3 Incompatibility.

The nineteen Inc test plasmids were used in attempts to determine the Inc group of the P factor. Since the P factor was not stably maintained in *E. coli* K-12 (see section 3.4), this necessitated transfer of the incompatibility test plasmids into *V. cholerae*. This also provided an opportunity to test the stability of these plasmids in *V. cholerae*.

E. coli K-12 strains harbouring the incompatibility plasmids were conjugated with *V. cholerae* both in liquid and on solid media. Thirteen of the nineteen incompatibility test plasmids were able to transfer to *V. cholerae*, but of these only six transferred at a detectable frequency in liquid matings (Table 3.1). Plasmids of Inc groups B, FI, FIV, H, K or V could not be transferred into *V. cholerae*.

After growth for 40 generations in antibiotic-free media, only R1-16 (Inc FII) and R446b (Inc M) were found to be unstable (18% and 85%, respectively) in *V. cholerae*. However, after 200 generations plasmids R1-16 (Inc FII) and TP114 (Inc I2), had been totally lost from strains harbouring them and plasmids R446b and R447b showed marked instability (42% and 35%, respectively). Plasmids R391 (Inc J, 98%) and Sa (Inc W, 88%) exhibited only a slight instability.

Since not all of the test plasmids could be transferred to *V. cholerae*, it was not possible to determine to which incompatibility group the P factor belonged. The P factor does not belong to the Inc

Table 3.1

TRANSFER OF INCOMPATIBILITY PLASMIDS INTO *V.CHOLERAE.*

Plasmid[a]	Inc Group	Transfer efficiency[b] in liquid mating into *E.coli* (P1848)	Transfer efficiency[b] in liquid mating into *V.cholerae* (V628)	Stability[c] of R factor in *V. cholerae* P-	Stability[c] of R factor in *V. cholerae* P+
P::Tn*3*-1	?	$<10^{-8}$	1.68×10^{-1}	100%	3.4%
P::Tn*10*-1	?	$<10^{-8}$	8.07×10^{-2}	100%	5.4%
R724	B	2.81×10^{-3}	-	-	-
R40a	C	1.07×10^{-6}	-*	100%	100%
R711b	D	4.81×10^{-4}	4.65×10^{-6}	100%	100%
R455-2	FI	1.70×10^{-4}	-	-	-
R1-16	FII	1.10×10^{-1}	2.42×10^{-2}	18%	-[d]
R124	FIV	4.69×10^{-3}	-	-	-
R726	H	8.00×10^{-7}	-	-	-
R144-3	I	7.25×10^{-2}	1.09×10^{-5}	100%	100%
TP114	I2	2.54×10^{-3}	-*	100%	-[d]
R391	J	7.44×10^{-5}	-*	100%	100%
R387	K	3.40×10^{-4}	-	-	-
R446b	M	1.13×10^{-3}	1.51×10^{-6}	85%	90%
R447b	N	1.10×10^{-4}	-*	100%	94%
R702	P	1.36×10^{-3}	1.76×10^{-4}	100%	100%
R401	T	3.00×10^{-3}	-*	100%	100%
R1460	U	1.29×10^{-3}	1.66×10^{-4}	100%	100%
R905	V	1.39×10^{-2}	-	-	-
Sa	W	1.79×10^{-6}	-*	100%	-[d]
R6K	X	2.79×10^{-4}	-*	100%	100%

Table 3.1 continued

a Incompatibility plasmids are in *E. coli* K-12 J53-1 (F^-. *pro*, *met* Rif^R, Nal^R) background except for R447b and R6K which are in J53 (F^-, *pro*, *met*, Rif^R, Nal^S)

b Transfer efficiency is the ratio of the number of transconjugants to the number of donors

c Stability of the R factor as determined after 40 generations. R-factor-containing strains were subcultured by successive 1 in 1000 dilutions into antibiotic free media, samples plated onto NA and replicated onto the appropriate antibiotic. Stability was scored as percentage of colonies which still retained the R factor.

d Transfer of the plasmids between *V.cholerae* strains could not be detected.

* No transconjugants were obtained in liquid media matings. However, transconjugants could be obtained if a filter mating technique was employed.

groups C, D, I, J, M, N, P, T, U or X. Plasmids of incompatibility groups FII, I2 and W can be maintained in *V. cholerae*. However, they are not able to transfer between *V. cholerae* strains. (All Inc plasmids which transferred into *V. cholerae* were able to transfer between strains with the exception of Inc FII, I2 and W plasmids.) The instability of the Inc M plasmid is probably not a reflection of incompatibility but merely the instability of R446b itself in *V. cholerae*.

The ease with which plasmid R1-16 can be transferred into *V. cholerae* and its marked instability may make this plasmid useful as a means of introduction of transposons into the genome of *V. cholerae*.

Homology between the P factor and the nineteen test Inc plasmids (Table 2.5) was examined by Southern hybridization using [^{32}P] labelled P factor DNA. Under stringent conditions no homologous sequences were observed.

3.4 Transfer of the P factor into other bacterial species

Some R factors found in Gram-negative bacteria have a broad host range, such as Inc P plasmids (Datta and Hedges, 1972), whereas other R factors like Inc T plasmids from *Proteus* have been isolated only from within this genus (Terawaki *et al.*, 1967; Coetzee *et al.*, 1972; Hedges, 1975).

Attempts were made to conjugate the transfer-proficient transposon-labelled P factor, P::*Tn3*-1 (Chapter 4) into *V. cholerae* 01 and non-01 strains, other *Vibrio* species, *Aeromonas* species, *E. coli* K-12 and *Salmonella typhimurium* strains. Matings were performed in liquid and on solid media (Table 3.2).

The P::*Tn3*-1 transferred to *V. cholerae* 01 and *V. cholerae* non-01 BV6 strain equally well and into other *Vibrio* species at a reduced

Table 3.2

TRANSFER OF THE P FACTOR TO OTHER BACTERIAL SPECIES

Recipient strain	Transfer efficiency[a] in liquid mating
V.cholerae-01[b]	
569B Cl In	1.10×10^{-2}
1621 ET Og	5.00×10^{-1}
017 ET Og	3.00×10^{-1}
non-01 #56	3.58×10^{-6}
non-01 #65	4.97×10^{-6}
non-01 BV6	7.76×10^{-3}
Vibrio	
V.fluvialis	2.30×10^{-5}
V.mimcus	4.03×10^{-5}
unidentified BV93	5.00×10^{-6}
Aeromonas	
A.caviae	2.00×10^{-6}
A.hydrophila	0*
A.sobria	1.30×10^{-5}
unidentified AB1	0*
unidentified AB3	3.00×10^{-6}
unidentified Pappu	0*

Table 3.2 (continued)

TRANSFER OF THE P FACTOR TO OTHER BACTERIAL SPECIES

Recipient strain	Transfer efficiency[a] in liquid mating
*E.coli*K-12	
LE 392	0*
DH1	0*
S.typhimurium	
# 4419	9.68×10^{-7}
# 4419, *galE*	3.87×10^{-7}

a = Donor strain was V135, El Tor Ogawa strain harbouring P::Tn^{3}-1
Liquid medium matings were for 90 min. at 37°C.
The efficiency of transfer is the number of transconjugants per cell (since there was an excess of recipients).

b = *V. cholerae*-01 strains
Cl In = Classical Inaba
ET Og = El Tor Ogawa

* = transfer of the P::Tn*3*-1 could not be detected on either solid or in liquid media.

frequency (100-1000 fold lower). Transfer into some *Aeromonas* species occurred at a 100-1000 fold lower frequency than into *V. cholerae*-01, however, *A. hydrophila* (A006) and the unidentified species AB1 and Pappu showed no detectable recipient ability in liquid or on solid media matings.

Restriction negative but modification positive strains of *E. coli* K-12 and *S. typhimurium* were selected as recipients in matings with transfer-proficient P::*Tn3*-1/*V. cholerae* donors. No transconjugants were detected with either liquid or filter matings with *E. coli* K-12 recipients (Table 3.2). Transformation of the P::*Tn3*-1 plasmid DNA into competent *E. coli* K-12 was also unsuccessful.

Transconjugants from a *V. cholerae* (donor) and *S. typhimurium* (#4419 or #4419 *galE*) mating occurred at a very low frequency (4×10^{-7}) and were found to be very unstable with loss of P::*Tn3*-1 within a few generations as determined by restreaking of the transconjugants onto selection media. When *S. typhimurium* transconjugants were examined for plasmid content, only the cryptic plasmid DNA characteristic of *S. typhimurium* was detected.

It appeared that the P factor was unable to replicate or be stably maintained in species like *E. coli* or *S. typhimurium* but was maintained/replicated in *Vibrio*-related species such as some *Aeromonas* spp..

3.5 Possible role of the large cryptic plasmid.

The large cryptic plasmid of *V. cholerae* strain V58 has been shown to be identical to the V factor, originally isolated from a *V. cholerae* non-01 strain (Chapter 5).

Examination of metal ion resistance (section 3.3 and Figure 3.4) revealed that the V factor did not encode resistance to arsenic, boron,

cadmium, chromium, copper, cobalt, lead, mercury, molybdenum, tungsten and zinc. This inferred that the lcp, too, did not encode resistance determinants to these metal ions. The V factor also does not play a role in the virulence of *V. cholerae* (Chapter 7).

V. parahaemolyticus is a natural inhabitant of estuarine and marine environments and is associated with sea-food-borne gastroenteritis (Fujino *et al.*, 1974; Blake *et al.*, 1980). A major virulence determinant of this *Vibrio* species is the thermostable direct haemolysin (TDH) (Sakurai et al., 1973) responsible for the Kanagawa phenomenon (Miyamoto *et al.*, 1980) which is β-haemolysis by this protein in Wagatsuma agar containing defibrinated human blood.

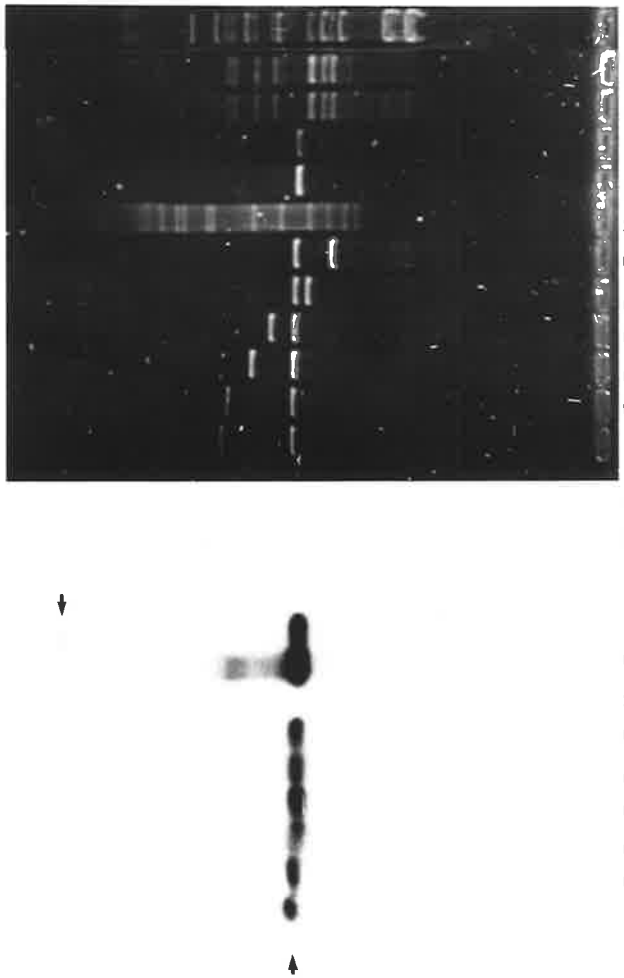
Using a 415 base pair probe to *tdh*, the structural gene for the thermostable direct haemolysin (TDH) of *V. parahaemolyticus*, it has been demonstrated that a haemolysin produced by some *V. cholerae* non-01 strains, NAG-rTDH (related to the TDH (Yoh *et al.*, 1986)) was present on a 33 kb plasmid (Honda *et al.*, 1986). This has lead to the examination of strain V58 containing the 34 kb lcp to see if it too contained DNA homologous to NAG-rTDH DNA.

Cellular extracts prepared from *E. coli* K-12 cells harbouring recombinant plasmids of *Eco*RI fragments of the lcp (pPM829 - pPM834; Chapter 5) were examined for the production of β haemolysis in blood agar. None of the subcloned *Eco*RI fragments exhibited haemolysis.

Possible homology between the P factor, the V factor and the lcp on the one hand, and the *V. parahaemolyticus* TDH gene on the other, was examined by Southern hybridization using pCVD518 which contains the 415 bp fragment of the structural gene. No homology was found to any of the plasmids examined nor to the recombinant plasmids harbouring fragments of the lcp (pPM829 - pPM834; Figure 3.5).

Figure 3.5.

Examination of homology between the thermostable direct haemolysin (TDH) of *V. parahaemolyticus* and the P factor and lcp. Plasmid pCVD518, containing a 415 bp fragment of the structural gene *tdh* in pBR322, was labelled with [^{32}P]-dCTP and used as a probe. The autoradiograph is shown on the right. Recombinant plasmids pPM829-pPM834 are *Eco*RI fragments of the lcp subcloned into pACYC184. All plasmids are digested with *Eco*RI. The arrow on the right indicates the position of the vector and in track pCVD518, the 0.415kb *Pst*I fragment is indicated. The size standard is SPP1 cut with *Eco*RI.


V58
KB9 V+
V58 P−
pCVD518
pBR322
SPP1/EcoRI
pPM829
pPM830
pPM831
pPM832
pPM833
pPM834
V58
KB9 V+
V58 P−
pCVD518
pBR322
SPP1/EcoRI
pPM829
pPM830
pPM831
pPM832
pPM833
pPM834

3.6 Possible role of the small cryptic plasmid.

Restriction and modification of DNA are mechanisms by which bacteria protect themselves against incoming foreign DNA (for example, bacteriophage DNA). When foreign DNA enters the cell it is cleaved by restriction endonucleases unless modified specifically for that strain. To protect the host DNA against restriction the cell encodes enzymes for modification by glycosylation (addition of sugars) or methylation of bases in the DNA. Restriction and modification systems are therefore processes which primarily limit inter-species recombination.

The occurrence of small Hsd (host specificity for DNA) plasmids is quite widespread in the *Salmonella-Shigella-E. coli* group (Yoshida and Mise, 1986). Restriction endonucleases of type II may be plasmid-borne or chromosomally-encoded (Arber, 1974). Genes encoding the restriction endonucleases *Eco*RI and *Eco*RII are on a large R plasmid (Rouland-Dussoix *et al.*, 1975) and the *Eco*RV gene is located on a small 6.2 kb plasmid (Bougueleret *et al.*, 1984). Many *hsd* genes are located on small plasmids, ranging in size from 4.4 to 12 kb (refer to table 5 in Yoshida and Mise, 1986).

The possibility that the small cryptic plasmid detected in the *V. cholerae* Classical strain V58 encoded a restriction-modification function was investigated.

To examine the likelihood that the smallest plasmid in strain V58 (4.7 kb) encoded an Hsd system, Classical phage VcIV and El Tor phages ET1 and CP-T1 were employed. The bacteriophage test system for demonstration of proficiency in foreign DNA restriction is based on the demonstration by Arber (1974) of differential efficiency of plating of bacteriophage lambda on restriction-proficient and restriction-deficient strains of *E. coli*. Bacteriophage CP-T1 has been reported to infect Classical and El Tor strains with equal efficiencies (Ogg *et al.*,

1978). Observations on phage specificities made in this laboratory, however, indicate that the bacteriophage CP-T1 has greater specificity for the El Tor rather than the Classical biotype (H.Ward, personal communication).

The eop of bacteriophage CP-T1 was determined on strains ±scp (Table 3.3A). The eop of CP-T1 on Classical strain V58 was approximately 10 fold lower than on an El Tor strain 1621 suggesting the possibility of restriction between biotypes (Imbesi and Manning, 1982). The El Tor strain H-1 harbours the small cryptic plasmid (discussed in Chapter 5) and CP-T1 plaquing on H-1 has an eop which is 4 fold lower than on El tor strains which lack the scp (1621 and 017). The presence of the P factor (V58 and V58 P^-) does not affect the eop of CP-T1, inferring that the P factor does not encode a restriction/modification function. Propagation of CP-T1 on strain H-1 (scp^+) does not alter the eop significantly on any strains except V58 and V58 P^- (Table 3.3B).

The eop of bacteriophages VcIV and ET1 was determined on both biotypes in the presence and absence of the scp.

Classical bacteriophage VcIV plaques equally well on Classical strains with or without the scp and, when propagated on Classical strains, subsequently plaques poorly on El Tor strains indicative of restriction between biotypes (Imbesi and Manning, 1982) (Table 3.4). When VcIV is propagated on El Tor and the eop then assessed on Classical strains the reverse situation is observed, such that it is restricted by the Classical biotype and not the El Tor. The El Tor bacteriophage ET1 behaves similarly, Table 3.5, exhibiting the same biotype specific restriction.

Propagation of either VcIV or ET1 on a strain containing the scp gives the same eop pattern as with propagation on a strain lacking the

Table 3.3

EFFICIENCY OF PLATING OF CP-T1

STRAIN	EOP[a]	SD[b]	SCP[c]
A: CP-T1 propagated on 1621			
1621	1.00		-
017	0.93	± 0.12	-
H-1	0.27	± 0.43	+
569B	0.80	± 0.09	-
Z17561	2.62×10^{-10}	± 1.77	+
V58 P-	0.08	± 0.03	+
V58	0.02	± 0.02	+
B: CP-T1 propagated on H-1			
1621	1.22	± 0.29	-
017	0.72	± 0.87	-
H-1	1.00		+
569B	0.62	± 0.62	-
Z17561	1.4×10^{-8}	± 1.4	+
V58 P-	0.26	± 0.25	+
V58	0.75	± 0.73	+

a eop = efficiency of plaquing is defined as the titre of the phage on the test strain compared with the titre of the phage on the propagation strain

b SD = standard deviation

c scp = small cryptic plasmid

Table 3.4

EFFICIENCY OF PLAQUING OF BACTERIOPHAGE VcIV

Strain	eop[a]	scp[b]
A: VcIV propagated on 569B		
569B	1.00	-
Z17561	1.82	+
V58 P-	1.49	+
V58	1.48	+
017	2.09×10^{-8}	-
H-1	3.65×10^{-8}	+
B: VcIV propagated on Z17561		
569B	1.26	-
Z17561	1.00	+
V58 P-	0.92	+
V58	1.1	+
017	2.0×10^{-9}	-
H-1	2.0×10^{-9}	+
C: VcIV propagated on H-1		
569B	1.77×10^{-7}	-
Z17561	4.25×10^{-8}	+
V58 P-	1.96×10^{-7}	+
V58	1.39×10^{-7}	+
017	0.38	-
H-1	1.00	+

Table 3.4 (continued)

a eop (efficiency of plaquing) is defined as the titre of the phage on the test strain compared with the titre of the phage on the propagation strain

b scp small cryptic plasmid

Table 3.5

EFFICIENCY OF PLAQUING OF BACTERIOPHAGE ET1

Strain	eop[a]	scp[b]
A: ET1 propagated on 017		
569B	2.82×10^{-9}	-
Z17561	2.28×10^{-9}	+
V58 P-	2.28×10^{-9}	+
V58	2.28×10^{-9}	+
017	1.00	-
H-1	3.3	+
B: ET1 propagated on H-1		
569B	5.59×10^{-11}	-
Z17561	5.95×10^{-11}	+
V58 P-	5.95×10^{-11}	+
V58	5.95×10^{-11}	+
017	0.08	-
H-1	1.00	+
C: ET1 propagated on Z17561		
569B	1.06	-
Z17561	1.00	+
V58 P-	1.30	+
V58	1.31	+
017	3.13×10^{-9}	-
H-1	3.13×10^{-9}	+

Table 3.5 (continued)

a eop (efficiency of plaquing) is defined as the titre of the phage on the test strain compared with the titre of the phage on the propagation strain

b scp small cryptic plasmid

Table 3.6

EFFICIENCY OF PLATING OF BACTERIOPHAGE LAMBDA

ON SUBCLONES OF THE SMALL CRYPTIC PLASMID

Strain/plasmid	eop[a]
LE392	1.00
+ pBR322	1.18
+ pPM851	0.96
+ pPM852	1.00
+ pPM853	0.92
C600	1.00
+ pBR322	1.34
+ pPM851	1.25
+ pPM852	1.16
+ pPM853	0.98

a eop = efficiency of plating is defined as the titre of the phage on the test strain compared with the titre of the phage on the parent strain

scp (Table 3.4 A, B, and Table 3.5.A, B), indicating that the scp is not influencing the plaquing efficiency of the bacteriophage.

The eop of bacteriophage lambda was determined on *E. coli* K-12 harbouring the subcloned fragments of the scp (Chapter 5) (Table 3.6). There was no variation in the eop of lambda plaquing on either the restriction-negative strain (LE392) or wild type (C600), with or without scp subclones, inferring that if a restriction/modification system exists on the scp, it is not functional in the recombinant plasmids (pPM851, pPM852 and pPM853) in *E. coli* K-12.

From the results presented, neither the scp nor the P factor encode restriction/modification systems.

3.7 Conclusions.

An attempt has been made in this chapter to investigate possible functions for the three plasmids detected in *V. cholerae* Classical strain V58. The molecular sizes of these three plasmids have been determined from electron microscopy measurements as 68 ±1.6 kb for the P factor, 34.1 ±0.13 kb for the large cryptic plasmid (lcp) (shown to be identical to the V factor in Chapter 6) and 4.7 ±0.13 kb for the small cryptic plasmid (scp).

Although serum resistance and resistance to metal ions are commonly associated with large conjugative plasmids, neither function could be attributed to P. Determination of the incompatibility group to which P belongs was hindered since the P factor could not be introduced into *E. coli* K-12 nor was it possible to transfer all test incompatibility plasmids into *V. cholerae*. But since replication and incompatibility are inter-related (Novick, 1987), several possibilities can be ruled out. It was possible to demonstrate that the P factor

does not belong to the Inc groups C, D, I, J, M, N, P, T, U, V or X (Table 3.1).

P is also unable to replicate or be stably maintained in species outside the *Vibrio* genus although it appears that it may be transferred to some (Table 3.2).

No homology was detected between the *tdh* (thermostable direct haemolysin) of *V. parahaemolyticus* and either the large cryptic plasmid (34 kb) or the P factor by Southern hybridization using plasmid pCVD518 containing a 415 bp fragment of the *tdh* gene (Figure 3.7) as a probe. Functional TDH activity was not detected in *E. coli* K-12 harbouring recombinant plasmids of the lcp.

No function could be ascribed to the small cryptic plasmid.

Chapter 4

Restriction analysis and physical mapping of the P factor.

4.1 Introduction

Restriction analysis of a plasmid facilitates its examination at a molecular and physical level. Subcloning of restriction fragments into plasmid vectors provides the basis for examining the proteins encoded by genes on the plasmid as well as providing probes for particular genes. Transposons can be used for site specific mutagenesis of DNA in plasmids to determine the physical location of particular genes.

Although the P sex factor has been previously described (Parker and Romig, 1972) it has not been subjected to detailed molecular analysis.

This chapter examines the P factor at the molecular level; both physical and molecular features are analysed.

4.2 Restriction endonuclease analysis of the P factor

P factor DNA extracted from strain V58 was subjected to digestion with 30 restriction endonucleases with 6 bp specificity. Figure 4.1 illustrates a selection of endonuclease cleavage patterns of the P factor. Thirteen of these enzymes do not cleave P, whereas the majority produce ten or more fragments. Table 4.1 summarizes the restriction data. Summation of fragment sizes for each restriction endonuclease ranged between 63 and 70kb, discrepancies would be due

Figure 4.1.

An 0.6% agarose gel showing a selection of restriction endonuclease cleavage patterns of the P factor. Indicated on the left is the position of uncut P factor DNA. Bacteriophage SPP1 DNA digested with *Eco*RI has been used as a size standard (Radcliff *et al.*, 1979; Franzon and Manning, 1986).

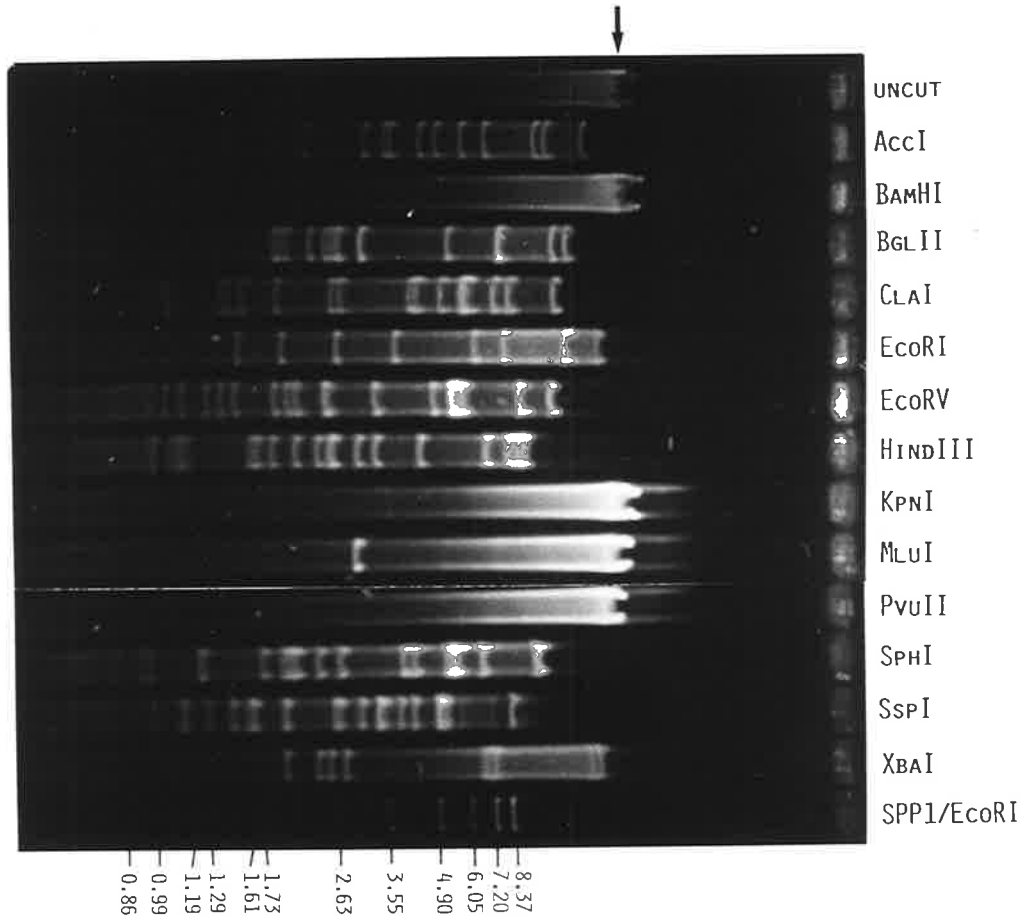

UNCUT
AccI
BamHI
BglII
ClaI
EcoRI
EcoRV
HindIII
KpnI
MluI
PvuII
SphI
SspI
XbaI
SPP1/EcoRI
0.86
0.99
1.19
1.29
1.61
1.73
2.63
3.55
4.90
6.05
7.20
8.37

Table 4.1

NUMBER OF RESTRICTION FRAGMENTS GENERATED

UPON DIGESTION OF THE P FACTOR

Restriction Enzymes	Number of fragments*	Sum of fragments (kb)	Restriction Enzyme	Number of fragments	Sum of fragments (kb)
*Acc*I	12	70	*Nru*I	0	-
*Aha*III	13	65	*Pst*I	0	-
*Ava*I	0	-	*Pvu*I	16	65
*Bam*HI	0	-	*Pvu*II	1	68
*Bcl*I	0	-	*Sal*I	0	-
*Bgl*I	6	69	*Sca*I	0	-
*Bgl*II	12	65	*Sma*I	0	-
*Cla*I	16	64	*Spe*I	2	68
*Eco*RI	10	68	*Sph*I	15	63
*Eco*RV	18	70	*Ssp*I	19	64
*Hin*dIII	16	67	*Sst*I	0	-
*Hpa*I	16	65	*Stu*I	0	-
*Kpn*I	0	-	*Xba*I	8	68
*Mlu*I	2	68	*Xho*I	0	-
*Nde*I	9	67	*Xma*I	0	-

* = Number of fragments represents the number which can be visualised on a 0.6% agarose gel stained with ethidium bromide (above 0.8 kb).

to inaccurate estimation of large fragments (>15kb). Accurate size determination of the P factor was verified from double digestions of the subcloned restriction fragments.

Several enzymes cleaved P at only a few sites and the sizes of the fragments generated upon digestion with *Eco*RI, *Xba*I, *Mlu*I, *Spe*I and *Pvu*II are summarized in Table 4.2, in which the fragments generated by each restriction endonuclease are numbered sequentially from the largest to the smallest fragment on agarose gels (e.g. *Eco*RI-1, *Eco*RI-2 etc.)

The five restriction endonucleases which cleaved the P factor at only a low frequency (Table 4.2), were used to construct a restriction map. From the electron microscopic measurements (Chapter3), the P factor was calculated to be 68 kb in size. However, the sum of the nine *Eco*RI bands was only 57 kb. A densitometer scan (Figure 4.2) of the DNA present in an *Eco*RI digestion revealed that the second *Eco*RI band was not present in an amount equimolar with the other fragments, but represented two fragments of the same size. These fragments have been designated as *Eco*RI-2A and *Eco*RI-2B and are each 11.5 kb in size.

The lack of cleavage sites for restriction endonucleases *Ava*I, *Bam*HI, *Bcl*I, *Kpn*I, *Nru*I, *Pst*I, *Sal*I, *Sma*I, *Sst*I, *Stu*I, *Xho*I and *Xma*I was confirmed by double digestions with the enzymes under examination and *Eco*RI and *Xba*I.

Double digests of the cloned *Eco*RI and *Xba*I fragments of P (section 4.4), and of the P factor itself allowed construction of a restriction endonuclease cleavage map (Figure 4.3). The single *Pvu*II site has been used as the zero co-ordinate on the map, which shows P to be of 68 kb in size. Other restriction endonuclease sites have been mapped in the individual subcloned *Eco*RI and *Xba*I fragments (section 4.4).

Figure 4.2.

Densitometer scan of the P factor DNA digested with *Eco*R1 after electrophoresis on an 0.6% agarose gel to separate the fragments. The *Eco*RI fragments of the P factor are labelled sequentially.

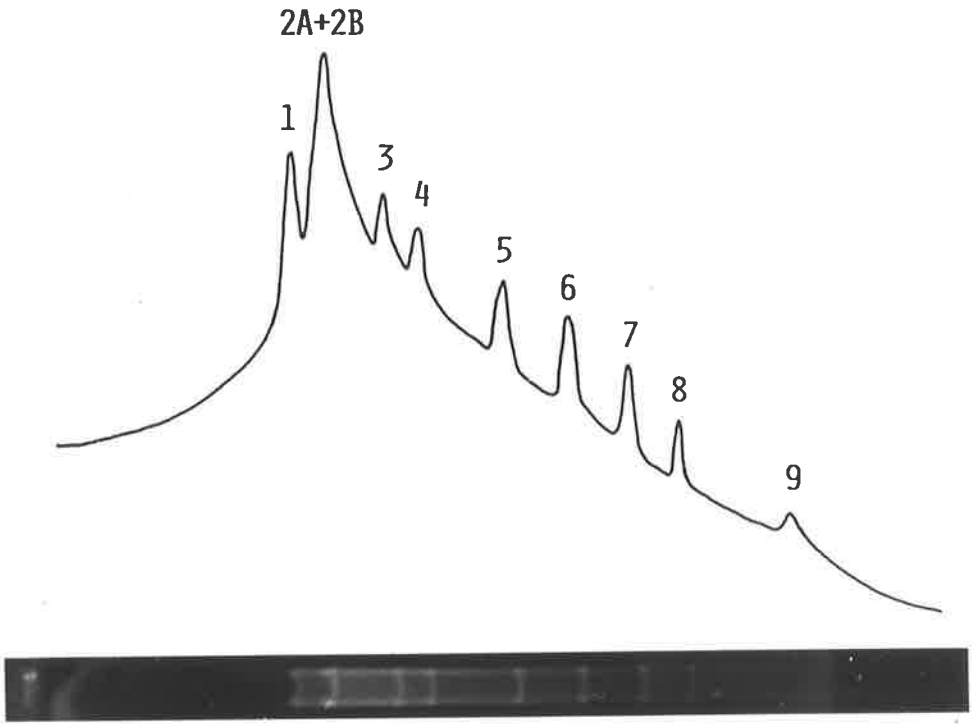

2A+2B
1
3
4
5
6
7
8
9

Table 4.2

RESTRICTION FRAGMENT SIZES OF THE P FACTOR

Fragment Number		Fragment size (kb)				
		*Eco*RI	*Xba*I	*Mlu*I	*Spe*I	*Pvu*II
1		24	45.5*	65.3*	54.5*	68*
2	A/B[a]	11.5	6.7	2.7	13.5	
3		6.5	6.3			
4		5.6	2.9			
5		3.5	2.7			
6		2.5	2.5			
7		1.9	2.1			
8		1.6	0.7			
9		0.8				

kb = kilobase

a A/B refers to the *Eco*RI-2 band which represents two fragments of the same size.

* Fragment size has been determined from double digestions.

Figure 4.3.

Restriction endonuclease cleavage map of the P factor. The map is calibrated in kilobases (kb), with the single *Pvu*II site as the zero co-ordinate. *Eco*RI and *Xba*I fragments were ordered from double digests of cloned fragments of P factor DNA and are drawn as concentric circles. As the enzymes *Mlu*I and *Spe*I each only cut once, they have been positioned on the outer circle.

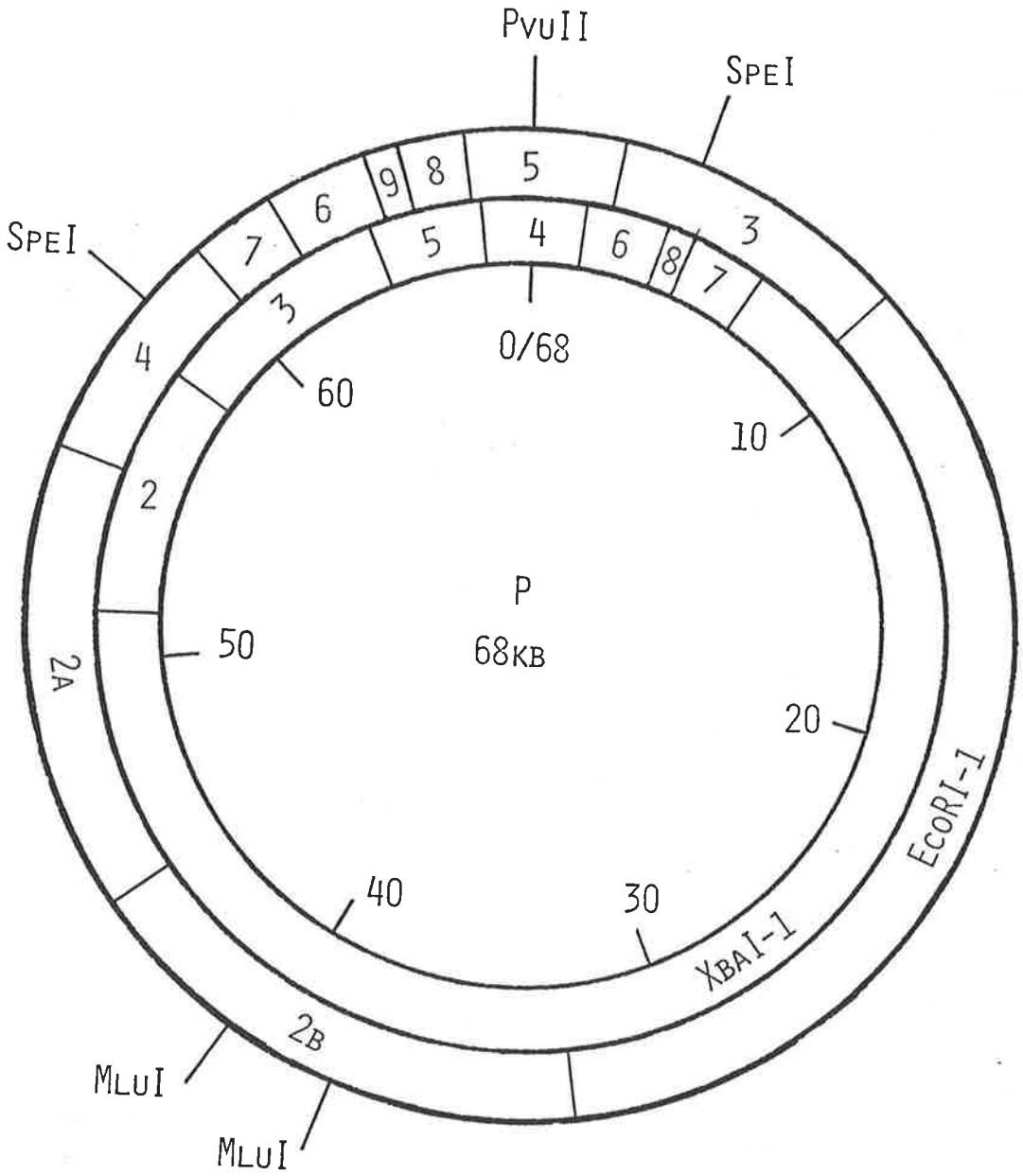

PvuII
SpeI
SpeI
9
8
5
6
7
5
4
6
8
7
3
3
4
2
2A
2B
0/68
60
10
20
30
40
50
P
68kb
EcoRI-1
XbaI-1
MluI
MluI

Figure 4.4

A detailed restriction endonuclease map of the *Eco*RI and *Xba*I fragments of the P factor. All subcloned fragments are shown in the same orientation with respect to the cloning vector.

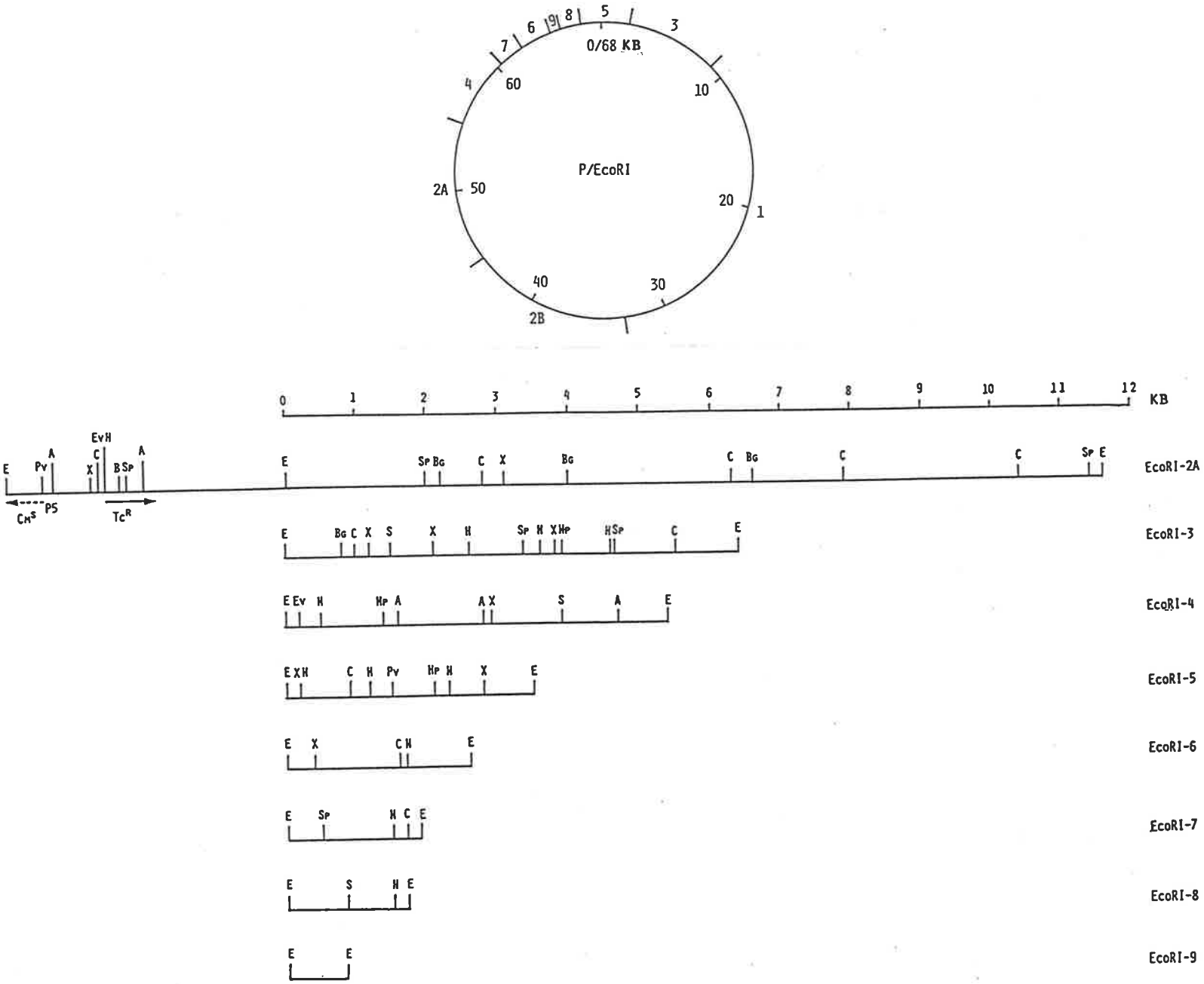
A: The *Eco*RI fragments subcloned into pACYC184.

B: The *Xba*I fragments subcloned into pJRD158b.

Recombinant plasmids pPM802-pPM809 are *Eco*RI-2A-*Eco*RI-9 fragments subcloned into pACYC184. The *Xba*I-2 fragment is pPM849 and *Xba*I-3 to *Xba*I-8 are recombinant plasmids pPM819-pPM824.

Restriction endonucleases: A-*Acc*I, B-*Bam*HI, Bg-*Bgl*II, C-*Cla*I, E-*Eco*RI, Ev-*Eco*RV, H-*Hind*III, Hp-*Hpa*I, M-*Mlu*I, Pv-*Pvu*II, S-*Spe*I, Sp-*Sph*I, X-*Xba*I.

The scale shown is in kilobases.


0/68 KB
P/EcoRI
1
2A
2B
3
4
5
6
7
8
9
10
20
30
40
50
60
KB
EcoRI-2A
EcoRI-3
EcoRI-4
EcoRI-5
EcoRI-6
EcoRI-7
EcoRI-8
EcoRI-9
PS

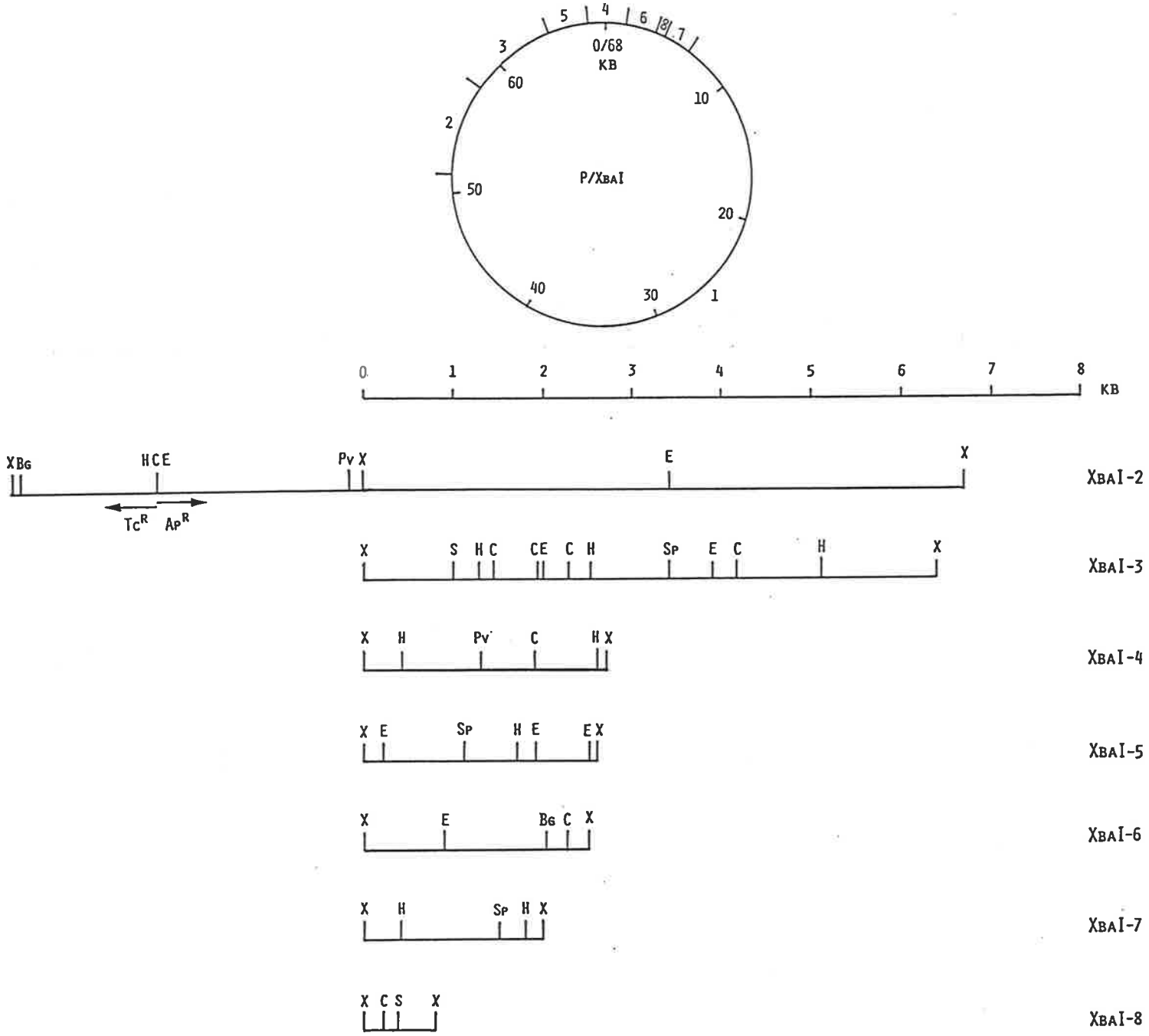

0/68
KB
P/XBAI
0
1
2
3
4
5
6
7
8
KB
XBG
HCE
PV X
E
X
TcR
ApR
XBAI-2
X
S H C
CE C H
SP
E C
H
X
XBAI-3
X H
PV
C
H X
XBAI-4
X E
SP
H E
E X
XBAI-5
X
E
BG C X
XBAI-6
X H
SP H X
XBAI-7
X C S X
XBAI-8

Figure 4.4 shows the individual subcloned fragments of the *Eco*RI and *Xba*I fragments and presents a more detailed restriction endonuclease pattern. All subcloned fragments are shown in the same orientation with respect to the cloning vector. Indicated on the cloning vector are promoters which may exert an effect on expression of putative proteins encoded on the subcloned fragments (section 4.5)

4.3 Physical mapping of the P factor

Conjugal transfer of DNA is a genetically complex process as evidenced by the size of the necessary transfer region of the F factor of *E. coli* K-12 which occupies about 33 kb of the total 100 kb (reviewed by Ippen-Ihler and Minkley, 1986). Consequently it is expected that a similar amount of DNA of the P factor might be required to encode its *tra* (transfer) region. There is also another sex factor encoded phenomenon, that of surface exclusion, in which a cell carrying a plasmid is reduced in its ability to act as a recipient with a donor carrying the same or a closely related plasmid (Zinder and Lederberg, 1952; Willetts and Maule, 1974). In order to estimate the size of and localize the regions involved in the conjugation process, transposon mutagenesis was undertaken.

The P factor was mutagenized using transposons Tn*3* (from R1-19), Tn*5* (from F'$_{ts}$*lac*::Tn*5*), Tn*10* (from R100-1) and Tn::*1732* (from pRU666). The transposon-carrying plasmids were conjugated from *E. coli* K-12 derivatives into *V. cholerae* with selection for the appropriate antibiotic resistance. The resultant exconjugants were screened for the loss of the plasmid by measuring loss of resistance to other antibiotic markers present on the plasmid. For example, the loss of the R1-19 plasmid could be monitored by the loss of Cm^R and Sm^R.

Donor strains carrying transposons at different sites in the P factor, or carrying the unmodified P factor, were tested for transfer and surface exclusion properties. Twenty seven independent transposon mutants derived here were screened as well as several imported transposon mutants (Table 4.3).

The normal range of transfer frequency was considered to be 10^{0} - 10^{-1}, and lower transfer frequencies were taken as indicative of *tra* mutations. Such lower frequencies observed ranged between 8.00 x 10^{-2} - 10^{-7}.

P::Tn*3*-1 and P::Tn*10*-1 derivatives were used to demonstrate that P^{+} strains were approximately 150 - 300 fold less efficient as recipients than P^{-} strains. P is able to express surface exclusion and this can be used to demonstrate the presence of P in other *V. cholerae* strains by their reduced ability to act as recipients. Plasmids expressing surface exclusion indices below 100 were considered as *sfx* mutants. The majority of the P::Tn isolates had surface exclusion indices which were greater than 100 (100 - 400), indicating that the transposon had not inserted into *sfx* gene(s), whereas only P::Tn*5-1* exhibited a significant increase in recipient ability.

Plasmid DNA was extracted from mutants which exhibited a decrease in transfer frequency or an increase in recipient ability and analysed by digestion with restriction endonucleases *Eco*RI and *Xba*I. Fragments were separated on agarose gels to determine which of the *Eco*RI and *Xba*I fragments were altered by transposon insertion. Further mapping of the mutant plasmids was performed with *Bam*HI, *Mlu*I, *Pst*I and *Spe*I. The transposon mutants appeared to map in several clusters (Figure 4.5). Insertions Tn*3*-1, pSJ5 and P::Tn*1* are transfer proficient (transfer frequencies of 1 - 8 x 10^{-1}). pSJ13 exhibits an intermediate transfer proficiency (transfer frequency of

Table 4.3

TRANSFER FREQUENCIES AND SURFACE EXCLUSION INDECES

OF TRANSPOSON MUTANTS OF THE P FACTOR

Transposon /Isolate Number	Transfer Frequency	Surface exclusion[a] index	*Eco*RI fragment affected
P	$1.00 \pm 0.3 \times 10^{-1}$	150	
Tn*3* -1	$1.68 \pm 1.3 \times 10^{-1}$	276	*Eco*RI-1
Tn*5* -1	$1.79 \pm 0.8 \times 10^{-1}$	4	*Eco*RI-2B
-3	$2.07 \pm 0.7 \times 10^{-3}$	76	*Eco*RI-1
-4	$8.54 \pm 0.5 \times 10^{-3}$	176	*Eco*RI-1
-7	$1.54 \pm 1.1 \times 10^{-2}$	380	*Eco*RI-1
-8	$3.92 \pm 0.9 \times 10^{-4}$	225	*Eco*RI-1
-10	$1.14 \pm 0.3 \times 10^{-1}$	427	*Eco*RI-2A
-11	$1.63 \pm 0.4 \times 10^{-3}$	375	*Eco*RI-1
-12	$2.05 \pm 0.8 \times 10^{-4}$	314	*Eco*RI-1
-13	$1.52 \pm 1.1 \times 10^{-2}$	450	*Eco*RI-1
-14	$1.51 \pm 0.9 \times 10^{-2}$	197	*Eco*RI-1
-15	$1.74 \pm 0.4 \times 10^{-1}$	190	*Eco*RI-2A
-16	$7.14 \pm 0.9 \times 10^{-1}$	147	*Eco*RI-1
-18	$1.93 \pm 1.1 \times 10^{-1}$	255	*Eco*RI-2A
-19	$3.75 \pm 0.7 \times 10^{-2}$	427	*Eco*RI-1
Tn*10*-1	$8.07 \pm 0.8 \times 10^{-2}$	151	*Eco*RI-1
-2	$1.05 \pm 1.3 \times 10^{-5}$	150	*Eco*RI-1
-3	$7.44 \pm 1.3 \times 10^{-7}$	190	*Eco*RI-1
-4	$1.13 \pm 0.9 \times 10^{-2}$	81	*Eco*RI-1
-5	$7.23 \pm 0.7 \times 10^{-7}$	250	*Eco*RI-1
-6	$6.92 \pm 1.1 \times 10^{-7}$	180	*Eco*RI-1
-8	$7.33 \pm 1.0 \times 10^{-7}$	230	*Eco*RI-1

Table 4.3 (continued)

TRANSFER FREQUENCIES AND SURFACE EXCLUSION INDECES

OF TRANSPOSON MUTANTS OF THE P FACTOR

Transposon /Isolate number	Transfer Frequency	Surface exclusion[a] index	*Eco*RI fragment affected
Tn*1732*-1	$1.30 \pm 0.3 \times 10^{-1}$	230	*Eco*RI-6
Imported P::Tn[b]			
pSJ5 (Tn*1*)	$8.78 \pm 0.7 \times 10^{-1}$	94	*Eco*RI-1
pSJ13 (Tn*1*)	$3.25 \pm 1.1 \times 10^{-3}$	38	*Eco*RI-1
P::Tn*1*	$1.38 \pm 0.9 \times 10^{-1}$	60	*Eco*RI-1

a = surface exclusion index is the ratio of the transfer frequency of the P::Tn into a P^- strain compared with the frequency of transfer of P::Tn into a P^+ strain.

b = transposon insertion in the P factor, constructed outside this study
pSJ5, pSJ13 (Johnson and Romig, 1979a)
P::Tn*1* (Khan *et al.*, 1985)

The transfer frequency and surface exclusion index were the mean of three experiments.

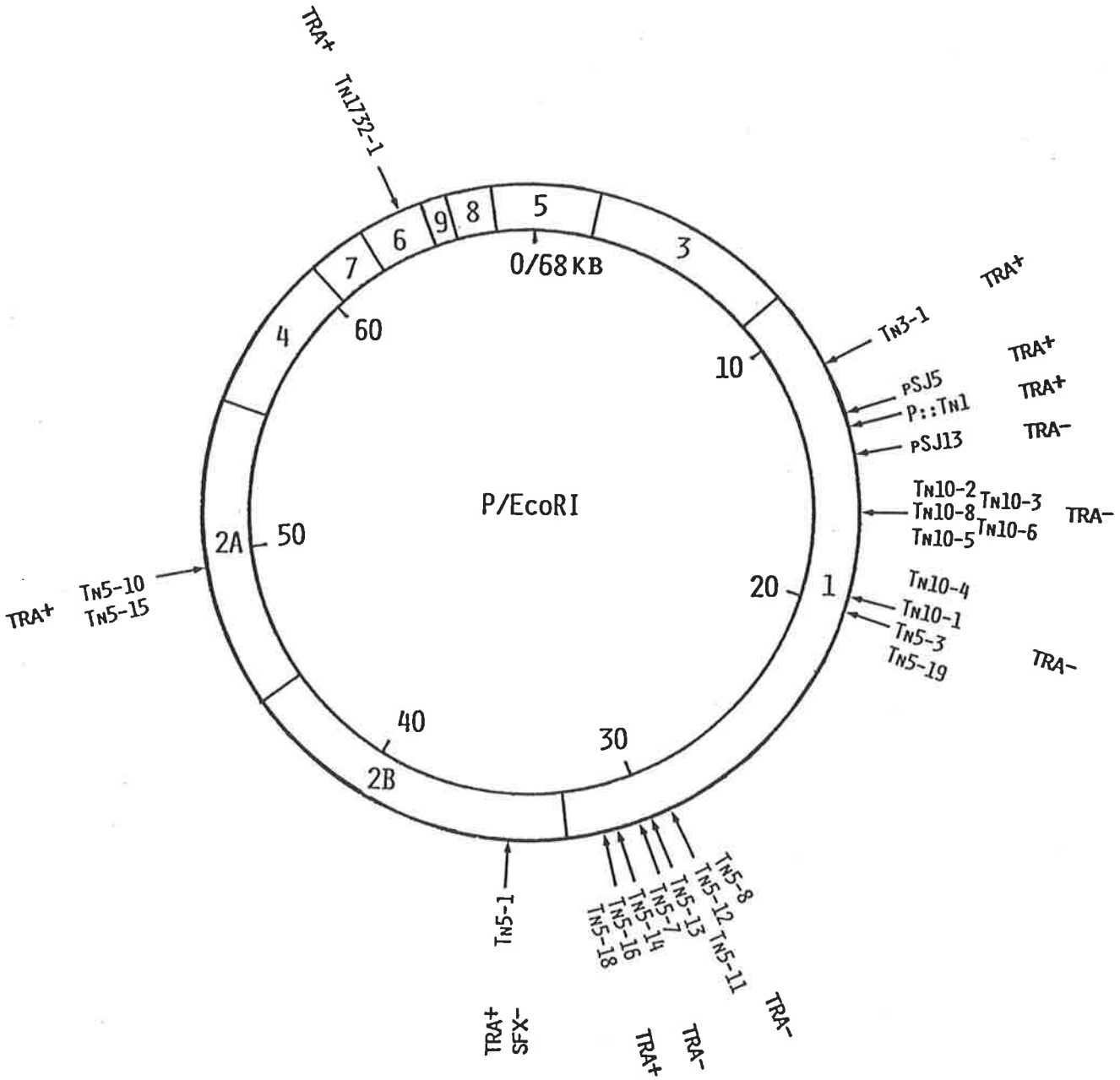
Figure 4.5.

Physical map of the transposon mutants of the P factor described in Table 4.3 which were used to determine the region(s) which are associated with the transfer (*tra*) and surface exclusion functions.

TRA$^+$ Transfer proficient (transfer frequencies range between 1.1–7.1 x 10^{-1}).

TRA$^-$ Transfer deficient (transfer frequencies range between 1.5 x 10^{-2}–7.0 x 10^{-8}).

SFX$^-$ Surface exclusion mutant. All other P::Tn mutants were SFX$^+$.


TRA+
Tn1732-1
0/68 KB
P/EcoRI
Tn3-1
TRA+
pSJ5
TRA+
P::Tn1
TRA+
pSJ13
TRA-
Tn10-2
Tn10-3
Tn10-8
Tn10-6
Tn10-5
TRA-
Tn10-4
Tn10-1
Tn5-3
Tn5-19
TRA-
TRA+
Tn5-10
Tn5-15
Tn5-1
TRA+
SFX-
Tn5-8
Tn5-12
Tn5-11
TRA-
Tn5-13
Tn5-7
Tn5-14
Tn5-16
Tn5-18
TRA+
TRA-
5
3
10
1
20
30
40
2B
50
2A
60
4
7
6
9
8

3.25 x 10^{-3}). Following these insertions are two clusters, the first which is considered tra$^-$ (Tn*10*-2, -3, -8, -6 and -5) with a transfer frequency of 10^{-7} - 10^{-8} and the second exhibiting poor transfer (10^{-2} - 10^{-3}) (Tn*10*-4, Tn*10*-1, Tn*5*-3 and Tn*5*-19) and mapping at the 20kb co-ordinate. Another cluster of mutants which map very close together are located within the *Eco*RI-1 fragment close to the *Eco*RI-2B (30 - 32kb co-ordinates) and consist of mutants which are either transfer proficient (Tn*5*-16 and Tn*5*-18, with a transfer frequency of 10^{-1}) or transfer deficient exhibiting a transfer frequency between 10^{-2} and 10^{-4} (Table 4.3). Transfer-deficient P::Tn derivatives consistently showed altered *Eco*RI-1 and *Xba*I-1 fragments to those of P (Figure 4.5) and appear to consist of at least two major regions (Tn*10*-2 to Tn*5*-19 and Tn*5*-8 to Tn*5*-14). The surface exclusion mutant P::Tn*5*-1 mapped in the *Eco*RI-2B fragment. Several P::Tn derivatives which did not have altered transfer or surface exclusion properties mapped outside of the *Eco*RI-1 fragment of the P factor.

4.4 Subcloning of the P factor

Restriction endonucleases *Eco*RI and *Xba*I cleave P factor DNA ten and eight times, respectively, as shown by electrophoresis in agarose gels (Figure 4.1, Table 4.1).

E. coli K-12 strain LE392 was used as the recipient for transformation of clones in order to avoid restriction of unmodified DNA from a *V. cholerae* background; eight of the ten *Eco*RI fragments of the P factor were cloned into the vector pACYC184 (Chang and Cohen,1978) (Figure 4.6A) and six of the eight *Xba*I fragments were cloned into the vector pJRD158b (Davison *et al.*, 1984) (Figure 4.6B). Together, these cloned fragments represent 48% of the P factor. It was

Figure 4.6.

Agarose gel electrophoresis of the P factor DNA and plasmids containing:

A: *Eco*RI fragments subcloned into pACYC184

B: *Xba*I fragments subcloned into pJRD158b.

The position of the vector is indicated by the arrow on the left and the P factor fragments are labelled on the left. Bacteriophage SPP1 DNA digested with *Eco*RI has been used as a size standard (Radcliff *et al.*, 1979; Franzon and Manning, 1986). The first *Xba*I fragment in P + *Xba*I represents the lcp (linear form) which is also present in V58 from which the P factor DNA was isolated.

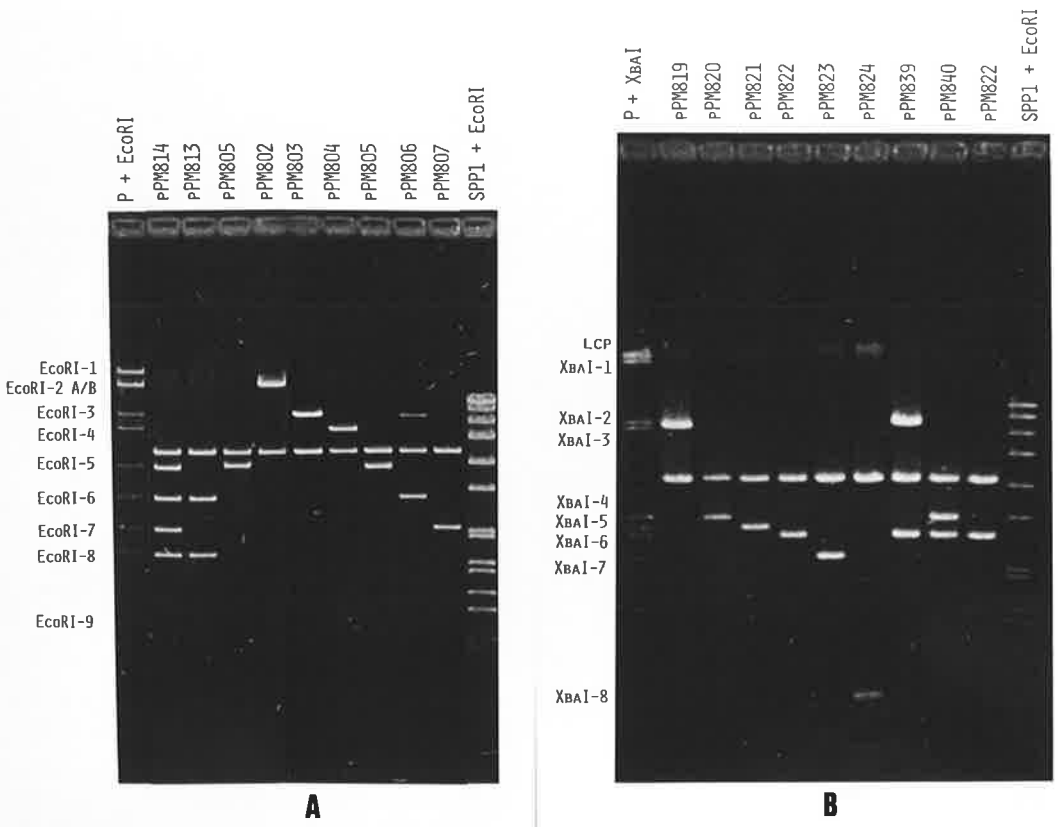
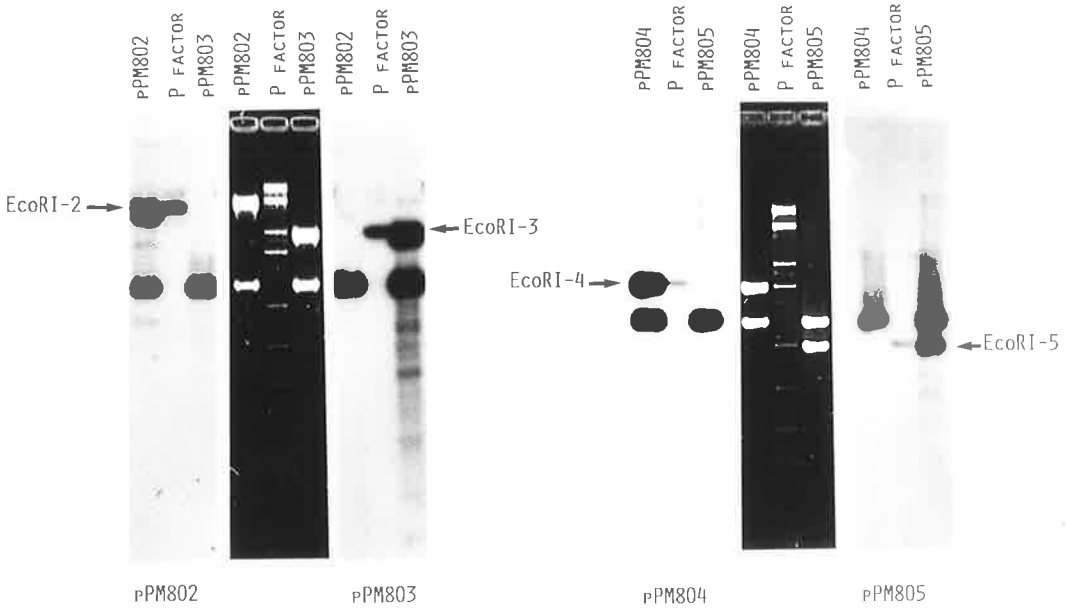

P + EcoRI
pPM814
pPM813
pPM805
pPM802
pPM803
pPM804
pPM805
pPM806
pPM807
SPP1 + EcoRI
EcoRI-1
EcoRI-2 A/B
EcoRI-3
EcoRI-4
EcoRI-5
EcoRI-6
EcoRI-7
EcoRI-8
EcoRI-9
A
P + XbaI
pPM819
pPM820
pPM821
pPM822
pPM823
pPM824
pPM839
pPM840
pPM822
SPP1 + EcoRI
LCP
XbaI-1
XbaI-2
XbaI-3
XbaI-4
XbaI-5
XbaI-6
XbaI-7
XbaI-8
B

Figure 4.7.

Southern hybridization analysis to confirm that subcloned fragments originate from the P factor. Subcloned *Eco*RI fragments 2A, 3, 4 and 5 were labelled with [^{32}P]dCTP by nick translation and used to probe *Eco*RI digested P factor DNA. The positions of the subcloned fragments are indicated.


pPM802
P FACTOR
pPM803
pPM802
P FACTOR
pPM803
pPM802
P FACTOR
pPM803
EcoRI-2
EcoRI-3
pPM804
P FACTOR
pPM805
pPM804
P FACTOR
pPM805
pPM804
P FACTOR
pPM805
EcoRI-4
EcoRI-5
pPM802
pPM803
pPM804
pPM805

not possible to clone the *Eco*RI-1, *Eco*RI-2B and *Xba*I-1 fragments (see below).

Southern DNA hybridizations, using nick translated cloned fragments, confirmed that the subcloned fragments originated from the P factor. Figure 4.7 shows an example of hybridizations using pPM802, pPM803, pPM804 and pPM805 as probes. Those DNA fragments containing P factor DNA showed homology with the P factor from strain V58, and not to the two cryptic plasmids also present in this strain, thus confirming the origin of the subcloned DNA.

4.5 Protein expression in *E. coli* K-12 of subcloned fragments of the P factor.

Whole cell extracts of *V. cholerae* strains Classical (V58) or El Tor (1621), in the presence or absence of the P factor do not reveal any new plasmid encoded proteins when stained with Coomassie blue following separation on SDS-PAGE (Figure 4.8).

E. coli K-12 derivatives harbouring the recombinant plasmids containing the cloned fragments of the P factor have been analysed for the expression of plasmid-encoded proteins in whole cell extracts and in minicells, in order to obtain an indication of the number of proteins encoded by the P factor.

4.5.1 Protein expression in whole cells.

Extracts of whole cells harbouring *Eco*RI and *Xba*I subclones were examined on a 15% SDS-polyacrylamide gel (Figure 4.9). The chloramphenicol acetyltransferase (CAT) present in pACYC184 (25 kDal) has been inactivated by insertion of DNA into the *Eco*RI site, hence this protein is absent in all subclones. Only one of the *Eco*RI subclones expressed new proteins which were detectable by staining. One plasmid, pPM804 (*Eco*RI-4 fragment in pACYC184) expressed

Figure 4.8.

Analysis of whole cell extracts of *V. cholerae* Classical strain V58 and El Tor strain 1621 with and without the P factor. Extracts were separated on SDS-PAGE and stained with Coomassie blue. Size standards are indicated on the right (in kDal).

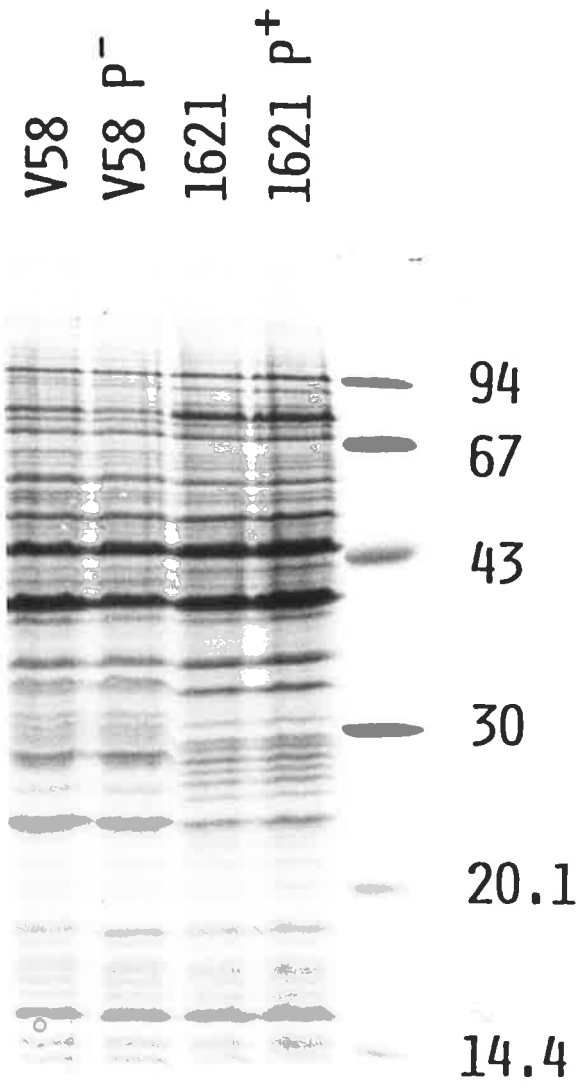

V58
V58 P⁻
1621
1621 P⁺
94
67
43
30
20.1
14.4

Figure 4.9.

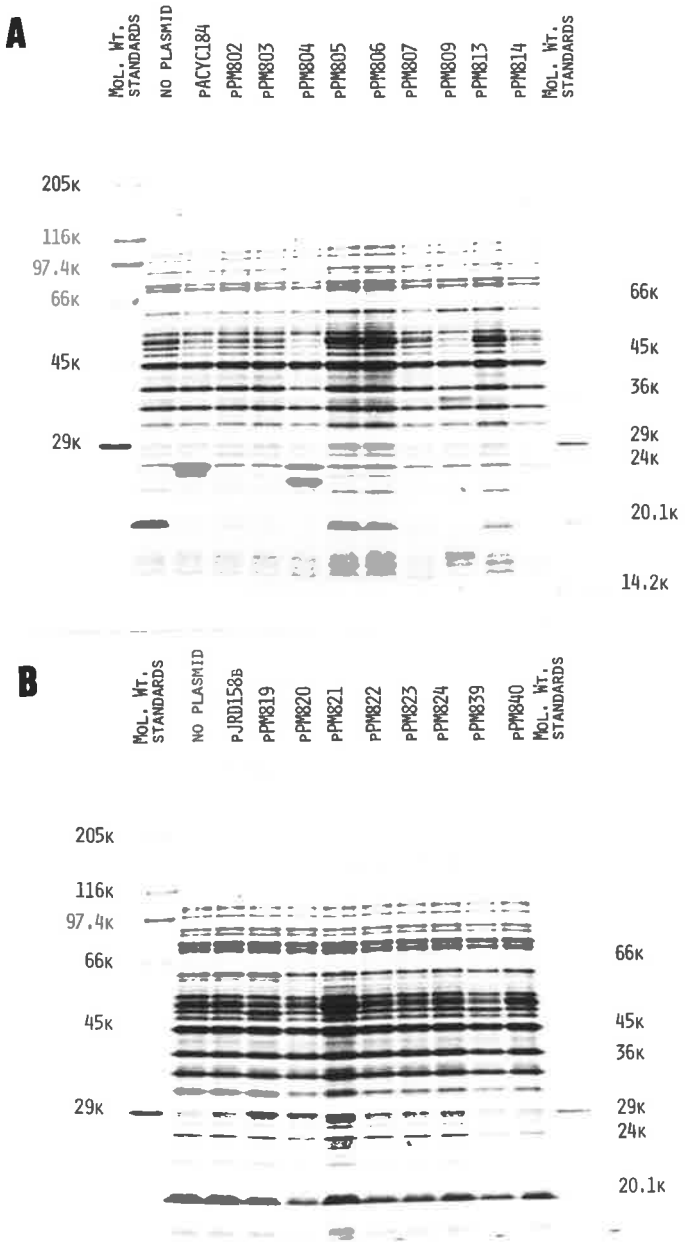
Analysis of whole cell extracts by SDS-PAGE followed by staining with Coomassie blue of *E.coli* K-12 derivatives harbouring plasmids containing:

A: the cloned *Eco*RI fragments

B: the cloned *Xba*I fragments

of the P factor.

The chloramphenicol acetyltransferase protein can be seen in the pACYC184 track, migrating at about 25 kDal.


A
MOL. WT. STANDARDS
NO PLASMID
pACYC184
pPM802
pPM803
pPM804
pPM805
pPM806
pPM807
pPM809
pPM813
pPM814
MOL. WT. STANDARDS
205K
116K
97.4K
66K
45K
29K
66K
45K
36K
29K
24K
20.1K
14.2K
B
MOL. WT. STANDARDS
NO PLASMID
pJRD158B
pPM819
pPM820
pPM821
pPM822
pPM823
pPM824
pPM839
pPM840
MOL. WT. STANDARDS
205K
116K
97.4K
66K
45K
29K
66K
45K
36K
29K
24K
20.1K

proteins of 24 kDal and 23 kDal which could be readily visualized (Figure 4.9).

4.5.2 Protein expression in minicells

The recombinant plasmids containing segments of P factor DNA were introduced into the *E. coli* K-12 minicell-producing strain DS410 (Dougan and Sherratt, 1977) for visualization of plasmid-encoded proteins.

Figure 4.10 is an autoradiograph of the [^{35}S] methionine-labelled proteins encoded by the *Eco*RI and *Xba*I recombinant plasmids. Table 4.4 lists the sizes of the potential P factor-encoded gene products identified in minicells and the predicted coding capacity required to encode these proteins.

Although synthesis of the tetracycline-resistance genes is constitutive (Backman and Boyer, 1983) protein bands other than those involved in mediating ampicillin-resistance are not seen in pBR322-containing minicells. Initially it was thought that a number of proteins were involved in mediating tetracycline resistance (Yang *et al.*, 1976; Curiale and Levy, 1982), however, fine deletion analysis of pBR322 has demonstrated that a single polypeptide of 43 kDal is able to confer resistance to tetracycline. This protein migrates anomalously on SDS-polyacrylamide gels and has an apparent size of 36.5 kDal (Backman and Boyer, 1983). No protein corresponding to either of these sizes was detected in minicells harbouring pJRD158b which is a derivative of pBR322 (differing only in a few restriction endonuclease sites) (Figure 4.10A). The 25 kDal CAT protein seen in minicells harbouring pACYC184 is eliminated from the protein profile of recombinants constructed with this plasmid as a result of insertion into the *Eco*RI site (Figure 4.9).

Figure 4.10.

Proteins expressed in *E.coli* K-12 minicells harbouring the *Eco*RI and *Xba*I fragments of the P factor cloned into pACYC184 and pJRD158b, respectively.

A: *Eco*RI subclones

B: *Xba*I subclones

The bands common to all *Xba*I clones at about 32 kDal and 30 kDal correspond to the precursor and mature form of the β-lactamase protein, encoded by the vector (Achtman *et al.*, 1979; Backman and Boyer, 1983). Minicells were solubilized in sample buffer and run in SDS PAGE on a 11-20% (w/v) polyacrylamide gradient gel which was autoradiographed at room temperature. Arrows mark the plasmid encoded proteins. Size standards (in kDal) are indicated.

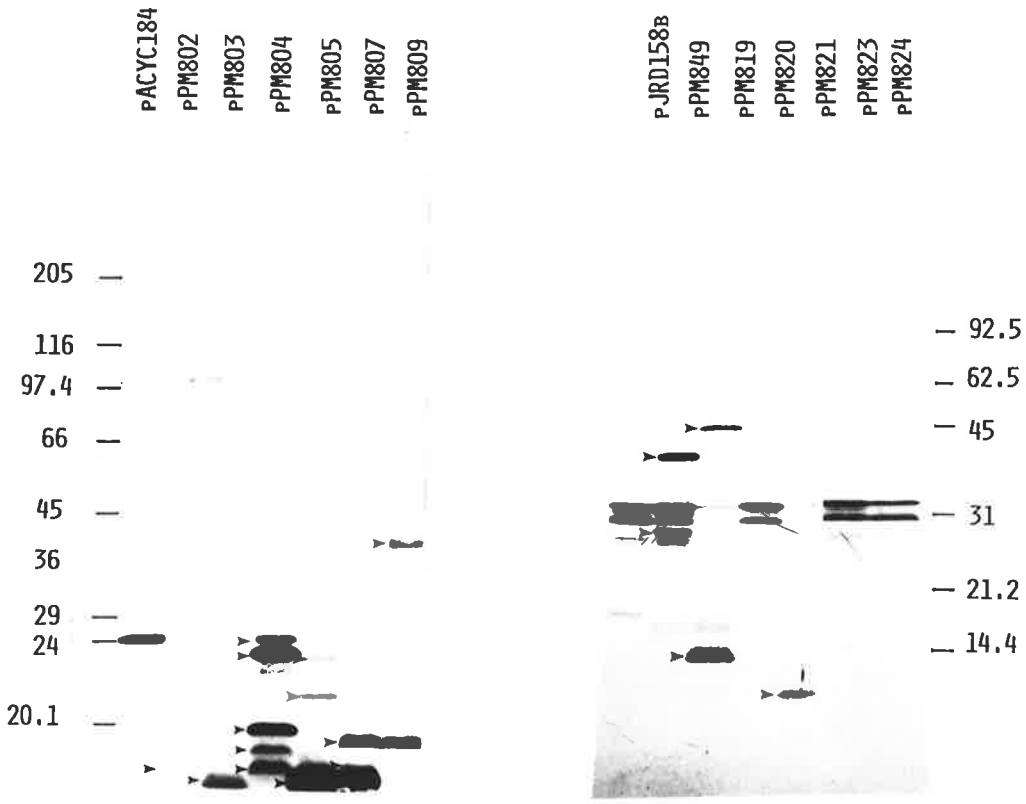

pACYC184
pPM802
pPM803
pPM804
pPM805
pPM807
pPM809
205
116
97.4
66
45
36
29
24
20.1
A
pJRD158B
pPM849
pPM819
pPM820
pPM821
pPM823
pPM824
92.5
62.5
45
31
21.2
14.4
B

Figure 4.11.

Autoradiograph of *E. coli* K-12 minicells harbouring plasmids pPM882-pPM890 which are *Eco*RI fragments 2A-9 subcloned into pSUP401 (Simon *et al.*, 1983). The band in common to all the tracks, migrating at 29 kDal corresponds to the aminoglycoside 3'-phosphotransferase protein (APH), encoded by the vector. The CAT (25 kDal) is only present in the pSUP401 track, since subcloning into the *Eco*RI site inactivates this *cat* gene. Minicells were solubilized in sample buffer and run in SDS-PAGE on a 15% polyacrylamide gel which was autoradiographed at room temperature. Size standards (in kDal) are indicated.

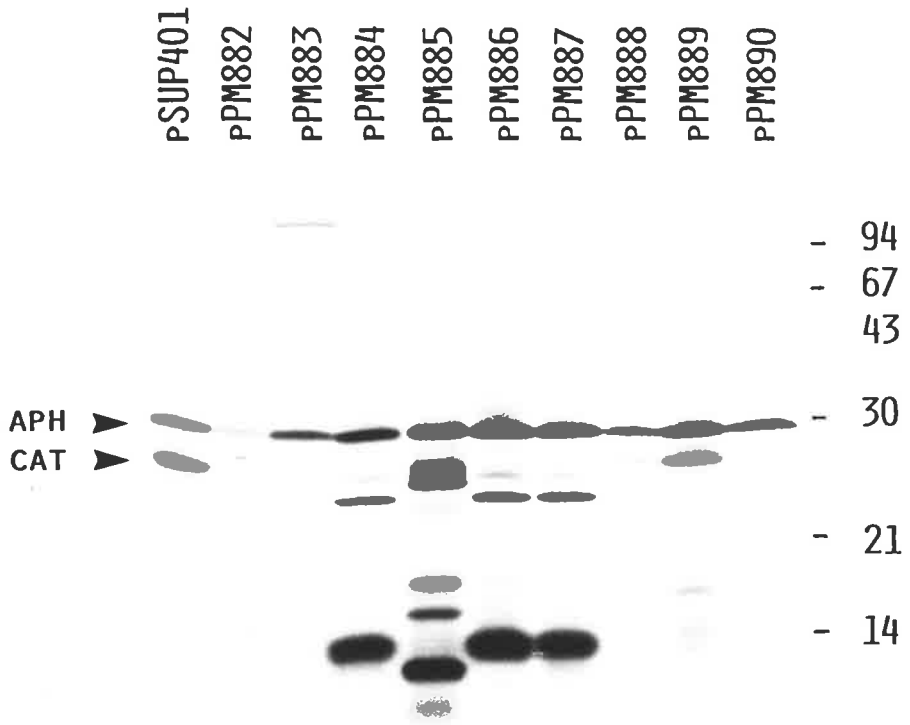

pSUP401
pPM882
pPM883
pPM884
pPM885
pPM886
pPM887
pPM888
pPM889
pPM890
APH
CAT
94
67
43
30
21
14

Table 4.4

PROTEINS DETECTED IN MINICELLS CONTAINING

CLONED RESTRICTION FRAGMENTS OF P FACTOR DNA

*Eco*RI Fragment	Plasmid	Fragment Size (kb)	Protein sizes (kDal)	Predicted Coding Capacity required (kb)
1	-	24	ND[a]	
2A	pPM802	11.5	105, 15	3.2
	pPM882		105, 15	3.2
	pPM883		105, 15	3.2
2B	-	11.5	ND	
3	pPM803	6.5	13.5	0.37
	pPM884		22.5, 13.5	0.98
4	pPM804	5.6	24, 23, 20, 17, 15.3	2.71
	pPM885		25, 24, 23, 17.5	
			16, 13	3.23
5	pPM805	3.5	22.5, 16.5, 13.5	1.43
	pPM886		22.5, 13.5	0.98
6	pPM806	2.5	-[b]	
	pPM887		22.5, 13.5	0.98
7	pPM807	1.9	18, 13.5	0.82
	pPM888		-	
8	pPM818	1.6	17	0.46
	pPM889		28, 15	0.77
9	pPM809	0.8	39, 18	1.55
	pPM890		-	
6,8,9	pPM813	4.9	23, 18	1.12
5,6,7 8,9	pPM814	10.3	18, 17	0.95

Table 4.4 (continued)

PROTEINS DETECTED IN MINICELLS CONTAINING

CLONED RESTRICTION FRAGMENTS OF P FACTOR DNA

*Xba*I Fragment	Plasmid	Fragment Size (kb)	Protein sizes (kDal)	Predicted Coding Capacity required (kb)
1	-	34	ND	
2	pPM849	6.7	40, 29	1.88
3	pPM819	6.3	45, 13	1.58
4	pPM820	2.9	-	
5	pPM821	2.7	13.2	0.36
6	pPM822	2.5	82, 11.5	2.55
7	pPM823	2.1	-	
8	pPM824	0.7	-	
2,6	pPM839	9.2	82, 40, 29, 11	4.42
4,6	pPM840	5.3	82, 11	2.54

a ND = not done as these fragments have not been subcloned

b - = no plasmid-encoded proteins detected

*Eco*RI fragments 2A - 9 were subcloned into the vector pSUP401 (Schmitt *et al.*, 1983) and analysed in minicells for the production of plasmid-encoded proteins (Figure 4.11). The CAT (25 kDal) has been inactivated and is only present in the vector track, whereas the aminoglycoside 3'-phosphotransferase (APH) responsible for Km^R is present in all the tracks, migrating at 29 kDal as reported by Beck *et al.* (1982). The 105 kDal and 15 kDal proteins of *Eco*RI-2A are expressed when subcloned in both orientations, inferring that their natural promoter is also present. The *Eco*RI-6 fragment in pPM887 has been subcloned in the opposite orientation into the *cat* gene, and in this orientation only expresses the early termination product of CAT (see below). Contiguous *Xba*I fragments 4 and 6 (pPM840) do not express any additional proteins from the individually cloned fragments.

The 13.5 kDal protein identified in plasmids pPM803, pPM805 and pPM807 may well be an early termination product of the CAT gene. The *Eco*RI site is approximately 400bp from the beginning of the CAT gene (Chang and Cohen, 1978) with the coding capacity for a maximum sized protein of 14.6 kDal. If there is a terminator present in the cloned P factor fragment, close to the EcoRI site, in frame with the *cat* gene, then it would be feasible to have a shortened CAT protein being synthesized. The size of this putative shortened CAT protein would be in agreement with the 13.5 kDal protein detected in pPM803, pPM805 and pPM807. These corresponding *Eco*RI fragments have also been subcloned into the cloning vector pSUP401 (pPM884, pPM886 and pPM888, respectively) (containing the same CAT gene as pACYC184) and minicells harbouring these recombinant plasmids (Figure 4.11) exhibit the same effect.

Plasmid pPM804 (with *Eco*RI-4 fragment of the P factor) expresses two proteins (24 kDal and 23 kDal) which are readily detectable in

both whole cell extracts (Figure 4.9A) and minicells (Figure 4.10A). A more detailed analysis of these proteins has been undertaken and this is discussed in section 4.7.

Plasmid pPM809 produces two proteins of 39 kDal and 18 kDal. These proteins would require a coding capacity of 1.5 kb which exceeds the 0.8 kb fragment actually cloned (Table 4.4). This could possibly be a fusion protein between the CAT (encoded by the vector pACYC184) and a P factor encoded protein. *Eco*RI subcloning into pACYC184 results in DNA being inserted after the promoter for CAT (Figure 4.4A). If this cloned DNA is in frame with a gene fusion could well result. When the *Eco*RI-9 fragment is subcloned into plasmid vector pSUP401 (Simon *et al.*, 1983) (pPM890) and minicells harbouring this recombinant plasmid are examined (Figure 4.11), no similar fusion product can be detected, indicating that either the *Eco*RI-9 fragment has been subcloned in the opposite orientation or not in frame with the *cat* gene. The latter possibility is not likely since the Cm^R gene in pSUP401 is identical to that in pACYC184.

Most of the subcloned fragments of the P factor express fewer proteins than expected for the coding capacity of the DNA (Table 4.4). The observation that only a small percentage (33%) of the total coding capacity of the subcloned *Eco*RI fragments (32.5 kb) results in detectable [^{35}S]methionine-labelled proteins could be due to a short half life of the proteins. Alternatively, the proteins encoded may have only a few or no methionine residues making them difficult to detect. Another possibility is that the genes have been removed from their natural promoters during cloning, or that the regulatory elements required for their expression are not present.

4.6 Attempts to subclone fragments *Eco*RI-1, *Eco*RI-2B and *Xba*I-1 of P.

The first attempts to subclone the *Xba*I-1 fragment or its constituent *Eco*RI-1 and *Eco*RI-2B fragments of the P factor utilizing the high copy number vectors pACYC184 and pJRD158b were unsuccessful. Low copy number vectors such as pSC101 (Tait and Boyer,1978), pLG339 (pSC101 derivative) (Stoker *et al.*, 1982) and pRK290 (Ditta *et al.*, 1980) were used in futile attempts to overcome a potential problem with over-expression of cloned determinants on high copy number vectors.

Several explanations are possible. The size of the fragments (*Xba*I-1 45 kb and *Eco*RI-1 24 kb), would result in very large recombinant plasmids and these might be particularly unstable. Large plasmids are also less readily transformed into competent cells. In the case of *E. coli* K-12, overproduction of outer membrane proteins or proteins lethal to the host (Manning et al., 1982) encoded by plasmids contributes to host instability or apparent poor transformability.

By transposon mutagenesis the *Eco*RI-1, *Eco*RI-2B region of the P factor was shown to be involved in transfer and surface exclusion functions (see section 4.3, Figure 4.5). Other workers (Skurray *et al.*, 1976) have shown that the cloning of analagous regions from the F factor or R6-5 in *E. coli* K-12 was difficult. The presence of partitioning (*par*) genes on these *Eco*RI fragments could contribute to instability and poor inheritance of the recombinant plasmids, as reported by Funnell (1988) when subcloning the *parB* region of P1 plasmid. The *par* region is very likely to map close to the *tra* region as for F (Ogura and Higara, 1983), hence possibly explaining the difficulty in cloning of these fragments.

4.7 Analysis of plasmid pPM804.

Plasmid pPM804 contains the *Eco*RI-4 fragment of P cloned in plasmid vector pACYC184. It has already been seen that this recombinant plasmid encodes at least two polypeptides of 24 kDal and 23 kDal which are readily detectable in whole cell extracts (Figure 4.9A) and in minicells (Figure 4.10A).

Fractionation of whole cells harbouring plasmid pPM804 permitted the localization of the encoded proteins to the cell envelope. Further separation of the cell envelope on sucrose gradients allows the outer and inner membranes to be separated. *E. coli* K-12 cells harbouring plasmid pPM804 were labelled with [^{35}S]-methionine, converted to sphaeroplasts and the cell envelope extracted. The inner and outer membranes of the cellular extracts were separated on a sucrose density gradient to localize the pPM804 proteins within the cell envelope. Separation of outer and inner membranes usually results in the appearance of discrete regions: H, M, L1 and L2 (Osborne *et al.*, 1972) which represent the outer membrane, unseparated envelope material and (the final two peaks) the inner membrane. A similar pattern can be observed with the separation of cell envelope material derived from cells harbouring pPM804. Figure 4.12 illustrates these results. Fractions A, B, and C represent the outer membrane (H band) banding at the higher sucrose density towards the bottom of the tube, fractions D and E unseparated membrane material (M band) and fractions F, G, H and I represent the inner membrane (the two L1 and L2 peaks). Figure 4.13 shows the distribution of protein within these fractions of cells containing pPM804. The pPM804 encoded proteins can be seen in the cell envelope sample prior to separation and also in the first three fractions (A,B,C) corresponding to the outer membrane (Figure 4.12).

Figure 4.12.

Graphic representation of the sucrose gradient used to separate the outer and inner membranes from the cell envelopes. The density of each fraction was measured to determine the linearity of the gradient (top section). The counts per minute (cpm) of each fraction was measured in order to determine the position of the outer and inner membranes (bottom section) (Osborne *et al.*, 1972).

H band = outer membrane

M band = unseparated membrane material

L_1 and L_2 = two peaks representing inner membrane

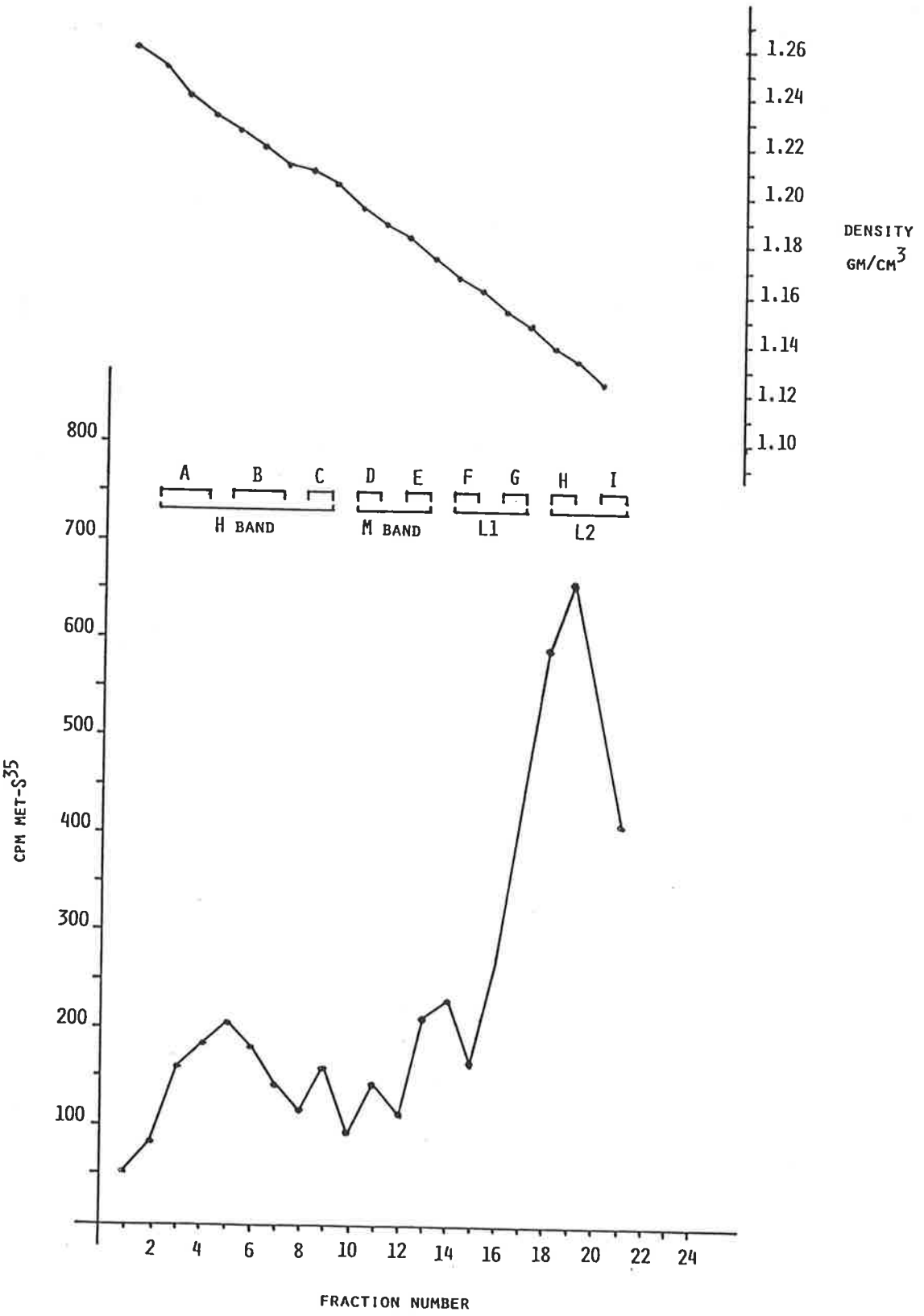
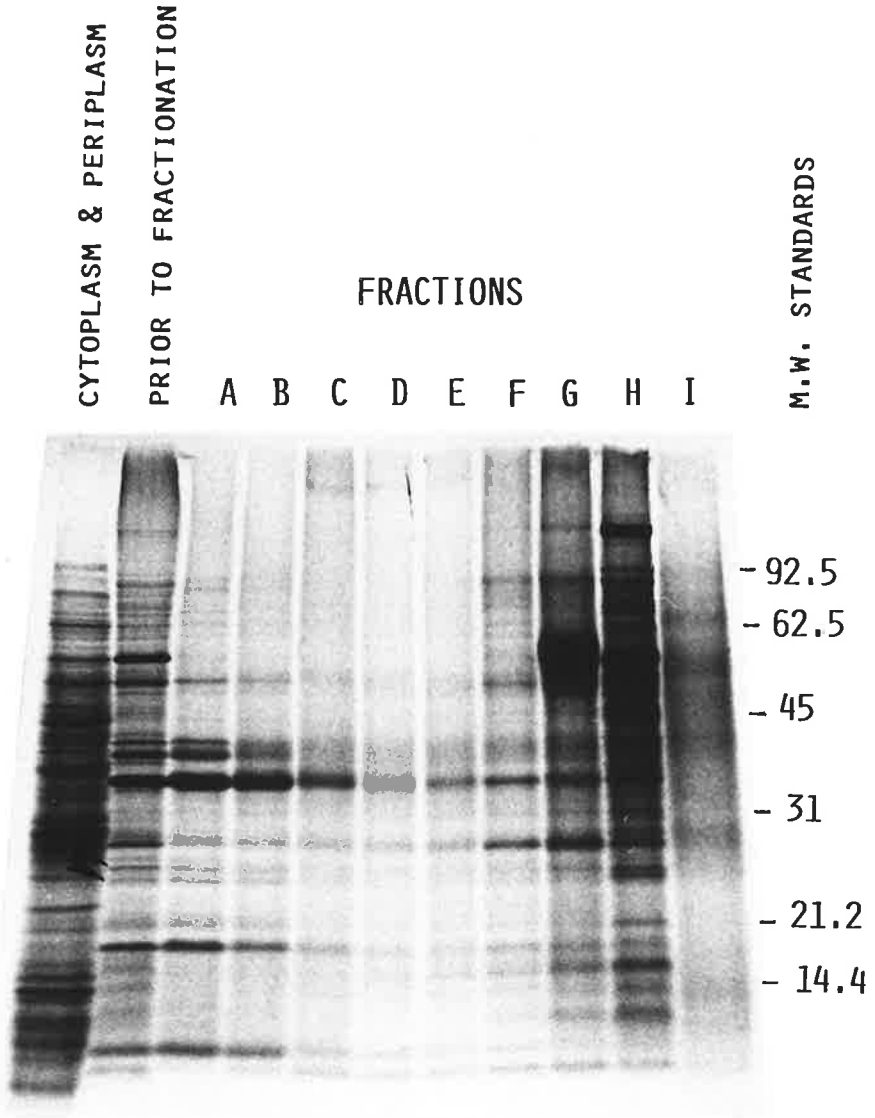

1.26
1.24
1.22
1.20
1.18
1.16
1.14
1.12
1.10
DENSITY
GM/CM3
A
B
C
D
E
F
G
H
I
H BAND
M BAND
L1
L2
800
700
600
500
400
300
200
100
CPM MET-S35
2
4
6
8
10
12
14
16
18
20
22
24
FRACTION NUMBER

Figure 4.13.

Autoradiograph of [^{35}S]-methionine-labelled cell envelopes of *E.coli* K-12 LE392 harbouring pPM804 after separation of inner and outer membranes on sucrose gradients. Fractions A, B and C represent the outer membrane (H band), fractions D and E represent unseparated membrane material (M band) and fractions F, G, H and I represent the inner membrane (L_1 and L_2 bands) (Osborne *et al.*, 1972). The two proteins encoded by pPM804 (24 and 23 kDal) are arrowed on the left. Fraction samples were run on SDS-PAGE (11-20% polyacrylamide) and autoradiographed at room temperature.


CYTOPLASM & PERIPLASM
PRIOR TO FRACTIONATION
FRACTIONS
A B C D E F G H I
M.W. STANDARDS
- 92.5
- 62.5
- 45
- 31
- 21.2
- 14.4

The *Eco*R1-4 fragment cloned in pPM804 has been inverted with respect to the vector pACYC184 to give pPM843. Both orientations of *Eco*RI-4 result in protein production in minicells and whole cells. Figure 4.14 shows the cellular envelope fractions from *E. coli* K-12 cells harbouring pPM804 and pPM843, with a greater expression in pPM843. The vector promoter (P5) which precedes the *cat* gene (Stüber and Bujard, 1981) could read through into the inserted DNA resulting in the higher levels of expression observed of the *Eco*RI-4 encoded proteins (24 kDal and 23 kDal). This interpretation would imply that the direction of transcription of both proteins was from left to right.

In order to map more precisely the position of the coding region on the *Eco*RI-4 fragment for these two proteins a series of deletions of the *Eco*RI-4 fragment were constructed (Figure 4.15). Examination of the protein profiles on SDS polyacrylamide gels of cell envelopes of *E. coli* K-12 harbouring these deletions (Figure 4.14) demonstrated that the proteins are encoded within the 2.5 kb *Eco*RI-*Xba*I DNA fragment. pPM846 has the 0.5 kb *Eco*RI-*Hin*dIII fragment deleted and this construct no longer expresses either protein. The plasmid pPM847 has the 0.2 kb *Eco*RI-*Eco*RV fragment deleted and does not express the 24 kDal protein and the 23 kDal in very reduced amounts, inferring that this 0.2 kb fragment is required for the expression of these proteins. Deletion of the 1.3 kb *Acc*I-*Acc*I fragment contained within the 2.5 kb *Eco*RI-*Xba*I fragment eliminates expression of both proteins, indicating that the end point of the coding region lies within this 1.3 kb *Acc*I-*Acc*I fragment. Plasmids pPM804 and pPM843 result in the disappearance of a 18 kDal protein (protein III; Henning *et al.*, 1973), however, deletions of the *Eco*RI-4 only cause a reduction in the amount of protein III being produced.

Figure 4.14.

Cell envelopes were obtained by fractionation of whole cells and the proteins separated in SDS on a 15% polyacrylamide gel followed by staining with Coomassie blue. The two proteins encoded on the *Eco*RI-4 P factor fragment (24 and 23 kDal) are arrowed on the left. Plasmids pPM804 and pPM843 have the *Eco*RI-4 fragment in opposite orientations in the vector pACYC184. Plasmids pPM841, pPM842, pPM846, pPM847 and pPM848 are deletion derivatives of *Eco*RI-4 (Figure 4.15).

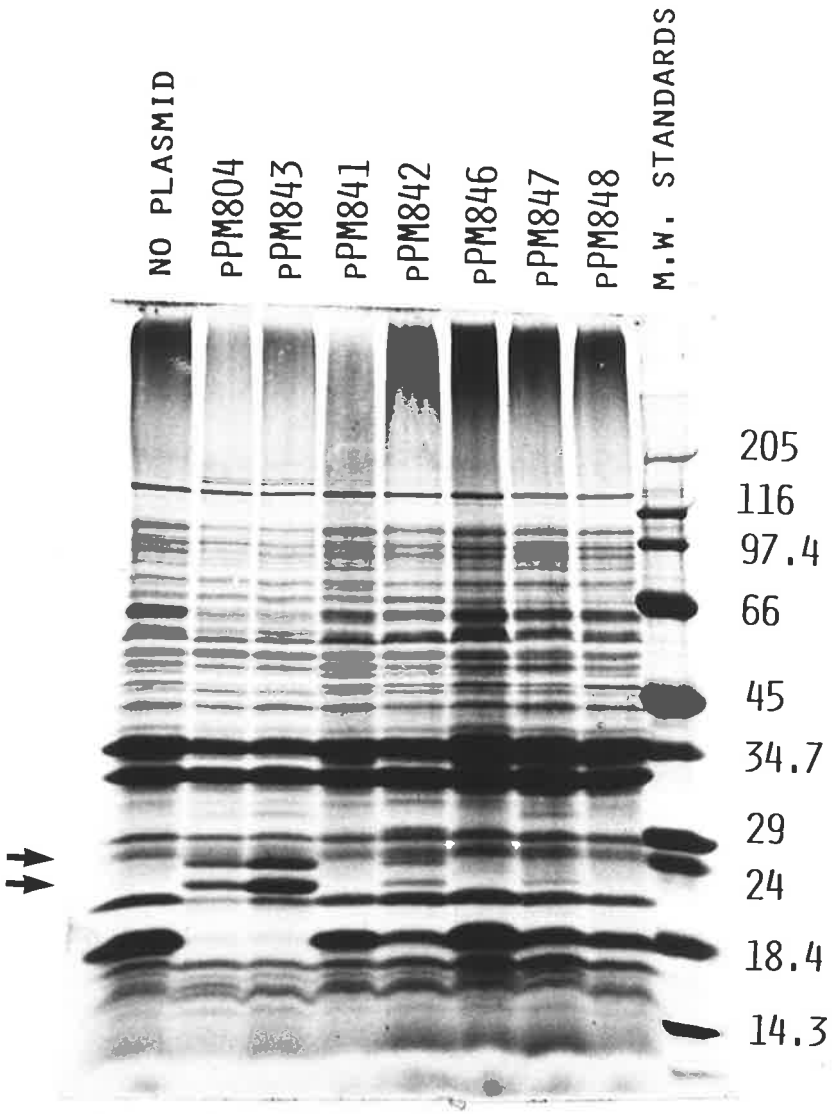

NO PLASMID
pPM804
pPM843
pPM841
pPM842
pPM846
pPM847
pPM848
M.W. STANDARDS
205
116
97.4
66
45
34.7
29
24
18.4
14.3

Figure 4.15.

Transposon insertions in the *Eco*RI-4 fragment of the P factor. Tn*1725* was used for mutagenesis of the 2.5 kb *Eco*RI-*Xba*I fragment (pPM842) and transposons Tn*3*, Tn*5* and Tn*1725* were used for mutagenesis of the entire *Eco*RI-4 fragment (pPM804). Plasmids pPM841, pPM842, pPM846, pPM847 and pPM848 are deletions of the *Eco*RI-4 fragment. The lines represent the extent of *Eco*RI-4 DNA retained in the plasmids.

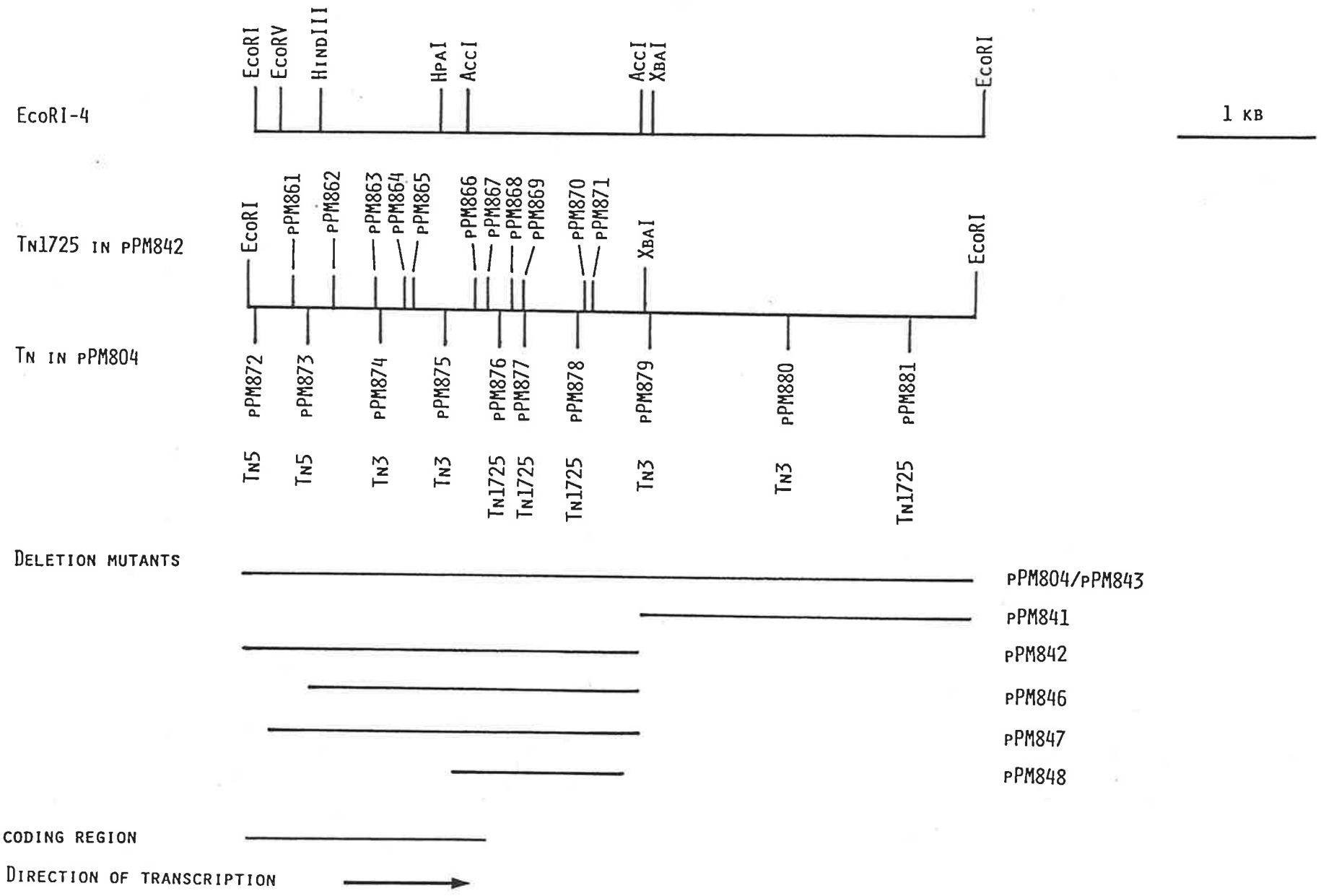

EcoRI-4
EcoRI
EcoRV
HindIII
HpaI
AccI
AccI
XbaI
EcoRI
1 kb
Tn1725 in pPM842
EcoRI
pPM861
pPM862
pPM863
pPM864
pPM865
pPM866
pPM867
pPM868
pPM869
pPM870
pPM871
XbaI
EcoRI
Tn in pPM804
pPM872
pPM873
pPM874
pPM875
pPM876
pPM877
pPM878
pPM879
pPM880
pPM881
Tn5
Tn5
Tn3
Tn3
Tn1725
Tn1725
Tn1725
Tn3
Tn3
Tn1725
Deletion mutants
pPM804/pPM843
pPM841
pPM842
pPM846
pPM847
pPM848
coding region
Direction of transcription

Figure 4.16

Cell envelopes of various transposon insertions in pPM842 were obtained by fractionation of whole cells and the proteins separated in SDS on a 11-20% (w/v) polyacrylamide gel followed by staining with Coomassie blue. The two proteins encoded on the *Eco*RI-4 fragment (23 and 24 kDal) and the *E. coli* K-12 encoded protein III (18 kDal; Henning *et al.*, 1973) are indicated on the right.

Plasmid pPM843 is the *Eco*RI-4 fragment cloned into pACYC184, pPM842, pPM846 and pPM847 are deletion derivatives of *Eco*RI-4 and pPM862-pPM871 are transposon insertions into pPM842 (Figure 4.15).

Size standards (in kDal) are indicated on the left.

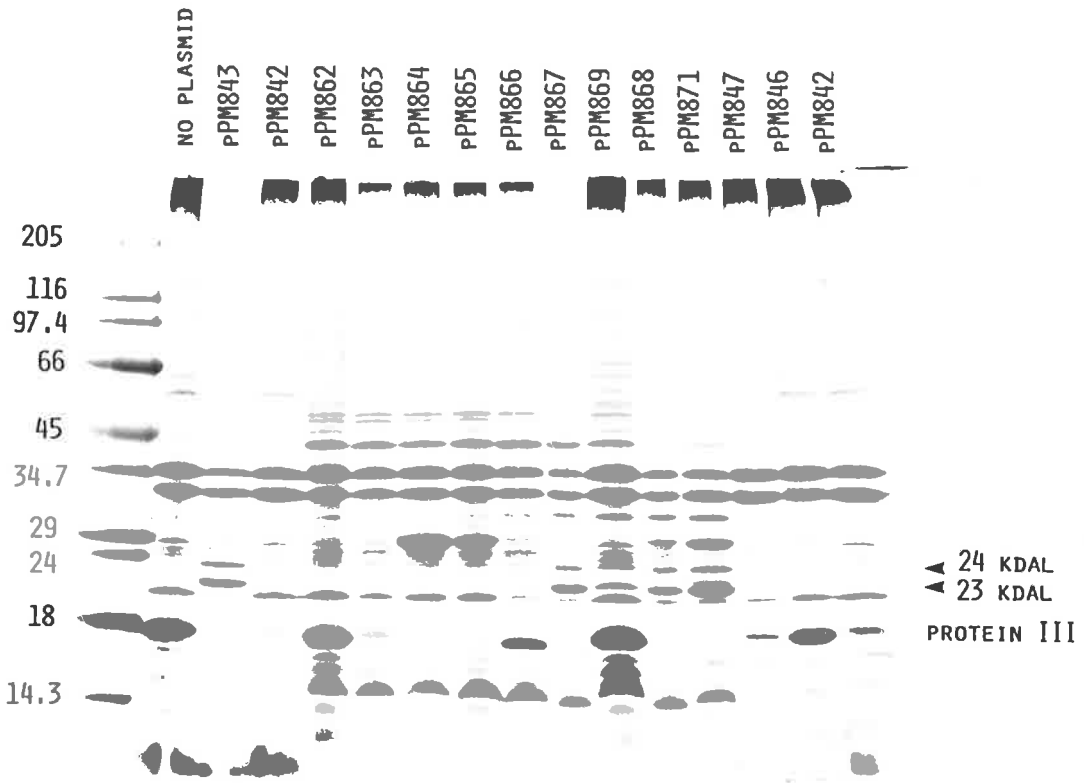

NO PLASMID
pPM843
pPM842
pPM862
pPM863
pPM864
pPM865
pPM866
pPM867
pPM869
pPM868
pPM871
pPM847
pPM846
pPM842
205
116
97.4
66
45
34.7
29
24
18
14.3
24 KDAL
23 KDAL
PROTEIN III

Table 4.5

TRANSPOSON INSERTIONS IN *Eco*RI-4 FRAGMENT OF THE P FACTOR

Plasmid	Protein alterations in 23 kDal	24 kDal	proteinIII[a]
no plasmid	-	-	+++
pPM843	+	+	-
Transposon insertion			
pPM861	-	-	+++
pPM862	-	-	+++
pPM863	-	-	++
pPM864	-	-	-
pPM865	-	-	+
pPM866	-	-	-
pPM867	+	+	-
pPM868	+	+	-
pPM869	+	+	-
pPM870	+	+	-
pPM871	+	+	-
pPM872	+red*	+red	-
pPM873	-	-	++
pPM874	-	-	++
pPM875	-	-	++
pPM876	+	+	-
pPM877	+	+	-
pPM878	+	+	-
pPM879	+	+	-
pPM880	+	+	-
pPM881	+	+	-

Table 4.5 (continued)

Plasmid	Protein alterations in 23 kDal	24 kDal	proteinIII[a]
Deletions			
pPM841	-	-	+++
pPM842	+red	+red	++
pPM846	-	-	++
pPM847	+red	-	++
pPM848	-	-	++

a protein III of *E. coli* K-12 (Henning *et al.*, 1973), 18 kDal number of + indicate the level of protein produced

* red = reduced amount of protein produced

The 2.5 kb *Eco*RI-*Xba*I restriction fragment was subcloned into the vector pJRD158b (pPM842) and subjected to transposon mutagenesis with Tn*1725*. Transposons Tn*3*, Tn*5* and Tn*1725* were also used to mutagenize the whole *Eco*RI-4 fragment (plasmid pPM804). Transposon insertions into the 2.5kb fragment result in a change of the membrane profile of *E. coli* K-12 cells harbouring these plasmids (Figure 4.16). The most marked effect is the disappearance or reduction in the amount of protein III being produced. In the presence of the 23 and 24 kDal proteins protein III disappears, suggesting that the two proteins encoded on the *Eco*RI-4 are displacing it in the membrane. The effects of the transposon insertions and deletions of *Eco*RI-4 on protein III are summarized in Table 4.5 and Figure 4.15 shows the sites of insertion of the different transposons in the *Eco*RI-*Xba*I fragment (top transposon insertions) and the entire *Eco*RI-4 fragment (lower transposon insertions). The 30 kDal protein present in many of the Tn*1725* insertions in pPM842 is likely to be due to overproduction of the β-lactamase (encoded on the vector, pJRD158b), which is then trapped in membrane vesicles.

The 23 and 24 kDal proteins appear to be part of one operon and are dependent on one another for their expression. Restriction analysis of the deletion and transposon insertion plasmids combined with analysis of the proteins expressed have been used to determine that the coding region for these two proteins (24 kDal and 23 kDal) lies within 1.7kb from the *Eco*RI site (Figure 4.16).

4.8 Conclusions

Cloning of 8 of the 10 *Eco*RI fragments and 6 of the 8 *Xba*I fragments has resulted in 48% of the P factor being subcloned. These subcloned fragments were examined for P factor encoded proteins in

both whole cell extracts and minicells. Only one third of the total coding capacity of the cloned DNA corresponding to approximately 19 proteins could be detected. It is feasible that the genes encoded by the F' factor have been removed from their natural promoters during the cloning, or that the regulatory elements responsible for their expession are not present. The synthesis of unstable proteins or proteins with short half lives may contribute to difficulties in the detection of proteins. Poor staining by Coomassie blue or few methionine residues within the protein may also hinder their detection.

The transfer (*tra*) region has been localized by transposon mutagenesis to the *Xba*I-1 and *Eco*RI-1 restriction fragments. Attempts to subclone this region have failed. A possible explanation is that this *tra* region would encode a large number of proteins, many of which would be located in the membrane and could well be lethal to the cell if over expressed or expressed in the absence of other proteins. These regions are very large fragments of DNA possibly resulting in a large unstable recombinant plasmid which is not easily transformed into competent cells.

Chapter 5

Restriction analysis and physical mapping of the two cryptic plasmids of *V. cholerae* strain V58.

5.1 Introduction.

V. cholerae strain V58 harbours three plasmids (Chapter 3), the P factor and two cryptic plasmids. The larger plasmid (lcp) is 34 kb and the smaller (scp) is 4.7 kb. These two plasmids have not been previously characterized and an analysis at a molecular and physical level is described here.

5.2 Restriction endonuclease analysis of the large cryptic plasmid.

Plasmid DNA from strain V58 contained both cryptic plasmids, with the smaller one usually difficult to visualise on agarose gels. This plasmid DNA was subjected to digestion with 23 different restriction endonucleases and the fragments were separated by electrophoresis on an 0.6% agarose gel (Figure 5.1).

A summary of the restriction data is shown in Table 5.1. No sites were detected for *Bcl*I, *Sca*I and *Xho*I in the large cryptic plasmid and seven restriction endonucleases cleave it only once; *Bam*HI, *Mlu*I, *Nde*I, *Pvu*II, *Sal*I, *Stu*I and *Xba*I. The sum of fragments for each of the restriction enzymes examined was calculated (Table 5.1). Discrepancies in total size of lcp range between 32-35kb and this would be due to inaccuracy of measurements of large fragments. The accurate size

Figure 5.1

A 0.6% agarose gel illustrating a selection of restriction endonuclease cleavage patterns of plasmid DNA isolated from *V. cholerae* strain V58 P-. The positions of the uncut lcp, the chromosome and the small cryptic plasmid (linear form at 4.7 kb and the uncut form just below it) are indicated on the left. Bacteriophage SPP1 DNA digested with *Eco*RI has been used as a size standard, and is indicated on the right (fragment sizes are in kb) (Radcliff *et al.*, 1979; Franzon and Manning, 1986).

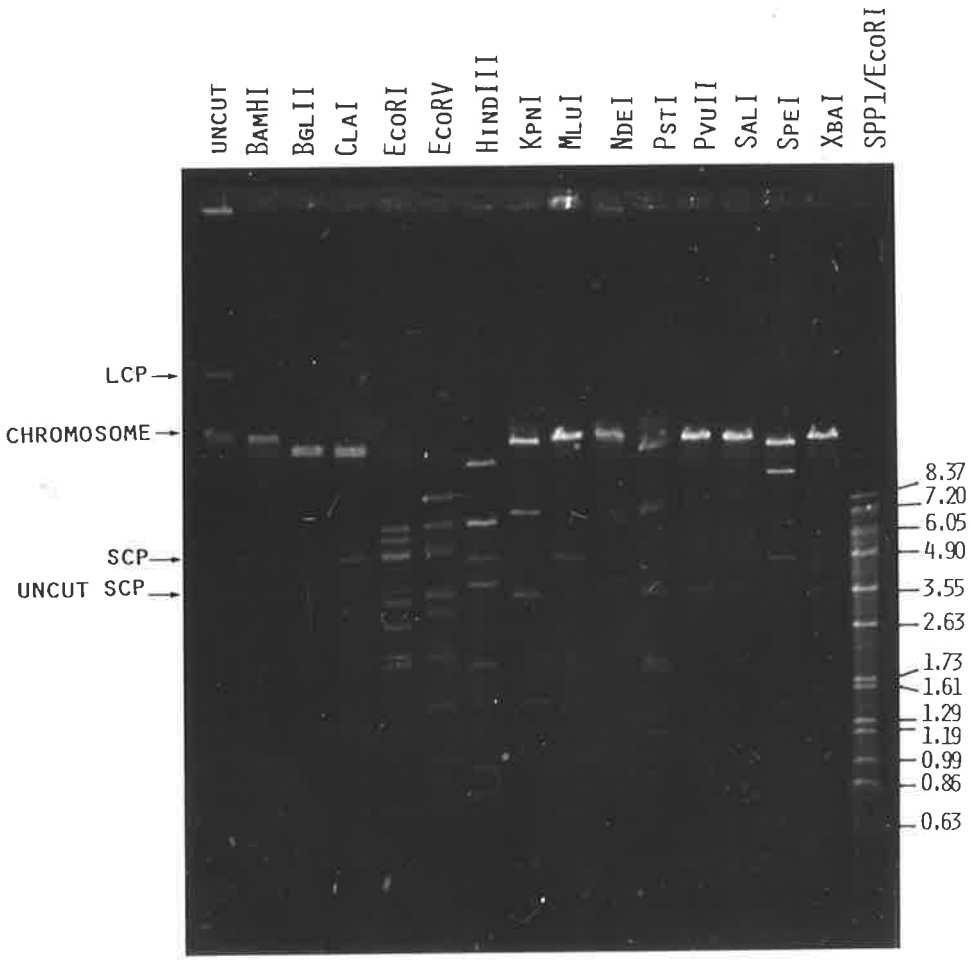

UNCUT
BAMHI
BGLII
CLAI
ECORI
ECORV
HINDIII
KPNI
MLUI
NDEI
PSTI
PVUII
SALI
SPEI
XBAI
SPP1/ECORI
LCP
CHROMOSOME
SCP
UNCUT SCP
8.37
7.20
6.05
4.90
3.55
2.63
1.73
1.61
1.29
1.19
0.99
0.86
0.63

Table 5.1

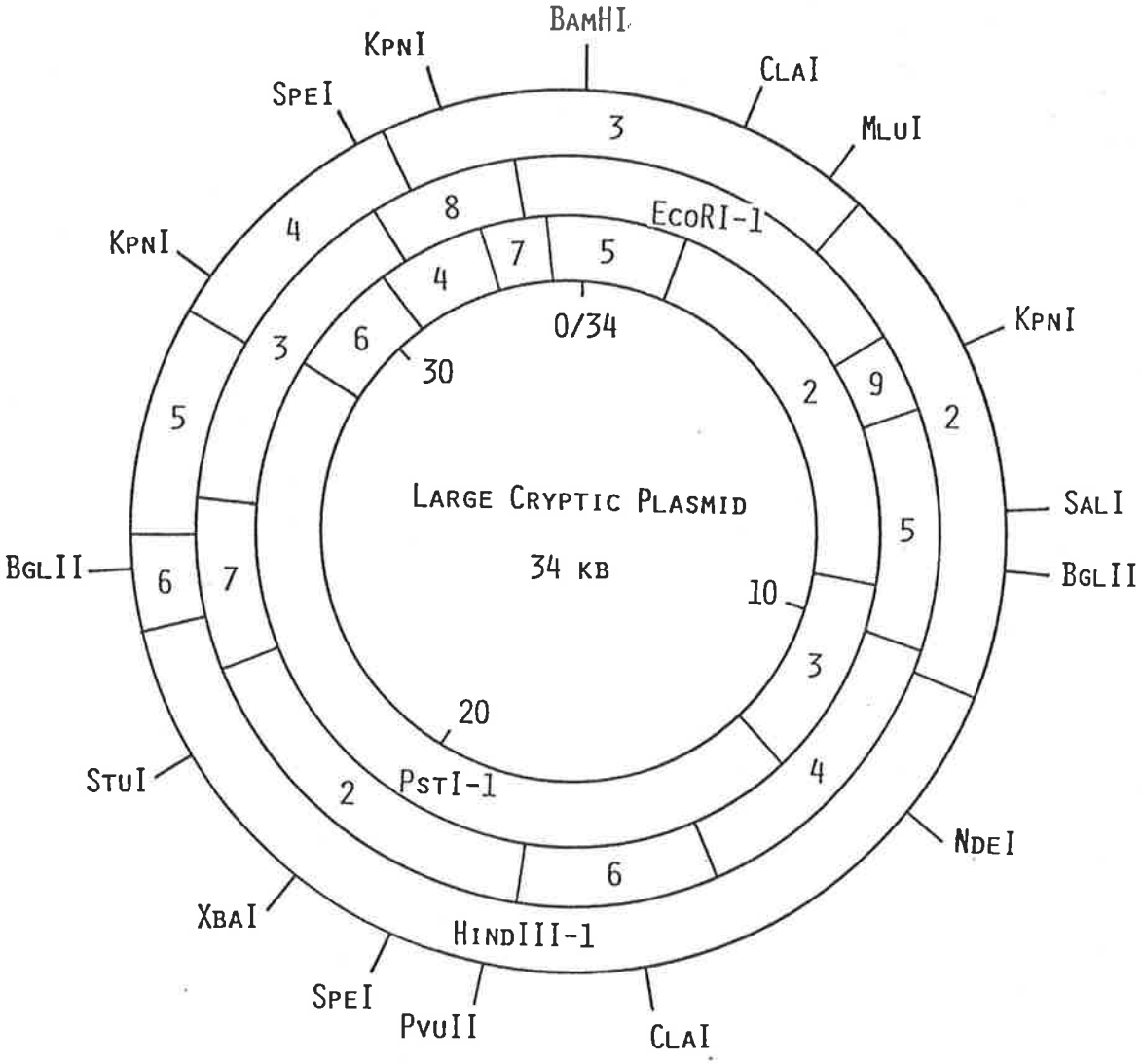
NUMBER OF RESTRICTION FRAGMENTS GENERATED UPON DIGESTION OF THE LARGE CRYPTIC PLASMID

Restriction Enzyme	Number of Fragments*	Sum of fragments (kb)	Restriction Enzyme	Number of Fragments	Sum of fragments (kb)
*Acc*I	6	35	*Nde*I	1	34
*Aha*III	5	35	*Nru*I	5	33
*Bam*HI	1	34	*Pst*I	7	34
*Bcl*I	0	-	*Pvu*II	1	34
*Bgl*II	2	34	*Sal*I	1	34
*Cla*I	2	34	*Sca*I	0	-
*Eco*RI	9	34	*Spe*I	2	34
*Eco*RV	12	32.5	*Ssp*I	3	35
*Hin*dIII	6	34	*Stu*I	1	34
*Hpa*I	5	35	*Xba*I	1	34
*Kpn*I	3	34	*Xho*I	0	-
*Mlu*I	1	34			

* number of fragments represents the number which can be visualised on a 0.6% agarose gel stained with ethidium bromide with a sensitivity limit of 0.8 kb.

Figure 5.2.

Restriction endonuclease cleavage map of the large cryptic plasmid (lcp) present in strain V58. The map is calibrated in kilobases (kb) with the *Bam*HI site as the zero co-ordinate. *Eco*RI, *Pst*I and *Hin*dIII fragments are drawn as concentric circles.


BamHI
KpnI
ClaI
SpeI
MluI
KpnI
EcoRI-1
KpnI
0/34
30
SalI
Large Cryptic Plasmid
34 kb
BglII
BglII
10
20
StuI
PstI-1
NdeI
XbaI
HindIII-1
SpeI
PvuII
ClaI

determination of lcp was calculated from double restriction endonuclease digestion of the subcloned fragments.

Single and double digests of plasmid DNA and of the subcloned *Eco*RI fragments of lcp were used to construct a cleavage map for fourteen restriction endonucleases, with the single *Bam*HI site as the zero co-ordinate (Figure 5.2).

5.3 Subcloning of DNA fragments of the large cryptic plasmid.

A mixed plasmid DNA preparation from strain V58 containing the P factor, lcp and scp was used as the source for subcloning the *Eco*RI fragments of the three plasmids into vector pACYC184. The scp is not cleaved by *Eco*RI (section 5.5), hence all subcloned *Eco*RI fragments would originate from either the P factor or the lcp. The subcloning of the *Eco*RI fragments from the P factor has been described (section 4.4). Six of the nine *Eco*RI fragments originating from the lcp have been cloned into the vector pACYC184 (Figure 5.3). Fragments *Eco*RI-1, *Eco*RI-3, *Eco*RI-5, *Eco*RI-6, *Eco*RI-7 and *Eco*RI-8 are present in recombinant plasmids pPM829-pPM834, respectively. Plasmid pPM836 contains two *Eco*RI fragments, *Eco*RI-1 and *Eco*RI-8. Fragments *Eco*RI-3 and *Eco*RI-4 comigrate when subjected to electrophoresis on an agarose gel. These two fragments can, however, be distinguished by treatment with *Kpn*I, which cleaves only *Eco*RI-3. These *Eco*RI fragments have been confirmed by Southern hybridization to originate from lcp and not the P factor. The cloned *Eco*RI fragments represent approximately 60% of the lcp and have facilitated the construction of a restriction map (Figure 5.2). A more detailed restriction analysis of the *Eco*RI subcloned fragments of the lcp is shown in Figure 5.4.

Figure 5.3.

Agarose gel electrophoresis of lcp DNA (isolated from strain V58 P$^-$) and plasmids containing the *Eco*RI fragments subcloned into pACYC184. The lcp fragments and the position of the vector (arrowed) are labelled on the left. Bacteriophage lambda DNA digested with *Hin*dIII has been used as a size standard, with sizes in kb indicated on the right.

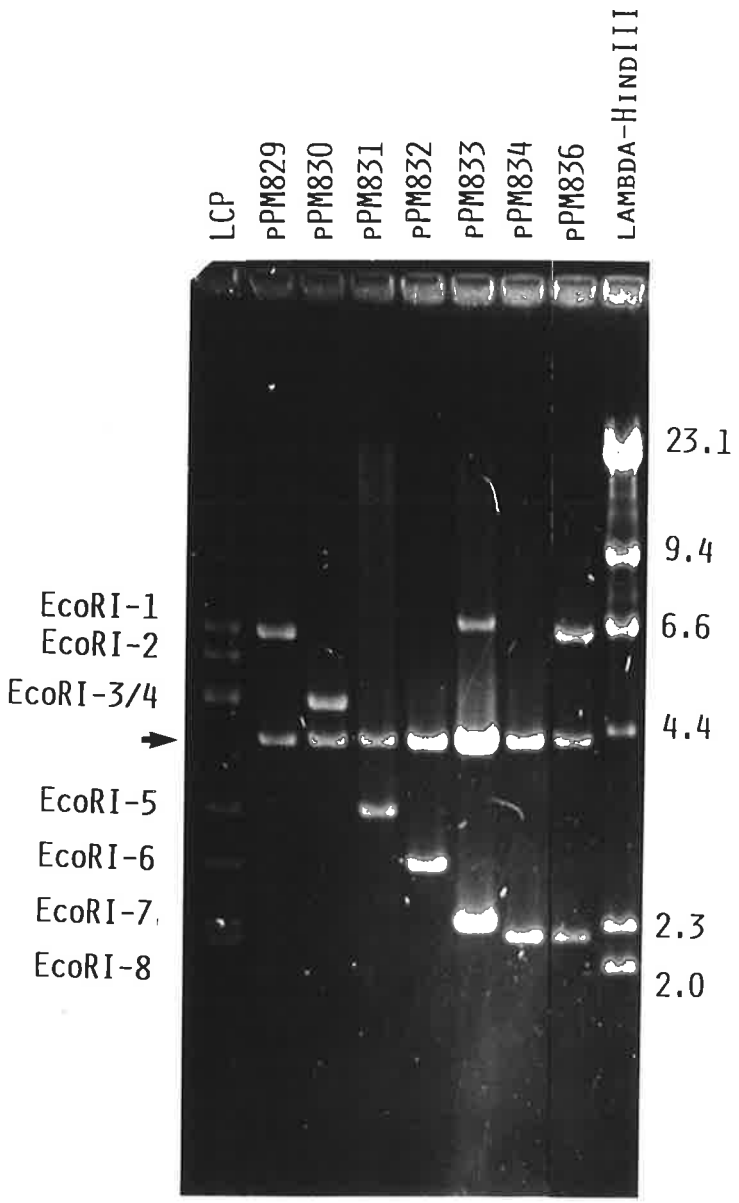
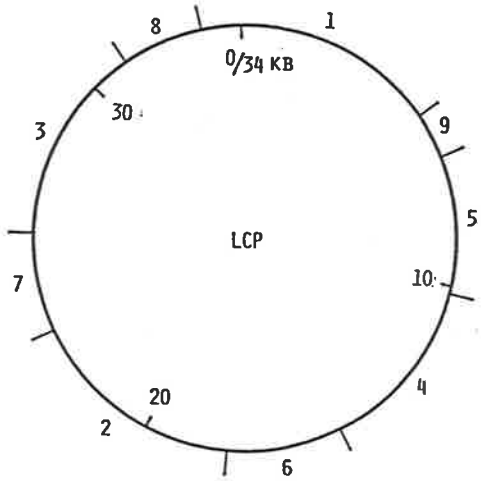

LCP
pPM829
pPM830
pPM831
pPM832
pPM833
pPM834
pPM836
LAMBDA-HindIII
23.1
9.4
6.6
4.4
2.3
2.0
EcoRI-1
EcoRI-2
EcoRI-3/4
EcoRI-5
EcoRI-6
EcoRI-7
EcoRI-8

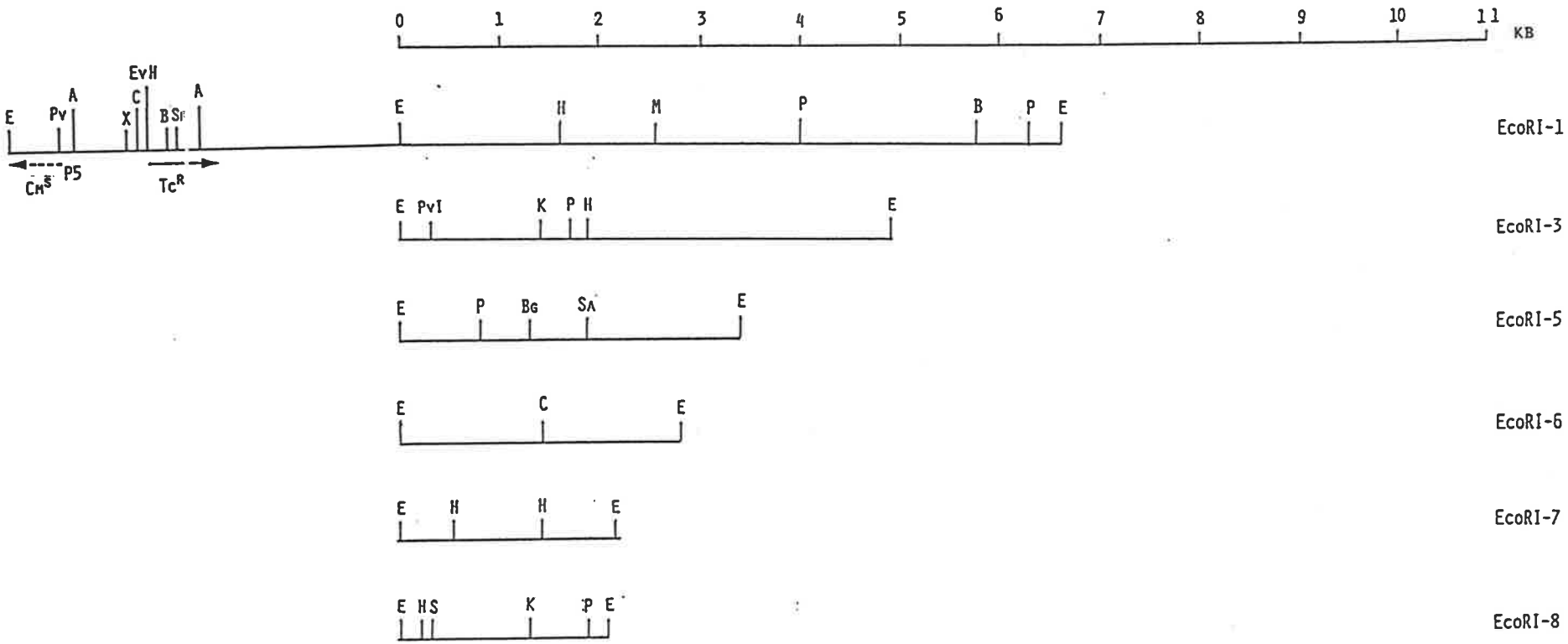
Figure 5.4.

A detailed restriction endonuclease map of the *Eco*RI fragments of the large cryptic plasmid subcloned into vector pACYC184. Those vector promoters which may affect the production of proteins encoded on the subcloned fragment are shown. The fragments are shown in the same orientation with respect to the cloning vector.

Restriction endonucleases: B-*Bam*HI, Bg-*Bgl*II, C-*Cla*I, E-*Eco*RI, H-*Hin*dIII, K-*Kpn*I, M-*Mlu*I, P-*Pst*I, PvI-*Pvu*I, Pv-*Pvu*II, Sa-*Sal*I, S-*Spe*I and X-*Xba*I.

EcoRI fragments -1, -3, -5, -6, -7 and -8 subcloned into pACYC184 are recombinant plasmids pPM829-pPM834, respectively.


0/34 KB
1
9
5
10
4
6
20
2
7
3
30
8
LCP


0 1 2 3 4 5 6 7 8 9 10 11 KB
E Pv A X C EvH B Sf A
CmS P5
TcR
E H M P B P E
EcoRI-1
E PvI K P H E
EcoRI-3
E P Bg Sa E
EcoRI-5
E C E
EcoRI-6
E H H E
EcoRI-7
E H S K P E
EcoRI-8

5.4 Tn*1732* labelling of the lcp.

The transposon Tn*1732* (Ubben and Schmitt, 1987) was used to insert a detectable marker onto the lcp. The lcp will be shown to be identical to the V factor (Chapter 6) and strain KB9 V^+ was used as the recipient for the transposon mutagenesis. The V factor transfers at a lower frequency (approximately 100 fold lower) than P (Bhaskaran and Sinha, 1971). Isolate V::Tn*1732*-1 had a transfer frequency of 1.68×10^{-3} and the Tn*1732* mapped in the *Hin*dIII-6 fragment. This isolate was used for P and V factor compatibility studies (section 6.6).

5.5 Analysis in *E. coli* K-12 of proteins produced by subclones of the large cryptic plasmid.

When the V factor was transferred into a Classical (569B) or El Tor (1621) strain, no new V factor-encoded proteins were detected on SDS-PAGE of whole cell extracts (Figure 5.5).

In order to obtain an indication of the number of proteins encoded by the lcp, *E. coli* K-12 derivatives harbouring recombinant plasmids pPM829-pPM834 and pPM836 have been analysed for the expression of plasmid proteins in both whole cells and in minicells.

5.5.1 Whole cell analysis.

Plasmids pPM829-pPM834 harbouring the *Eco*RI fragments of the lcp cloned into pACYC184 have been examined for the expression of lcp encoded proteins. Whole cell extracts of *E.coli* K-12 harbouring these six recombinant plasmids were examined by SDS-PAGE (Figure 5.6). The (CAT) encoded by pACYC184 (25 kDal) has been inactivated by insertion of DNA into the *Eco*RI site, hence it is absent in all of the subclones.

Figure 5.5.

Analysis of whole cell extracts of *V. cholerae* Classical strains V58, V58 P^-, 569B, 569B V^+ and El Tor strains 1621 and 1621 V^+ by SDS-PAGE followed by staining with Coomassie blue. Size standards are indicated on the right (in kDal).

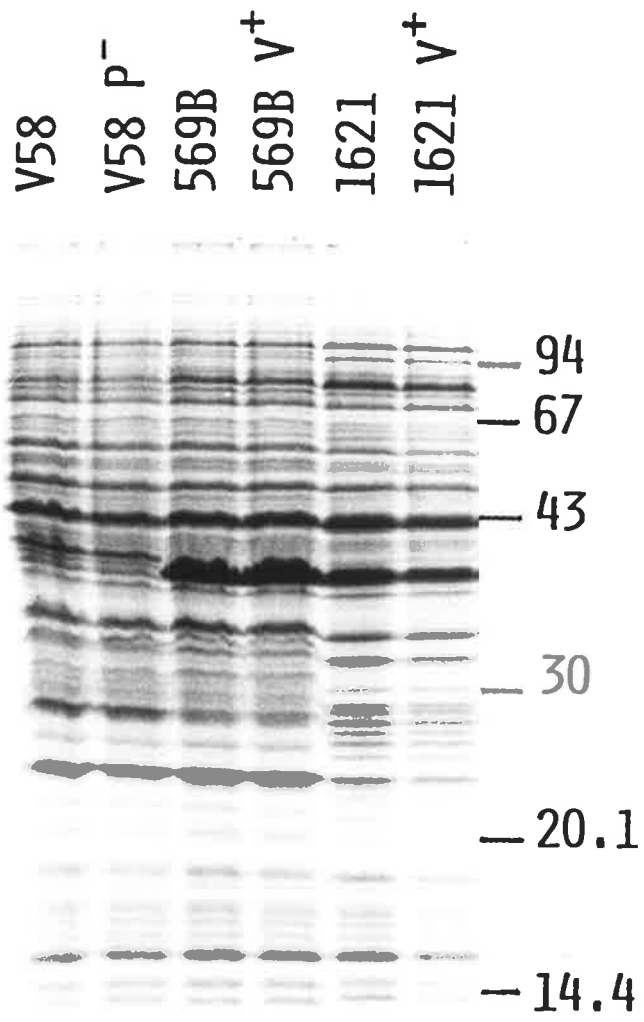
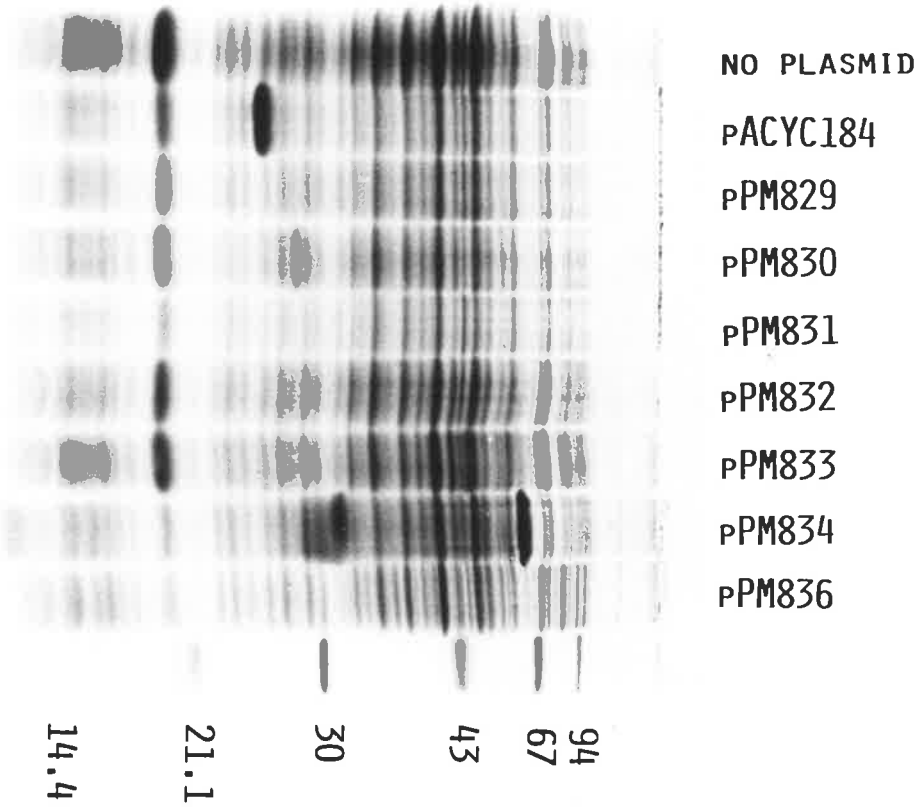

V58
V58 P⁻
569B
569B V⁺
1621
1621 V⁺
94
67
43
30
20.1
14.4

Figure 5.6.

Analysis of whole cell extracts of *E.coli* K-12 derivatives harbouring plasmids containing cloned *Eco*RI fragments of the large cryptic plasmid (lcp) (pPM829-pPM836), by SDS-PAGE on a 15% linear gel followed by staining with Coomassie blue.


NO PLASMID
pACYC184
pPM829
pPM830
pPM831
pPM832
pPM833
pPM834
pPM836
14.4
21.1
30
43
67
94

Plasmid pPM830 produces a protein of 30 kDal and plasmid pPM834 two proteins (92 kDal and 34 kDal) which can be detected by Coomassie blue staining and upon fractionation are localized in the cell envelope fraction. Plasmid pPM836 (which contains the contiguous fragments *Eco*RI-1 and *Eco*RI-8) does not encode the synthesis of any plasmid-encoded proteins which can be visualized by Coomassie blue staining.

5.5.2 Minicell analysis

An autoradiograph of [^{32}S]-methionine labelled minicells harbouring the plasmids pPM829-pPM834 is shown in Figure 5.7 and Table 5.2 lists the lcp-encoded gene products as well as the predicted coding capacity required to encode these proteins. The 92 kDal protein from pPM834 which was readily detectable in whole cell extracts of cells harbouring pPM834 (Figure 5.6) cannot be detected in minicells harbouring pPM834, however, the smaller 34 kDal protein can be detected. The 92 kDal protein may not have any methionine residues present and hence, may not be detectable in the [^{35}S]-methionine-dependent minicell system. Plasmid pPM836 contains the contiguous *Eco*RI fragments 1 and 8. This plasmid, however, did not encode any new proteins visible in this system, inferring that the *Eco*RI-8 fragment did not contain any promoters for genes on *Eco*RI-1. Very little of the potential coding capacity has been accounted for in these subclones. As with the P factor, it may be that few proteins are expressed because the genes have been removed from their natural promoters during the subcloning, the proteins synthesized are very unstable, have a short half life, or have few or no methionine residues.

Figure 5.7

Autoradiograph of proteins expressed in minicells harbouring *Eco*RI-subcloned fragments of the large cryptic plasmid (lcp). Minicells harbouring these recombinant plasmids were separated on 20% sucrose gradients, labelled with [^{35}S]-methionine (Kennedy *et al.*, 1977; Achtman *et al.*, 1979), solubilized in sample buffer, separated in SDS on a 11-20% polyacrylamide gradient gel and autoradiographed at room temperature. The plasmid encoded proteins are indicated by arrows. Size standards (kDal) are marked on the right.

pPM829
pPM830
pPM831
pPM832
pPM833
pPM834
pACYC184
-14.4
20.1
-30
-43
-67
-94

Table 5.2

PROTEINS DETECTED IN MINICELLS CONTAINING

CLONED RESTRICTION FRAGMENTS OF LARGE CRYPTIC PLASMID (lcp) DNA

*Eco*RI Fragment	Plasmid	Fragment Sizes (kb)	Protein Sizes (kDal)	Predicted Coding Capacity required (kb)
1	pPM829	6.6	16	0.44
2	-	5.9	NA	
3	pPM830	4.9	43, 22	1.77
4	-	4.7	NA	
5	pPM831	3.4	-	-
6	pPM832	2.8	21	0.57
7	pPM833	2.15	21	0.57
8	pPM834	2.10	34, 33	1.83
9	-	1.00	NA	
1,8	pPM836	8.7	34, 33, 16	2.26

NA = not available

5.6 Restriction endonuclease analysis of the small cryptic plasmid.

Only 5 of the 16 restriction endonucleases tested cleave the scp; enzymes *Cla*I, *Hind*III, *Mlu*I and *Spe*I cleave it once, while *Sph*I cuts three times. The enzymes *Bam*HI, *Bcl*I, *Eco*RI, *Kpn*I, *Nhe*I, *Nru*I, *Pst*I, *Pvu*II, *Sac*I, *Sst*I and *Xba*I do not cleave at all. Using single and double digests and with the *Spe*I site as the zero co-ordinate it has been possible to construct a restriction map of scp (Figure 5.8).

5.7 Subcloning of the small cryptic plasmid.

Plasmid DNA isolated from strain V58 P^- was subjected to digestion with restriction endonuclease *Sph*I (which cleaves the scp three times) and fragments ligated to *Sph*I digested pBR332 DNA. Two of the three *Sph*I fragments were cloned to give pPM851 (*Sph*I-2, 1.8 kb) and pPM852 (*Sph*I-1, 2.25 kb). The whole scp has been subcloned into the *Cla*I site of pBR322 to give pPM853.

The subcloned *Sph*I fragments pPM851 and pPM852 have been confirmed to originate from the scp by Southern hybridization (Figure 5.9) and pPM853 has been confirmed by detailed restriction analysis.

5.8 Analysis of the proteins encoded by the small cryptic plasmid.

5.8.1 Whole cell analysis.

Whole cell extracts of *E.coli* K-12 derivatives harbouring pPM851, pPM852 and pPM853 were analyzed by SDS-PAGE. No proteins encoded by the subclones were detected upon staining with Coomassie blue.

Figure 5.8.

Restriction endonuclease cleavage map of the 4.7 kb small cryptic plasmid (scp) present in strain V58. The map is calibrated in kilobases (kb) with the *Spe*I site as the zero co-ordinate.

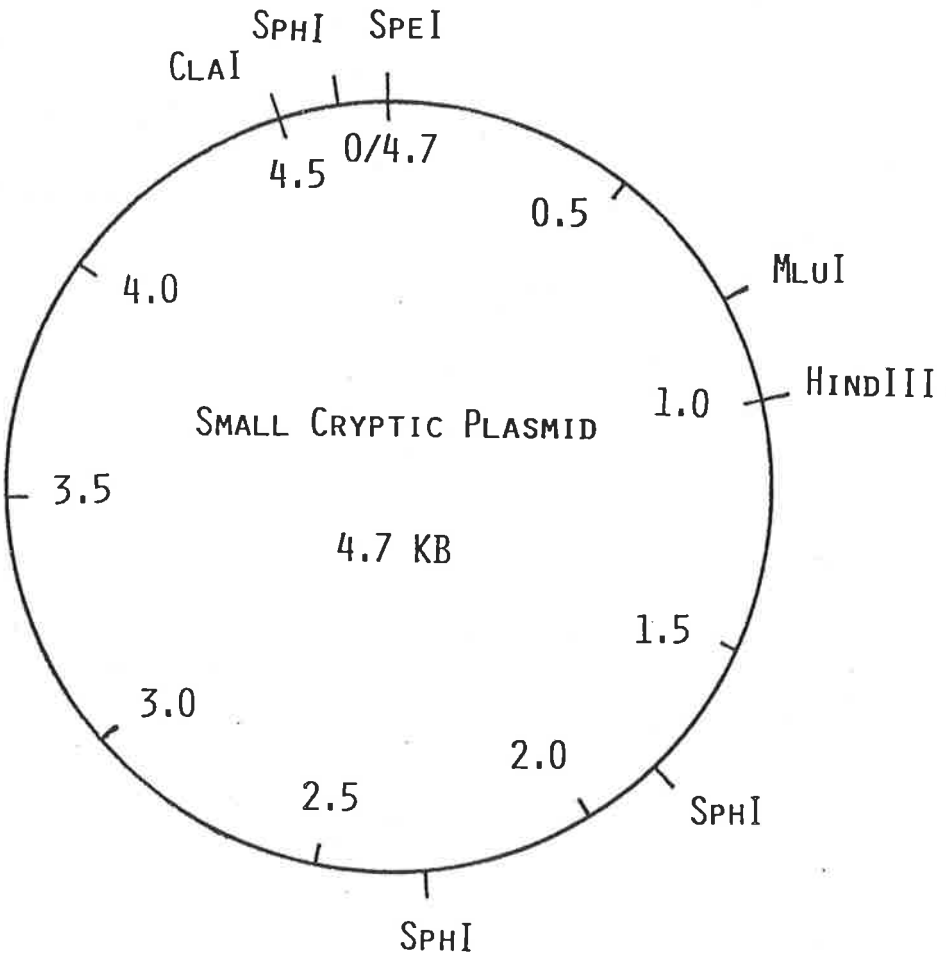

SphI
SpeI
ClaI
0/4.7
4.5
0.5
MluI
4.0
HindIII
1.0
Small Cryptic Plasmid
3.5
4.7 KB
1.5
3.0
2.0
2.5
SphI
SphI

Figure 5.9.

Southern hybridization analysis to confirm that *Sph*I subcloned fragments into pBR322 originate from the scp present in *V. cholerae* strain V58. DNA extracted from V58 (scp^+) and plasmids pPM851 and pPM852 were digested with *Sph*I, fragments separated by electrophoresis through a 0.6% agarose gel, transferred to nitrocellulose and probed with [^{32}P]-dCTP labelled plasmids pPM851 and pPM852. The position of the subcloned *Sph*I fragment is indicated as well as the vector, pBR322.

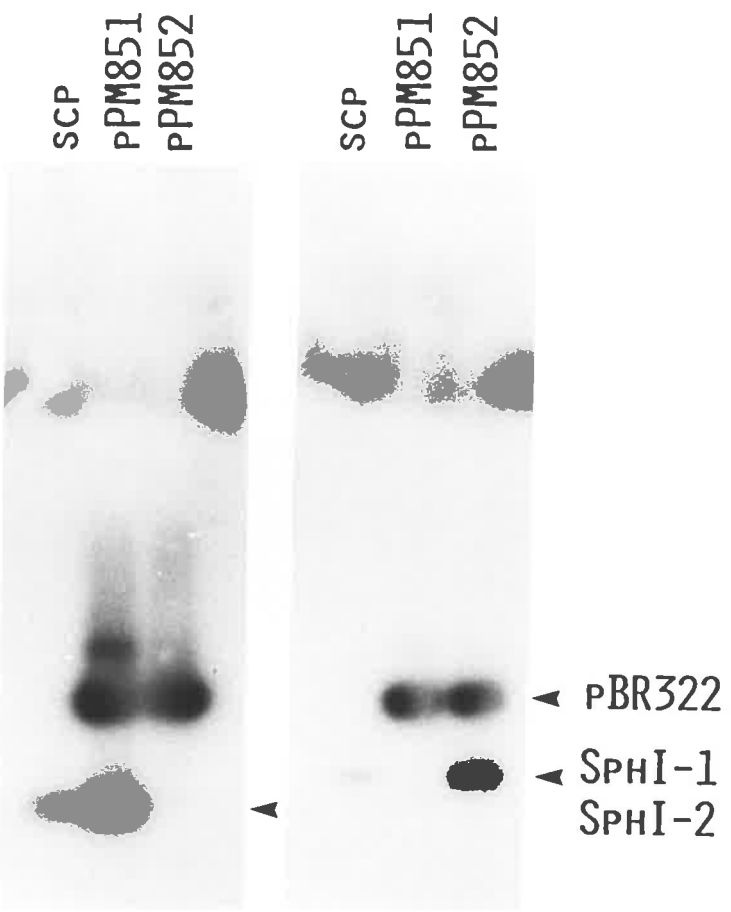

SCP
pPM851
pPM852
SCP
pPM851
pPM852
pBR322
SphI-1
SphI-2
pPM851
pPM852

Figure 5.10

Autoradiograph of proteins expressed in *E.coli* K-12 minicells harbouring subcloned fragments of the small cryptic plasmid (scp) following separation in SDS on a 11-20% polyacrylamide gradient gel. Minicells harbouring the recombinant plasmids were separated from whole cells on a 20% sucrose density gradient, labelled with [^{35}S]-methionine, and solubilized in sample buffer. The vector encoded β-lactamase proteins (precursor and mature forms) are present in all the tracks. pPM853 is the entire scp cloned into the *Cla*I site of pBR322 and pPM851 and pPM852 are *Sph*I fragments cloned into pBR322. Size standards (in kDal) are indicated on the left. Plasmid encoded proteins are arrowed.

94 -
67 -
43 -
30 -
20.1
14.4
pPM853
pPM851
pPM852
pBR322

Table 5.3

PROTEINS DETECTED IN MINICELLS CONTAINING CLONED

RESTRICTION FRAGMENTS OF THE SMALL CRYPTIC PLASMID DNA

Restriction Fragment	Fragment Size (kb)	Protein Sizes (kDal)	Predicted Coding Capacity required (kb)
*Cla*I (pPM853)	4.7	28	0.7
		13.5	0.37
*Sph*I-1 (pPM851)	1.4	28	0.7
*Sph*I-2 (pPM852)	2.25	13.5	0.37
		25	0.68

5.8.2 Minicell analysis.

Plasmids pPM851, pPM852 and pPM853 were also introduced into the *E. coli* K-12 minicell producing strain (Figure 5.10). Table 5.3 lists the scp-encoded gene products identified in minicells as well as the predicted coding capacity required to encode these proteins. The vector encoded β-lactamase and its precursor form (30 kDal and 32 kDal, respectively) are present in all samples. Plasmid pPM853 encodes two proteins of 28 kDal and 13.5 kDal which are encoded separately on the two *Sph*I subclones. In addition pPM852, encodes another protein 25 kDal (which becomes prominent following a longer exposure). This protein was not encoded by pPM853, possibly indicating that the *Cla*I site lies within the gene for this protein and that the gene is anticlockwise from the *Sph*I site at 4.6kb.

5.9 Conclusions

Restriction analysis of the two cryptic plasmids (lcp and scp) has confirmed the sizes determined by electron microscopic measurements (Chapter 3) as 34 kb and 4.7 kb, respectively.

The cloned *Eco*RI fragments of the large cryptic plasmid constitute approximately 60% of the plasmid. Examination of plasmid encoded proteins has revealed only five proteins corresponding to approximately one fifth of the potential coding capacity, possibly due to removal of natural promoters during the subcloning, or cloning with *Eco*RI may have interrupted coding sequences.

The entire scp has been subcloned into the *Cla*I site of pBR322, resulting in pPM853, and two plasmid-encoded proteins (28 kDal and 13.5 kDal) were detected by minicell analysis.

Since these two plasmids appear cryptic, this may account for the limited number of plasmid encoded proteins. The lcp is a conjugative

plasmid, inferring that a transfer region should result in membrane-encoded proteins (Willetts and Skurray, 1987). This however is not seen with the cloned *Eco*RI fragments, suggesting that the *tra* region would lie over several *Eco*RI fragments.

Chapter 6

Molecular epidemiology of the plasmids of *V. cholerae* Classical strain V58 and relationships to other plasmids

6.1 Introduction

A limited number of plasmids have been identified in *V. cholerae* including: several R factors which usually belong to the IncC group (Hedges and Jacob, 1975; Yokota and Kuwahara, 1977; Arai *et al.*, 1985; Davidson and Oliver, 1986), the P factor (Bhaskaran, 1958), the V factor (originally isolated in a non-01 *V. cholerae*), L factors (which are presumed to be plasmids, although this has not been confirmed (Smigocki and Voll, 1986)) and two plasmids which have consistently been identified in *V. cholerae* strains from the sixth pandemic, (Cook *et al.*, 1984).

Newland *et al.* (1984b) have examined a number of *V. cholerae* 01 strains and shown that only 2% of clinical and environmental isolates of such strains examined harboured plasmids, whereas 25% of clinical and environmental isolates of the non-01 *V. cholerae* strains harboured plasmids.

In this chapter, the occurrence of the P factor, the large (lcp) and small (scp) cryptic plasmids chararacteristic of strain V58 and their relationship to other plasmids among clinical, environmental and laboratory strains of *V. cholerae* has been investigated.

6.2 Plasmid profile of *Vibrio* species.

A collection of 76 strains of several *Vibrio* species including laboratory, environmental and clinical isolates was examined for the presence of plasmids by a rapid plasmid isolation method. Figure 6.1 shows an example of Queensland non-01 *Vibrio* isolates of which nine out of nineteen strains harbour low molecular weight plasmids. Figure 6.2 shows a sample of clinical isolates which harbour plasmids, one of which, AA14041, a Classical Inaba isolate from Bangladesh, was demonstrated to harbour the P factor (section 6.3). Table 6.1 summarizes the incidence of plasmids in the strains examined. No plasmids were identified in any of the *V. cholerae*-01 laboratory strains representing older isolates, whereas all the recent clinical isolates of *V. cholerae*-01 from Bangladesh and Calcutta contained at least one plasmid. The *V. fluvialis* strain examined harboured a plasmid of low molecular weight and one of the five unidentified *Vibrio* species harboured a plasmid.

6.3 Probing *Vibrio* strains for the presence of the P factor, lcp and scp by colony hybridization.

Examination of the 76 *Vibrio* strains for the presence of the P factor, large cryptic plasmid (lcp) and the small cryptic plasmid (scp) was carried out by colony hybridization using [^{32}P]-dCTP labelled probes specific for each of the three plasmids. Specific probes for each plasmid were: pPM802 (*Eco*RI-2A of P) for the P factor; pPM831 (*Eco*RI-5 of lcp) for the lcp and pPM851 (*Sph*I-2 of scp) for the scp (see sections 4.4, 5.9, 5.10 respectively).

Strain V58 hybridizes with all three plasmid probes. None of the non-01 isolates nor *V. mimicus* or *V. fluvialis* possess either P factor,

Figure 6.1.

Plasmid profiles of *V.cholerae* non-01 isolates from Queensland. Plasmid DNA was extracted by a rapid SDS lysis method (Meyers *et al.*, 1976) and subjected to electrophoresis through a 0.6% agarose gel. Controls for high and low molecular weight plasmids were R144 (100 kb) and pBR322 (4.3 kb), respectively.

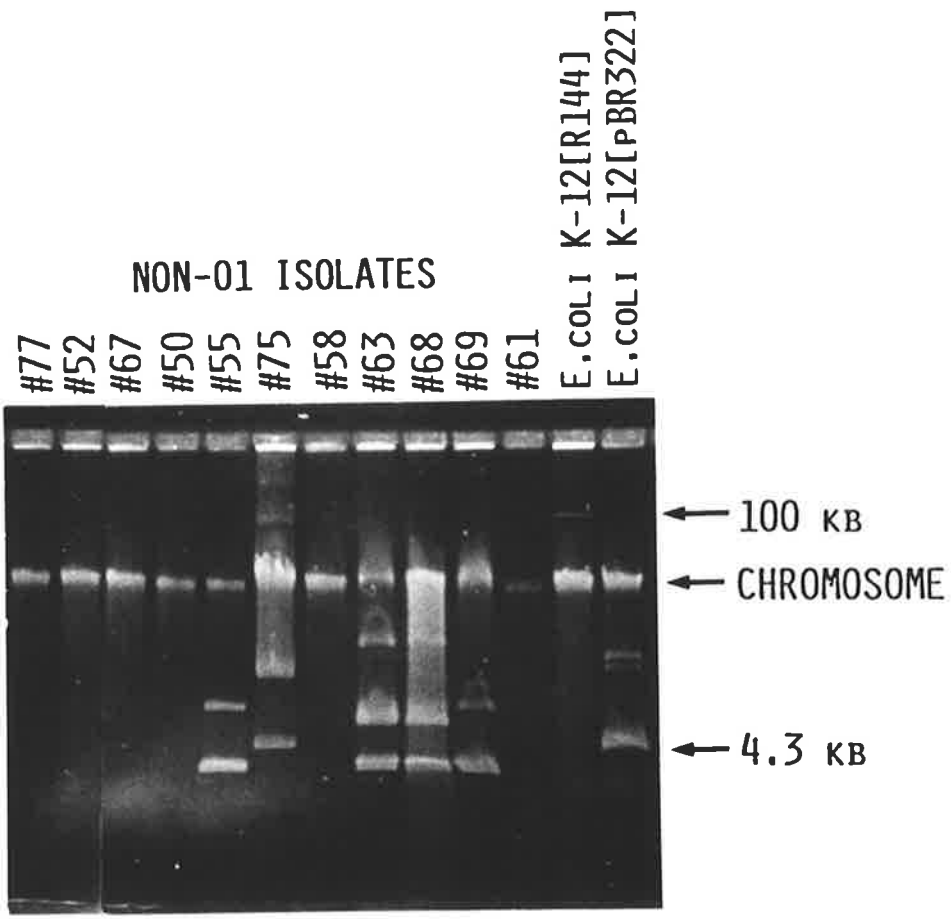

NON-01 ISOLATES
#77
#52
#67
#50
#55
#75
#58
#63
#68
#69
#61
E.coli K-12[R144]
E.coli K-12[pBR322]
100 kb
CHROMOSOME
4.3 kb

Figure 6.2.

Plasmid profiles of recent clinical isolates of *V. cholerae* 01 from Bangladesh and Calcutta, the non-01 strains (#68, 69 and 61) and 017 P^+ and 017 showing the presence of the P factor in isolate AA14041. Plasmid DNA was extracted by a rapid SDS lysis method (Meyers *et al.*, 1976) and subjected to electrophoresis through a 0.6% agarose gel. Plasmids R64 (100 kb) was used as a size standard.

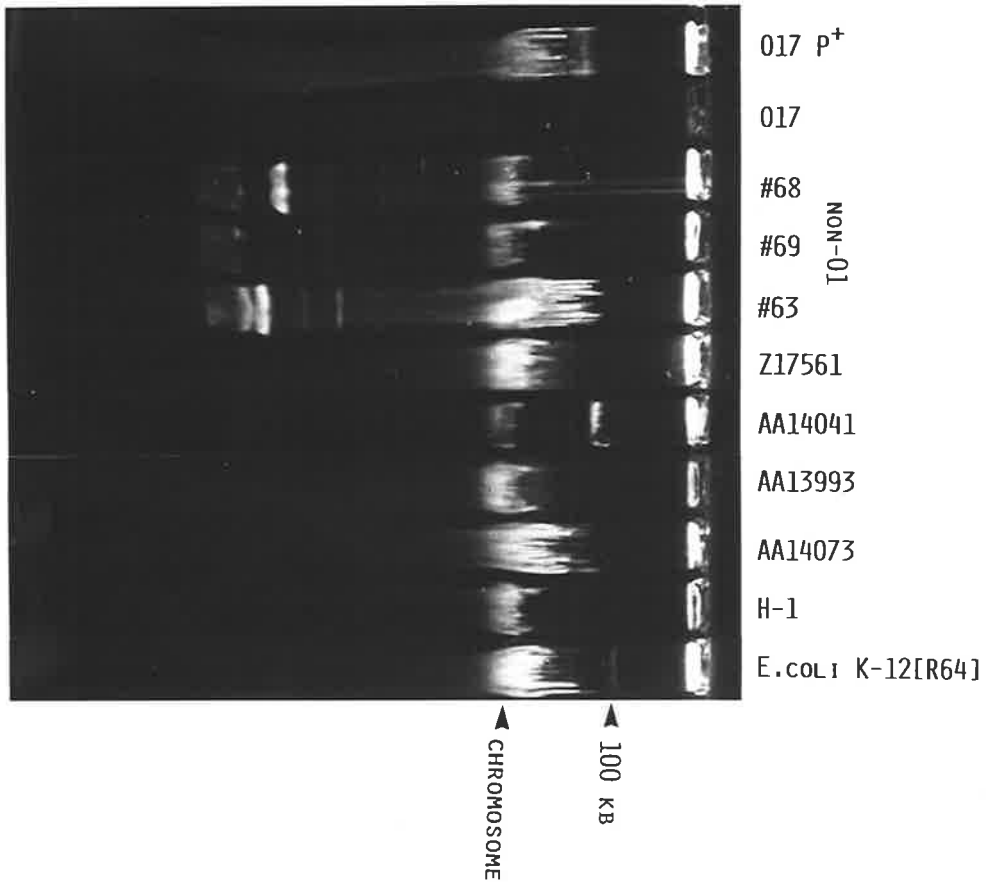

O17 P+
O17
#68
#69
#63
Z17561
AA14041
AA13993
AA14073
H-1
E.coLi K-12[R64]
NON-O1
CHROMOSOME
100 KB

Table 6.1

PLASMIDS DETECTED IN CLINICAL *VIBRIO* ISOLATES

Vibrio species[a]	Number of strains screened	Number of strains with detectable plasmids[b]	Colony hybridization to[c] P	lcp	scp
V.mimicus	1	0			
V.fluvialis	1	1			
V.parahaemolyticus	1	0			
V.cholerae 01					
Classical	14	0			
El Tor	9	0			
not biotyped	11	0			
Recent *V.cholerae* 01 clinical isolates					
Bangladesh Cl In	1	1			+
" Cl Og	1	1	+		+
" ET In	1	1			+
" ET Og	1	1			+
Calcutta ET Og	1	1			+
non-cholerae *Vibrio*	10	2			
Vibrio species-unidentified	5	1			
Queensland non-01 *Vibrio*	19	9			

Table 6.1 (continued)

a = Unidentified *Vibrio* species were obtained from M.J. Voll

Queensland isolates were from S.C. Pal

Cl Classical biotype

ET El Tor biotype

In Inaba serotype

Og Ogawa serotype

b = All strains were screened using a rapid plasmid DNA isolation method adapted from Meyers *et al.* (1976) and scaled down to 3 ml.

c = P = P factor: the probe was whole pPM802 containing the 11.5 kb (*Eco*RI-2A) fragment of P in pACYC184.

lcp = large cryptic plasmid: the probe was whole pPM831 containing the 3.4 kb (*Eco*RI-5) fragment of lcp in pACYC184.

scp = small cryptic plasmid: the probe was whole pPM851 containing a 1.8 kb (*Sph*I-2) fragment of scp in pBR322.

+ = hybridizes with probe.

Figure 6.3.

Colony hybridizations of various *Vibrio* strains with probe specific for P, or lcp or scp. V58 P^- contains the large and small cryptic plasmids and V58 contains all three plasmids. The control strains containing the three plasmids (P, lcp and scp) are in the second row (left side) and the fifth row (right side). A diagrammatic representation of the colony blot is shown at the top of the figure. *Vibrio* strains included are:

Vm = *V. mimicus*

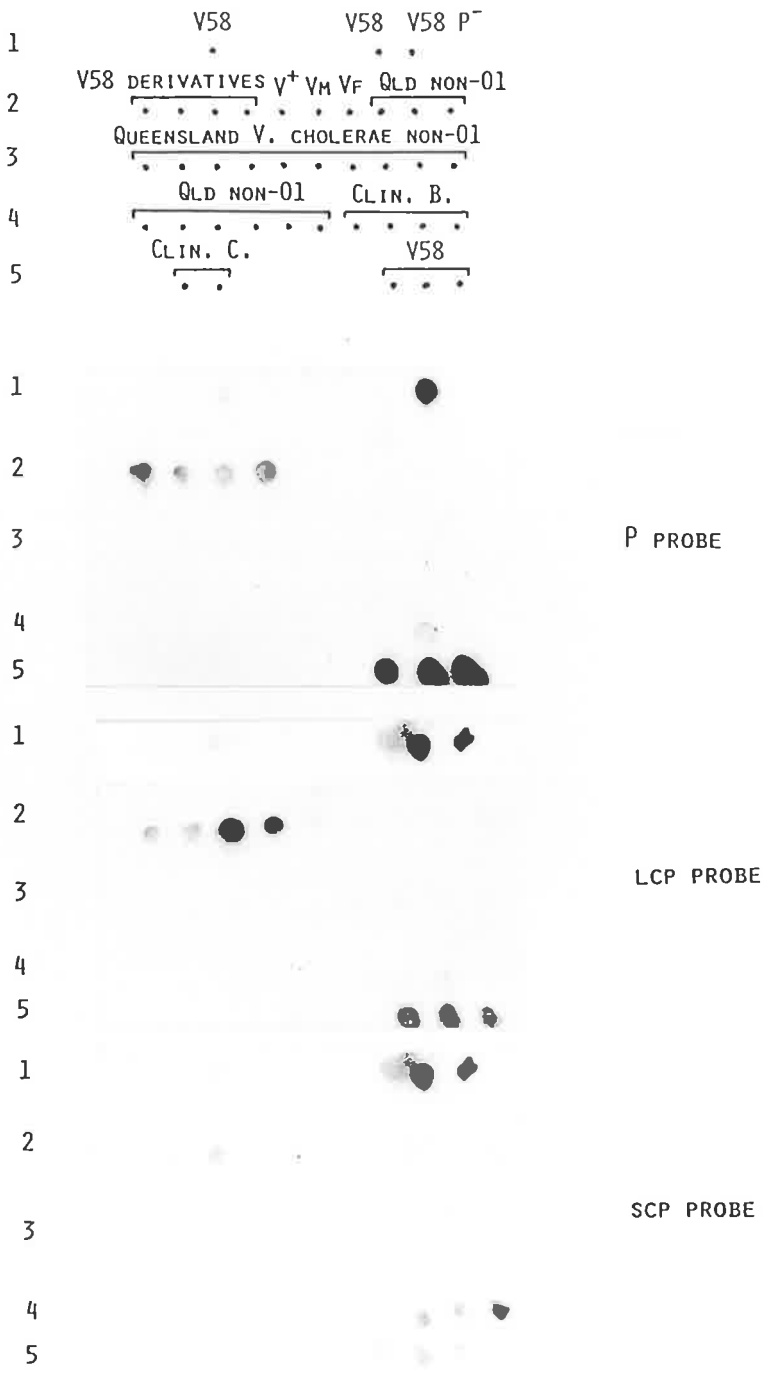
Vf = *V. fluvialis*

Cl V58 = Classical strain V58

Clin. B. = Clinical isolates from Bangladesh

Clin. C. = Clinical isolates from Calcutta

Control strains are V507, V508, V509 and V510


1
2
3
4
5
V58
V58 V58 P⁻
V58 DERIVATIVES V⁺ VM VF QLD NON-01
QUEENSLAND V. CHOLERAE NON-01
QLD NON-01
CLIN. B.
CLIN. C.
V58
P PROBE
LCP PROBE
SCP PROBE

lcp or scp, but the five clinical isolates from Bangladesh and Calcutta have the small cryptic plasmid and one of the strains (AA14041) has the P factor (Figure 6.3).

6.4 Southern hybridization to detect the presence of the P factor and the scp in clinical isolates.

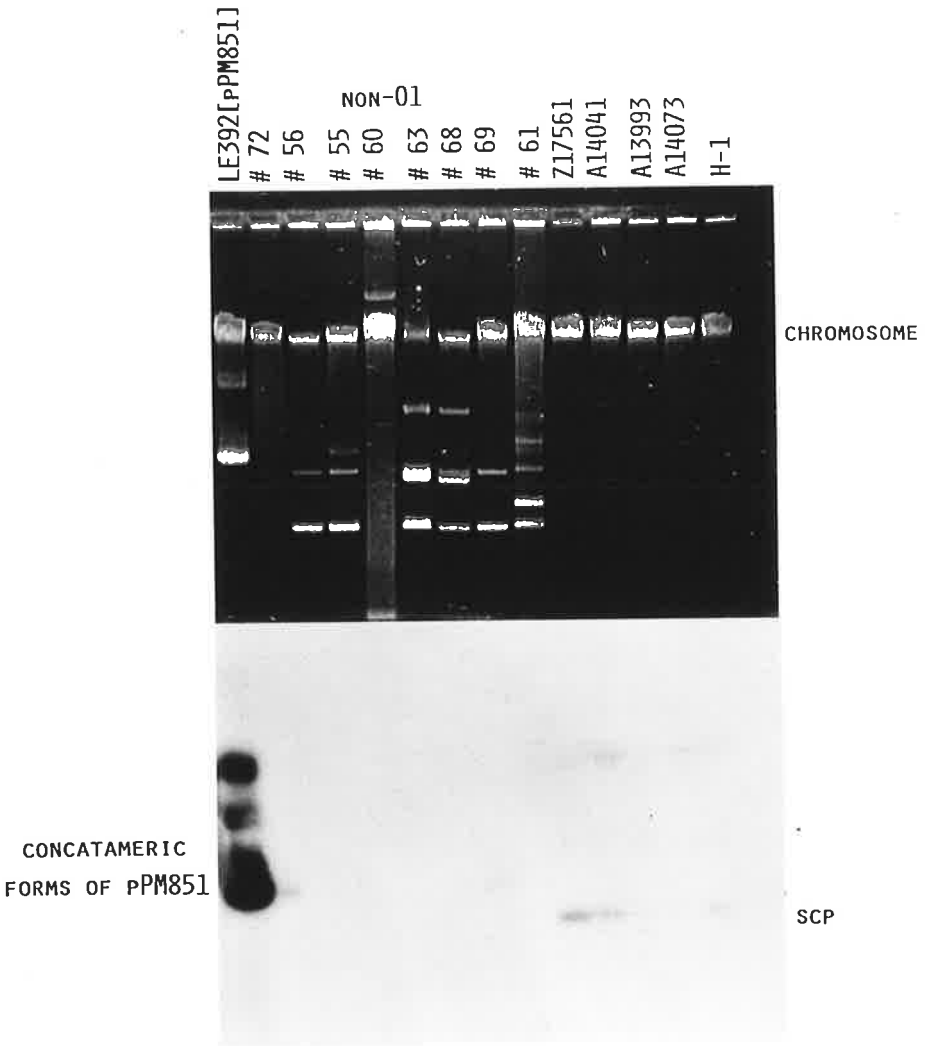
The presence of the P factor and the scp in the *V. cholerae*-01 clinical isolates from Calcutta and Bangladesh has been confirmed by Southern hybridization using the same probes as were used above (P factor: pPM802 and scp: pPM851) (section 6.3). The small cryptic plasmid is often difficult to visualize on agarose gels (probably due to its low copy number) by comparison with the plasmids found in the non-01 isolates, however, it can be detected by probing (Figure 6.4). The Queensland isolates of *V. cholerae* non-01 strains harbouring plasmids were included in the Southern hybridization and probing with pPM851 confirmed that none of these plasmids had homology to the scp (Figure 6.4, lanes 2 - 9). The recent clinical isolate AA14041 from Bangladesh possesses the P factor as demonstrated by probing with pPM802 and restriction analysis of isolated plasmid. The plasmid can also be functionally detected by the lacunae assay.

6.5 Homology between the V factor and the large cryptic plasmid.

The V factor is a transmissible factor which was identified in a non-cholera *Vibrio* by Bhaskaran and Sinha (1971). This V factor is compatible with the P factor but exhibits a lower rate of conjugative transfer and fails to mobilize chromosomal markers under the usual mating conditions. Like P, the presence of the V factor can be

Figure 6.4.

Southern hybridization of *V. cholerae* non-01 isolates from Queensland and clinical isolates from Bangladesh and Calcutta, using a probe specific for the small cryptic plasmid. DNA was separated on an 0.8% agarose gel, transferred to nitrocellulose and probed with [^{32}P]dCTP labelled pPM851 (scp). The autoradiograph shows the presence of the small cryptic plasmid (arrowed) which is not easily visualized in the ethidium bromide stained agarose gel (top panel). Plasmid pPM851 serves as a control for the probing and various concatameric forms are present.


LE392[pPM851]
72
56
55
NON-01
60
63
68
69
61
Z17561
A14041
A13993
A14073
H-1
CHROMOSOME
CONCATAMERIC
FORMS OF pPM851
SCP

demonstrated by the lacunae assay, (Figure 6.5A). The distinctive plaque-like clearings around the V^+ colonies are easily visible on the background of a lawn of V^- bacteria. The lacunae produced by the V factor are larger than those seen with the P factor (Figure 6.5B).

Plasmid DNA extracted from the strain KB9 V^+ and from strain V58 P^- which contains the lcp was compared by digestion with restriction endonucleases *Hin*dIII, *Pst*I and *Eco*RI (Figure 6.6).

These two plasmids have identical restriction endonuclease patterns, strongly inferring that the lcp from the strain V58 P^- is the same as the V factor from strain KB9 V^+. Plasmid pPM831 which contains a 3.4 kb *Eco*RI (*Eco*RI-5) fragment of the lcp was labelled with [^{32}P]-dCTP by nick-translation and used to probe plasmid DNA isolated from various lcp^-, lcp^+, V^- and V^+ *V. cholerae* strains. Figure 6.7 shows the agarose gel of the *V. cholerae* plasmids and the corresponding autoradiograph. The probe, pPM831, hybridizes to both the lcp and the V factor, confirming that these two plasmids have homology.

Plasmid DNA extracted from both KB9 V^+ and V58 P^-, and digested with restriction endonuclease *Eco*RI, was probed with the six subcloned *Eco*RI fragments of lcp, pPM829-pPM834 (section 5.9). Utilizing the same six probes it has been demonstrated that there is no homology between P and lcp (inferring that the P and V factors do not have homologous DNA sequences).

Cleavage of the large cryptic plasmid and the V factor by the restriction endonucleases *Eco*RI, *Hin*dIII and *Pst*I has demonstrated identical restriction patterns for the two plasmids. Southern hybridization has confirmed these two plasmids to be indistinguishable.

The 31.5kb (21 Mdal) plasmid identified by Cook *et al.* (1984) appears to be the same as the V factor (lcp). Restriction fragments

Figure 6.5.

Plates showing the lacunae assay using, as donors, strains KB9 V^+ and V58 harbouring the V factor (A) and the P factor (B), respectively and, with CA411 as the recipient strain. The recipient forms a confluent lawn of bacteria with clearings or lacunae around the donor colonies (Lacunae are thought to represent areas of killing probably due to lethal zygosis (Skurray and Reeves, 1974).

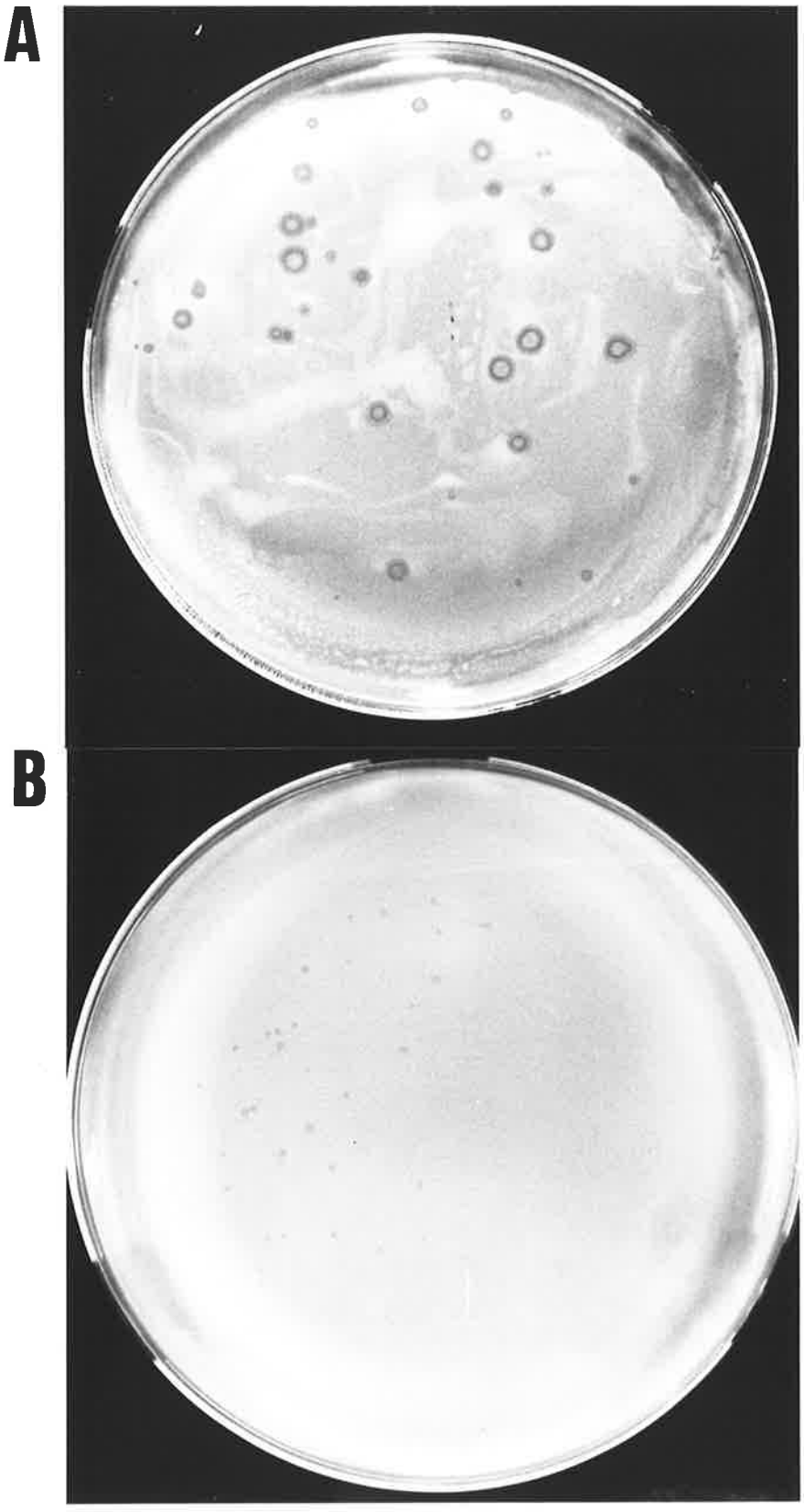

A
B

Figure 6.6.

DNA digested with restriction endonucleases *Hind*III, *Pst*I and *Eco*RI was used to compare lcp (from V58 P^-) and V factor (from KB9 V^+). The fragments were separated by electrophoresis on a 0.7% agarose gel. The size standard was SPP1 DNA digested with *Eco*RI and the mobility of the different fragments and their sizes (in kb) are indicated on the left (Radcliff *et al.*, 1979; Franzon and Manning, 1986).

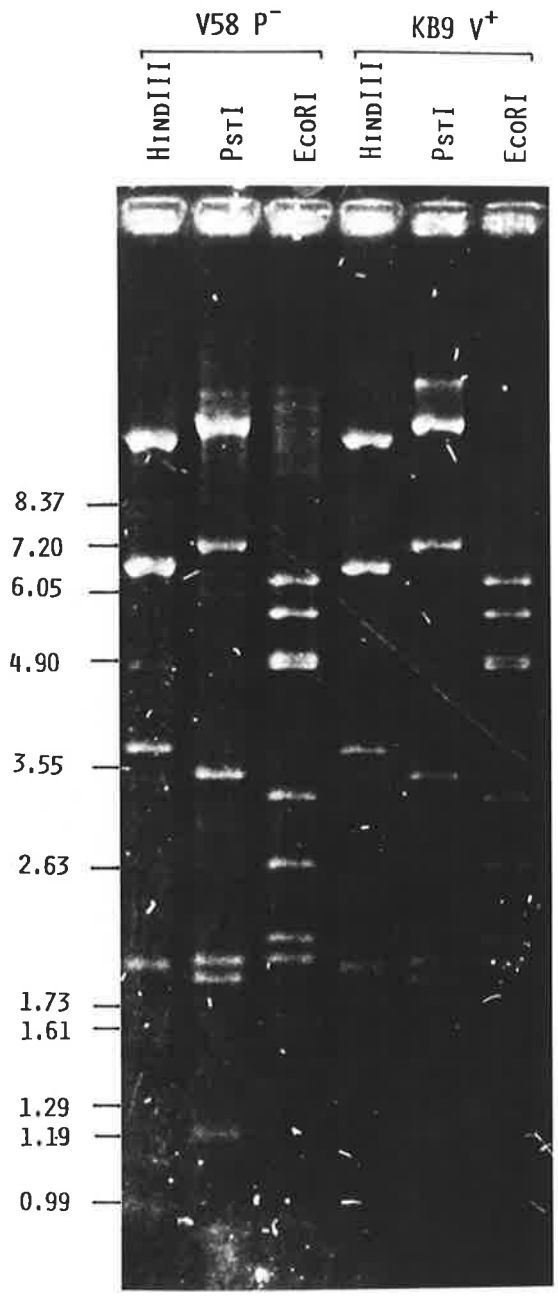

V58 P⁻
KB9 V⁺
HindIII
PstI
EcoRI
HindIII
PstI
EcoRI
8.37
7.20
6.05
4.90
3.55
2.63
1.73
1.61
1.29
1.19
0.99

Figure 6.7.

Southern hybridization analysis of plasmids isolated from various lcp$^+$, P$^+$ and V$^+$ strains. [^{32}P]-labelled plasmid pPM831 was used as a probe for the lcp and pPM802 for the P factor. The centre panel depicts the 0.7% agarose gel where plasmid extracts have been separated by electrophoresis, and the autoradiographs on the left and right represent the results obtained probing for lcp and P factor, respectively.

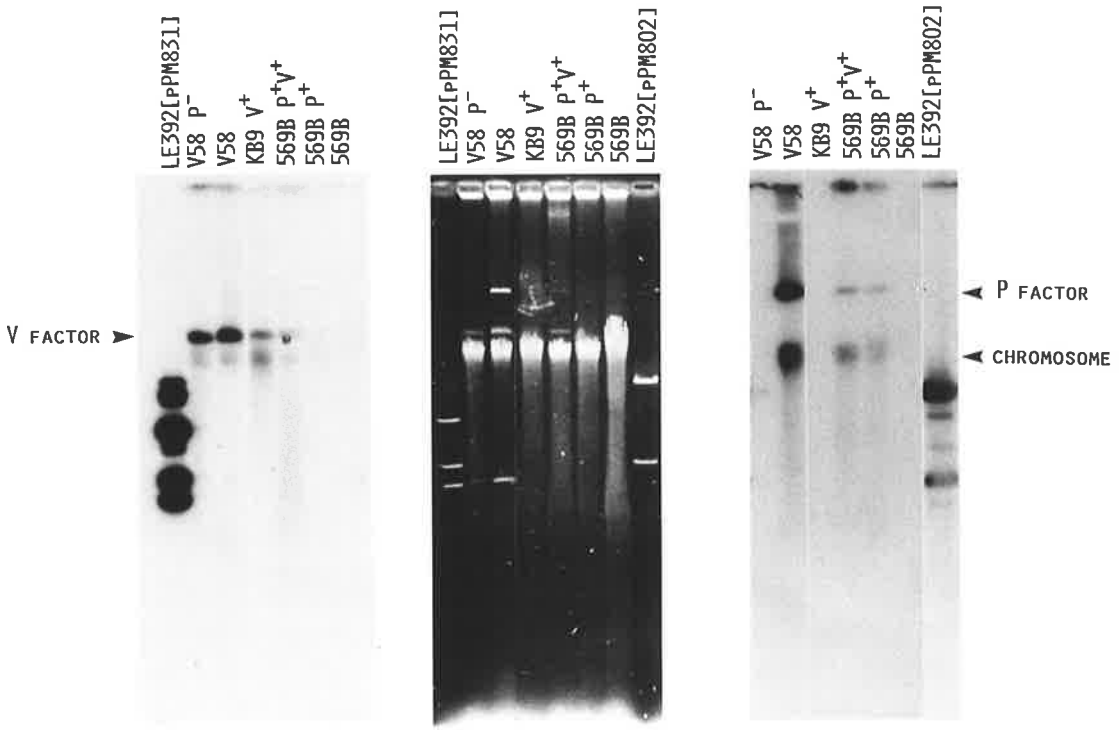
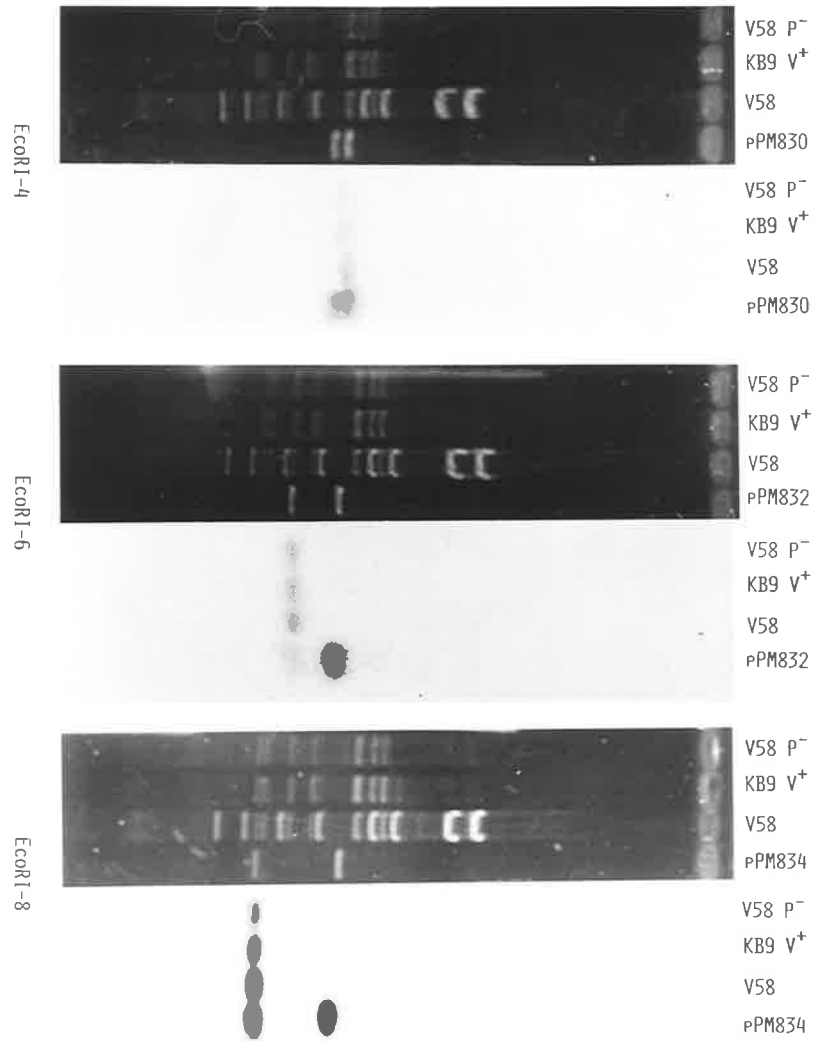

LE392[pPM831]
V58 P⁻
V58
KB9 V⁺
569B P⁺V⁺
569B P⁺
569B
V FACTOR
LE392[pPM831]
V58 P⁻
V58
KB9 V⁺
569B P⁺V⁺
569B P⁺
569B
LE392[pPM802]
V58 P⁻
V58
KB9 V⁺
569B P⁺V⁺
569B P⁺
569B
LE392[pPM802]
P FACTOR
CHROMOSOME
LCP PROBE
P FACTOR PROBE

Figure 6.8.

0.6% agarose gel with the corresponding autoradiograph of *Eco*RI digested isolated plasmid DNA of V factor (KB9 V^+), lcp (V58 P^-) and P factor (V58) probed with the *Eco*RI-4 (pPM830), *Eco*RI-6 (pPM832) and *Eco*RI-8 (pPM834) fragments of the lcp.


EcoRI-4
V58 P⁻
KB9 V⁺
V58
pPM830
V58 P⁻
KB9 V⁺
V58
pPM830
EcoRI-6
V58 P⁻
KB9 V⁺
V58
pPM832
V58 P⁻
KB9 V⁺
V58
pPM832
EcoRI-8
V58 P⁻
KB9 V⁺
V58
pPM834
V58 P⁻
KB9 V⁺
V58
pPM834

generated on cleavage by *Hin*dIII of the V factor (and hence lcp) are of the same size as seen by Cook *et al.* (1984) (Table 6.2 and Figure 6.8).

The agarose gel from Cook *et al.* (1984) shows a *Hin*dIII digestion of plasmid from strains RV31 and X22501 which was shown to harbour two plasmids of sizes 3 and 21 Mdal. The scp has a single *Hin*dIII site (Chapter 5) resulting in a band at 4.7 kb which can be seen in RV31 and X22501 (tracks 2 and 3, Figure 6.9) as well as in V58 P$^-$ plasmid *Hin*dIII digestions (Figure 6.9). From these observations it can be inferred that the 21 Mdal plasmid commonly detected in Classical strains from the sixth cholera pandemic is identical to the lcp of strain V58 and the V factor. The scp is probably identical to the 3 Mdal plasmid.

6.6 Compatibility of the V and P factors.

In the presence of R100, the F factor of *E. coli* K-12 is repressed for transfer which occurs at a 100-1000 fold lower efficiency than in the absence of R100 (Finnegan and Willetts, 1971).

Transfer proficient transposon derivatives of the P and V factors (P::Tn*3*-1 (Chapter 4) and V::Tn*1732*-1 (Chapter 5)) were used to examine compatibility and fertility inhibition between the two plasmids.

P::Tn*3*-1 and V::Tn*1732*-1 were introduced into the same strain (Classical V628) and the stability of both plasmids examined over 200 generations without antibiotic selection. Both plasmids were retained at 100% efficiency.

To investigate whether either of the two plasmids represses the transfer of the other when both are present in the same host strain, the frequency of transfer of P::Tn*3*-1 and V::Tn*1732*-1 into a plasmid free strain was examined. The frequency of transfer of P::Tn*3*-1 in the presence or absence of the V factor was identical. The frequency

Figure 6.9.

The V (lcp) plasmid has been compared with the plasmids described by Cook *et al.* (1984) using *Hin*dIII digestions. The panel shows a section of a gel of *Hin*dIII digests reproduced from published data (Cook *et al.*, 1984). Lane 2: strain RV31 (harbouring the 3 and 21 Mdal plasmids), lane 3: strain X22501 (harbouring the 3 and 21 M dal plasmids, lane 4: lambda phage DNA cleaved with *Hin*dIII as a size standard. The left panel shows V factor DNA from KB9 V^+ digested with *Hin*dIII and separated by electrophoresis through a 0.7% agarose gel, with *Hin*dIII-digested lambda phage DNA as a size standard. The *Hin*dIII fragments of V (lcp) are labelled 1-6, and the corresponding fragments of the plasmids of Cook *et al.* (1984) are indicated.

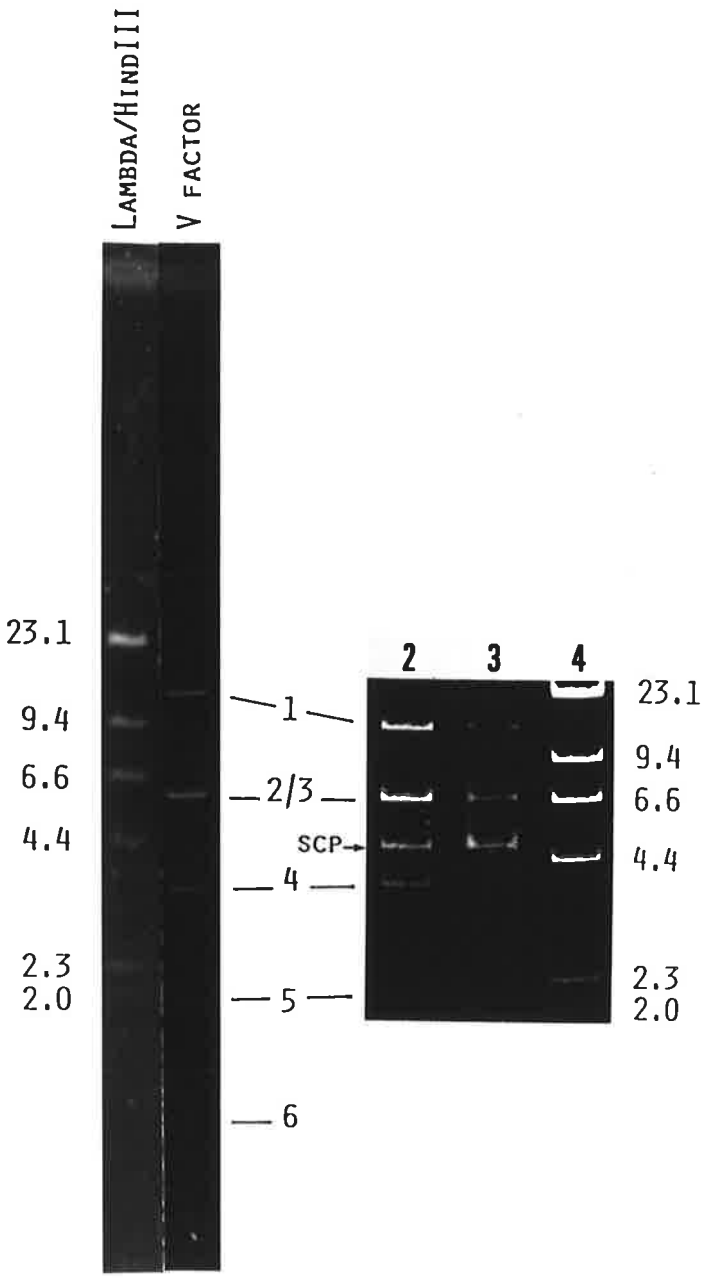

LAMBDA/HINDIII
V FACTOR
23.1
9.4
6.6
4.4
2.3
2.0
1
2/3
SCP
4
5
6
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0

Table 6.2

*Hin*dIII FRAGMENTS FROM V FACTOR AND THE PLASMIDS OF COOK *et al* (1984)

*Hin*dII fragment	Size (kb)[a]	from 21 MDal[b]
1	15.0	15.0
2	6.4	6.5
3	6.4	6.5
4	3.6	3.7
5	2.0	2.1
6	0.9	c

a = Sizes for the *Hin*dIII fragments have been determined from the sizes of fragments generated by digestion with *Hin*dIII and *Eco*RI. The source of DNA was KB9 V^+.

b = Sizes have been calculated from an agarose gel published by Cook *et al.* (1984), which has been reproduced in Figure 6.9.

c = A fragment of this size would not be detected on the published agarose gel; fragments smaller than 2.0 kb have eluted.

Table 6.3

TRANSFER OF P OR V FROM THE SAME HOST CELL

Plasmid	Host strain	Transfer frequency*
P::Tn*3*-1	V697	$1.5 \pm 0.3 \times 10^{-2}$
P::Tn*3*-1	V697[V::Tn*1732*-1]	$1.7 \pm 0.2 \times 10^{-2}$
V::Tn*1732*-1	V697	$1.68 \pm 0.1 \times 10^{-3}$
V::Tn*1732*-1	V697[P::Tn*3*-1]	$2.39 \pm 0.5 \times 10^{-3}$

* Transfer frequency is the ratio of the number of transconjugants to the number of donors.

of transfer of V::Tn*1732*-1 in the presence or absence of the P factor was also identical (Table 6.3). This indicates that neither plasmid is exerting a fertility inhibition effect.

6.7 Conclusions.

The presence of the P factor, lcp and scp in a collection of *Vibrio* strains from laboratory, environmental and clinical sources was examined by colony hybridization using subcloned fragments from each of the plasmids. Seventy-six *Vibrio* strains were examined. Some 47% (9/19) of *V. cholerae* non-01 Queensland strains harboured low molecular weight plasmids whereas none of the *V. cholerae*-01 laboratory strains harboured plasmids. The P factor was detected in one recent clinical isolate (AA14041) of *V. cholerae*-01 from Calcutta. The small cryptic plasmid was identified in the five Clinical isolates of *V. cholerae*-01 from Calcutta and Bangladesh. The large cryptic plasmid was not detected in any of the *Vibrio* strains examined, with the exception of the strains harbouring the V factor.

The V factor has been demonstrated to be a plasmid. Using Southern hybridization and restriction endonuclease analysis it has been possible to demonstrate that the lcp is identical to the V factor and most likely also to the 31.5kb (21 Mdal) plasmid described by Cook and coworkers (1984). The lcp (and V factor) do not contain any DNA homologous with the P factor (Figure 6.8). The scp appears to be identical to the 4.5kb (3 Mdal) plasmid consistently present in strains isolated by Cook *et al.* (1984).

The P and V factors are compatible and stably maintained when present together in the same host strain. Neither plasmid exhibits a fertility inhibition upon the other.

Chapter 7

The role of the P and V factors in the virulence of *V. cholerae*

7.1 Introduction

Sinha and Srivastava (1978) and Khan *et al.* (1985) observed a 10-fold decrease in cholera toxin (CT) production, by Classical Inaba strain KB365 (a Sm^R derivative of 569B) in the presence of the P and V factors, and proposed that these plasmids encoded regulatory functions which affected the biosynthesis of CT. In contrast, Hamood *et al.* (1986) reported that the presence of the P factor had no effect upon fluid accumulation induced by *V. cholerae* in infant mouse intestines, nor did it diminish *in vitro* toxin production (as measured by cytopathy of mouse Y1 adrenal cells). A slight decrease (2 fold) in colonization ability was observed in the presence of this factor. It was concluded that if the P factor was attenuating, its influence lay in alterations to virulence attributes other than toxin production. Khan *et al.* (1985) also proposed that plasmid-borne genes were involved in the regulation of toxin biosynthesis, observing that reacquisition of the P factor by a cured strain resulted in the suppression of toxin production.

This chapter investigates the capacity of the P and V factors to affect the major virulence determinants; motility, colonization and toxin production in *V. cholerae* 569B.

7.2 Methods employed.

Several approaches were adopted to investigate this phenomenon. At a cellular level, fluid accumulation in ileal loops of adult rabbits following administration of *V. cholerae*, and pathogenicity in the infant mouse model, were assessed. At a molecular level, the effect of the P and V factors on the cholera toxin promoter was examined.

7.2.1 Strain construction.

The Classical Inaba strain, 569B was employed as the parent strain, and P^+, V^+, P^+V^+ derivatives were constructed. The presence of the plasmids was confirmed by the rapid small scale SDS lysis method (2.7.1). As motility is important for virulence (Attridge and Rowley, 1983a), all strains were checked for motility by swarming in soft agar as well as for their chemotactic responses to glucose and methionine by a capillary test (Freter *et al.*, 1981a). All strains were verified as equally motile by these two tests.

7.3 Fluid accumulation in adult rabbit ileal loops.

The fluid accumulation in ligated ileal loops of adult rabbits following administration of 569B, 569B P^+, 569B V^+ and 569B P^+V^+ was investigated. Samples of each of the strains were introduced into ligated ileal loops in four rabbits. The fluid accumulation ratio (FA) was calculated as a mean of the four rabbits (Table 7.1). The P^+V^+ loop showed a slightly lower FA (Figure 7.1), however, this was not significantly different from the other loops as seen in the standard deviation (Table 7.1) and calculation of the P value using the student's t test (Roscoe, 1975) which was greater than 0.1, inferring that there was no significant difference between the values obtained..

Figure 7.1.

Fluid accumulation was examined in ligated ileal loops of adult rabbits, essentially as described by Sinha and Srivastava (1978). Classical strain 569B (1-2 x 10^5 bacteria/ml) in the presence and absence of the P and V factors was injected into the closed ileal loops. Autopsies were performed after 18 hours and the accumulation of fluid was observed with a virulent strain and scored as positive and loops without fluid as negative. Shown is one series of loops where the P^+V^+ ileal loop illustrates slightly less fluid accumulation.

BHI = Brain Heart Infusion broth

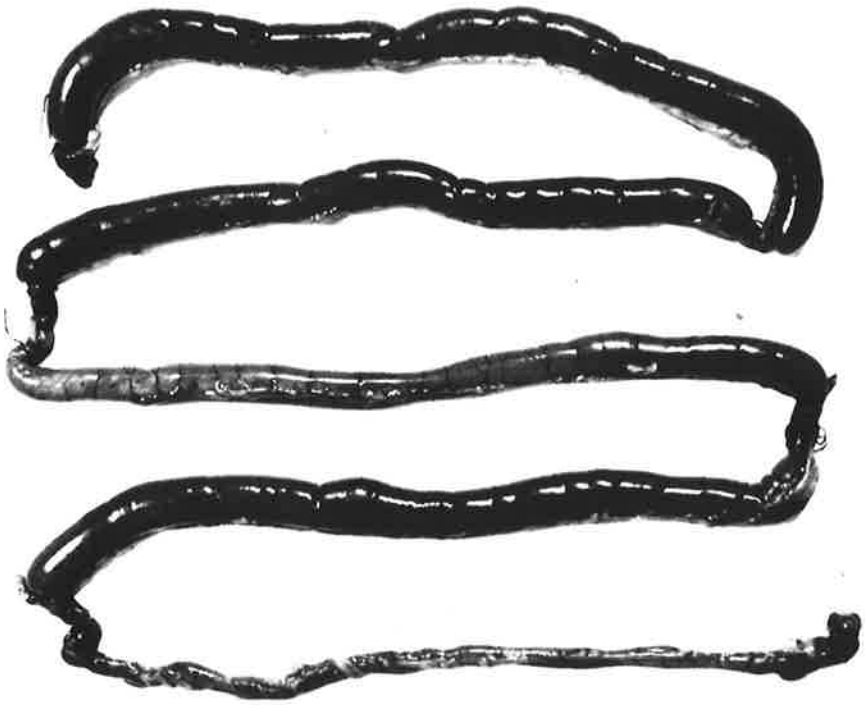

569B P+
569B V+
569B P+V+
569B
BHI

Table 7.1

FLUID ACCUMULATION IN RABBIT ILEAL LOOPS

Strain/plasmid	Fluid accumulation ratio ml/cm	SD
BHI	0	± 0
569B	1.56	± 0.33
569B P^+V^+	1.49	± 0.40
569B P^+	1.65	± 0.30
569B V^+	1.57	± 0.33

SD = standard deviation (mean calculated from four rabbits)

7.4 Cholera toxin.

Preliminary evidence failed to reveal any significant difference in fluid accumulation in ligated ileal loops of adult rabbits, examination of the effect of the P and V factors on CT was carried out.

The GM_1 ganglioside ELISA assay (Holmgren, 1973) was used to determine whether there was a difference in the amount of CT produced by the Classical *V. cholerae* 569B strain series (Table 7.2). The amount of CT produced in the presence or absence of the P and V factors was comparable to that of the parent strain 569B (Table 7.2) (P value greater than 0.1). The strains were cultured as suggested by Taylor *et al.* (1987) in TB medium containing 66mM NaCl at 30°C with an initial of pH6.5. These cultural conditions lead to optimal production of CT. The amount of CT produced under these conditions are as those observed by Miller *et al.* (1987), Mishra and Holmes (1987) and Taylor *et al.* (1987). No significant differences were observed (Table 7.2).

7.5 Cholera toxin promoter.

A study of the strength of the CT promoter in strains with or without the P and V factors has been undertaken.

Cholera toxin is one of many secreted proteins in *V. cholerae* and is an essential virulence factor of this organism. The genes for the production of CT, the *ctxAB* operon, have been cloned into the plasmid pBR322 on a 5.1 kb *Pst*I fragment (Mekalanos *et al.*, 1983), resulting in pJM17. This cloned fragment includes the promoter and regulatory region for these genes.

7.5.1 Constructions.

In order to analyse the possible effects of the P and V factors on the *ctx* promoter, the *ctxAB* operon including the promoter was

Table 7.2

CONCENTRATION OF CHOLERA TOXIN PRODUCED BY 569B AND PLASMID DERIVATIVES

Strain/plasmid	CT (μg/ml)[a]
569B	1.2 ± 0.13
569B P^+V^+	1.4 ± 0.10
569B P^+	1.5 ± 0.12
569B V^+	1.5 ± 0.12

a concentration of CT produced is determined by the GM_1 ganglioside ELISA assay (Holmgren, 1973)

The sensitivity of the assay was 0.02 μg of CT/ml

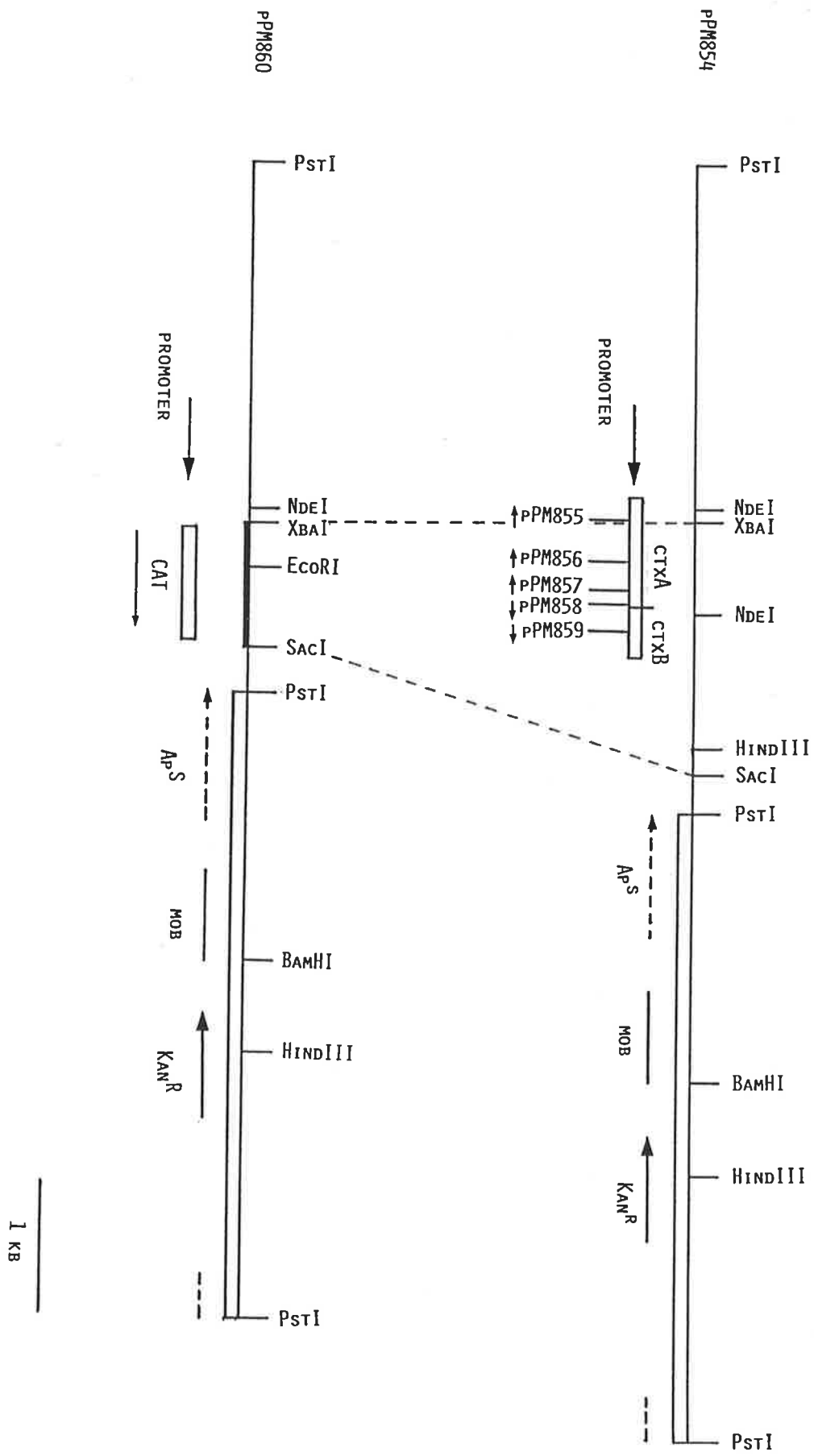
subcloned into the mobilizable vector pSUP301 (Simon *et al.*, 1983) resulting in the recombinant plasmid pPM854. A segment of the CT genes was deleted and a promoterless CAT cartridge inserted between the *Xba*I and *Sac*I sites after the *ctx* promoter (pPM860) such that expression of the CAT was dependent on the *ctx* promoter (Figure 7.2)

The transposable promoter probe Tn*1736Tc* (Ubben and Schmitt, 1987) was used to obtain insertions in the region near the *ctx* promoter. Five promoter fusions located within the *ctxAB* operon were isolated and are shown (Figure 7.2) (pPM855-pPM559). Restriction analysis was used to orientate the Tn*1736Tc* insertions. Insertions pPM858 and pPM859, which are approximately 570 and 880 bp downstream from the *ctx* promoter, are located in *ctxA* and *ctxB*, respectively. They are orientated such that CAT expression is from left to right, hence under the control of the *ctx* promoter. pPM855, pPM856 and pPM857 are oriented such that expression occurs from right to left, most likely from the Bla promoter in the vector which would be reading in this direction. Since the construction of the plasmid pPM854 resulted in inactivation of the Bla gene, there would not necessarily be a terminator for this promoter.

Insertion of Tn*1736Tc* into the *ctxAB* sequence may possibly result in the artificial production of a promoter. This construct would produce CAT from the newly constructed promoter and not from the *ctxAB* promoter. Computer analysis has allowed the examination of the sequences flanking the CAT gene from Tn*1736Tc* insertions in *ctxAB* for possible -35 and -10 regions. A potential -10 region, 10bp in from the end of the Tn*1736Tc* transposon (CAGAAT compared with TATAAT, the consensus sequence) has been identified. If the Tn*1736Tc* inserts into a site within 5-7bp of a potential -35 region within the *ctxAB* gene, an artificial promoter may be constructed. This analysis revealed

Figure 7.2.

Construction of plasmids which contain the chloramphenicol transacetylase (CAT) gene under the control of the *ctx* promoter. pPM854 is a recombinant plasmid with the 5.1 kb *PstI–Eco*RI fragment encoding the *ctxΛB* operon including the promoter subcloned into the mobilizable vector pSUP401 (Simon *et al.*, 1983). The transposable promoter probe Tn*1736Tc* (Ubben and Schmitt, 1987) was used to obtain insertions in the region near the *ctx* promoter (pPM855–pPM859). The orientation of the CAT cartridge in the transposon Tn*1736Tc* is denoted by an arrow. pPM860 was constructed by deleting the *Xba*I – *Sac*I segment of pPM854 (thick segment) and replacing it with the promoterless CAT cartridge, following the *ctx* promoter.


pPM860
pPM854
PstI
PstI
PROMOTER
PROMOTER
NdeI
XbaI
EcoRI
SacI
pPM855
pPM856
pPM857
pPM858
pPM859
CTxA
CTxB
NdeI
XbaI
NdeI
CAT
HindIII
SacI
PstI
PstI
ApS
ApS
MOB
MOB
BamHI
BamHI
KanR
KanR
HindIII
HindIII
1 KB
PstI
PstI

three potential -35 regions. Two -35 regions, 930 bp and 950 bp from the *Nde*I site in *ctxAB*, which precedes the pPM859 insertion by 80 - 100 bp indicating potential promoter sites in this region. If the mapping of pPM859 is out by approximately 50 - 100 bp this may present problems in that the CAT gene is under the control of the artificially constructed promoter and not the *ctx* promoter (see below). The third -35 region (565 bp from the *Nde*I site), by the pPM858 insertion may result in a promoter as seen from examination of the sequence, however, if this were to be the case, a relatively high level of CAT in *E. coli* K-12 should be produced. This is, however, not the case, since the amount of CAT produced in *E. coli* K-12 S17-1[pPM858] is 20 times lower than for S17-1[pSUP401] (CAT^+) (Table 7.3). This is also observed in the MIC for these strains (Table 7.4) inferring that this potential promoter in pPM858 is unlikely to be functional. Similar computer analysis confirms that potential promoters would not be generated in the construction of pPM860.

7.5.2. Effects on the *ctxAB* promoter in the presence or absence of the P and V factors.

The final constructs, plasmids pPM854-pPM860, were mobilized into Classical *V. cholerae* strains 569B and CA401 in the presence and the absence of the P and V factors. *V. cholerae* strain 569B has a defective *toxS* (Taylor *et al.*, 1988b) but was included since all animal experiments utilized this strain. The amount of CAT as a result of each of the plasmids was assayed spectrophotometrically (Shaw, 1975) in *E. coli* K-12 (strain S17-1) as well as in *V. cholerae* (569B and CA401) (Table 7.3).

Plasmid pSUP401 is a mobilizable vector as is pSUP301 and encodes Cm^R. It was included as a control for the CAT assay and produces more CAT in *E. coli* K-12 than in *V. cholerae*. The amount of CAT

Table 7.3

DETERMINATION OF AMOUNT OF CAT[a] PRODUCED IN *E. coli* K-12 AND *V. cholerae* (± P AND V FACTORS) HARBOURING VARIOUS PLASMIDS

Plasmid	*E. coli* K-12[b]	*V. cholerae*-01[c]			
		-	P^+V^+	P^+	V^+
no plasmid	2	3	3	3	3
pSUP401	256	160	138	179	130
pSUP301	1	3	5	4	4
pPM854	2	2	3	3	4
pPM855	10	3	4	3	3
pPM858	12	10	10	11	12
pPM859	15	8	8	6	9
pPM860	20	40	48	48	40

a CAT assay was performed as described in materials and methods and calculated as μmol/min/total protein (mg)
Total protein was determined by the method of Bradford (1976)

b *E. coli* K-12 strain used was S17-1

c *V. cholerae* strain used was V697 (CA401 Rif^R)

Table 7.4

MINIMAL INHIBITORY CONCENTRATION OF CHLORAMPHENICOL UNDER THE CONTROL OF THE *ctxAB* PROMOTER

	MIC[a] (µg/ml)								
	E.coli K-12	*V. cholerae*							
Plasmid		569B				CA401			
		-	P+V+	P+	V+	-	P+V+	P+	V+
no plasmid	1	1	1	1	1	1	1	1	1
+ pSUP401	500	50	50	50	50	100	100	100	100
+ pSUP301	1	1	1	1	1	1	1	1	1
+ pPM854	1	1	1	1	1	1	1	1	1
+ pPM855	10	10	10	10	10	10	10	10	10
+ pPM856	25	10	10	10	10	ND*			
+ pPM857	10	10	10	10	10	ND			
+ pPM858	50	25	25	25	25	50	50	50	50
+ pPM859	10	25	25	25	25	25	25	25	25
+ pPM860	100	50	50	50	50	100	100	100	100

a minimal inhibitory concentration (µg/ml)

* ND not done

produced by pSUP401 was comparable to those reported by Bauer *et al.* (1988), who examined the strength of several different promoters. This effect is also noted when the minimal inhibitory concentration (MIC) is determined for these strains harbouring the recombinant plasmids (Table 7.4). This lower resistance to Cm by *V. cholerae* has also been observed by Ouellette *et al.* (1988) and by Young and Amyes (1986) on plasmid transfer from *Vibrio* to *E. coli* K-12. As expected, neither pPM854 (the *ctxAB* operon subcloned into pSUP301) nor pSUP301 exhibited any resistance to Cm. Surprisingly, the level of Cm^R in *V. cholerae* CA401 was slightly higher than in 569B.

Plasmids pPM855, pPM856 and pPM857 confer a low level of Cm^R in both *E. coli* K-12 and *V. cholerae*. Insertions of Tn*1736Tc* in the correct orientation under the control of the *ctxAB* promoter (pPM858 and pPM859) did not exhibit an increase in MIC in *V. cholerae*. In *E. coli* K-12, the resistance of pPM858 to Cm is significantly lower than for pSUP401, indicating that the potential promoter detected by computer analysis (section 7.5.1) is not functional in plasmid pPM858. Plasmid pPM860 exhibits the highest level of CAT of this series, inferring that the CAT is under the control of the *ctx* promoter. The CAT expression in pSUP401 is under the control of a strong promoter (P5) (Stüber and Bujard, 1981), hence the high level of CAT produced. None of the plasmids pPM858, pPM859 or pPM860 exhibit an effect in the presence or absence of the P and V factors, suggesting that the P factor is not suppressing virulence by affecting the transcription of cholera toxin. The P and V factors, when present in 569B, did not exhibit an effect on the level of Cm^R, inferring that these plasmids are not contributing to *ctx* control.

Goldberg and Mekalanos (1986) have inserted a Km^R gene into the *Xba*I site in the ctxAB gene on pJM17 *ctxABN4*Km^R) and then

recombined this *ctxABN4*Km^R) into the chromosome. As a single copy in the chromsome Km^R was only observed at 150 µg/ml and the plating efficiency dropped markedly as the concentration of Km was increased. A derivative, which could grow in the presence of 3 mg/ml, was isolated at a frequency of 9.4 x 10^{-8} due to duplication and amplification of the *ctx* genetic element (this only occurs in the presence of *recA*) and was only stable under the selective pressure. Such amplification would not occur under the conditions in this experiment, since the entire 7kb fragment on which the *ctxAB* is located in the chromosome is not present.

7.6 Infant mouse virulence model.

Examination of the LD_{50} of the 569B derivatives in the infant mouse virulence model (Attridge and Rowley, 1983a) demonstrated a decrease of virulence in 569B P$^+$ and 569B P$^+$V$^+$ (Table 7.5). Classical strain 569B had an LD_{50} of 2.0 ± 1.5 x 10^5, and this is not altered by the presence of the V factor (Table 7.5). However, the P factor increases the LD_{50} by more than 10^2 in the presence or absence of the V factor. This suggests that P but not V is responsible for the attenuation of 569B.

Since neither motility nor *in vitro* CT production is affected by the presence of the P factor, the effect of this plasmid upon colonization was next evaluated.

7.7 Colonization.

The colonization capacity of various 569B strains in the presence, absence or combination of the P and V plasmids was examined in infant mice (Table 7.6).

Table 7.5

LD_{50} (48 HOURS) USING THE INFANT MOUSE VIRULENCE MODEL

Strain/plasmid	LD_{50}
569B -	$2.0 \pm 1.5 \times 10^5$
569B P^+V^+	$> 10^8$
569B P^+	$> 10^8$
569B V^+	$2.1 \pm 0.9 \times 10^5$

Data are geometric mean ± SD from two to four determinations.

Table 7.6

COLONIZATION OF THE SMALL INTESTINE OF BABY MICE USING 569B IN THE PRESENCE AND ABSENCE OF P AND V FACTORS

Strain/plasmid[a]	GM ± SE[b]
569B -	7.77 ± 0.05
569B P^+V^+	2.20 ± 0.89*
569B P^+	2.27 ± 0.67*
569B V^+	6.98 ± 0.63

a dose fed to baby mice ranged between 4.0 - 5.8 x 10^6 organisms

b raw data was converted to $\log_{10}$ and the geometric mean (GM) and standard error (SE) were determined on log recovery from six mice.

* one mouse in the group of six showed no colonies on a neat plate; one colony was used for statistical purposes

Groups of 6 baby mice were fed 5 x 10^6 organisms, a dose which would not kill the mice after 24 hours. At this time, the mice were sacrificed, their small intestines carefully excised, and bacterial contents enumerated by homogenizing the tissue and plating appropriate dilutions on NA. In the presence or absence of the V factor, the P factor had a dramatic effect on persistence of the 569B strain (Table 7.6). Calculation of the P value (569B P^+V^+, P^+ <0.0005 and 0.1 for 569B V^+)(Roscoe, 1975) demonstrated that there was a significant difference between colonization of 569B in the presence of the P factor. In the absence of P, the mean total recovery of organisms was ~10^7 for both V^+ and V^- strains, whereas in the presence of P the recoveries were ~200. These data strongly suggest that the P factor in some way inhibits the colonization or *in vitro* growth of the 569B strain.

7.7.1 Growth rates of the 569B P/V series.

To eliminate the possibility that either the P or V factors are influencing the growth rate of the strain 569B, the doubling times of the 569B derivatives were examined in limiting medium (minimal medium (MM)) by spectrophotometrically monitoring the increase in bacterial numbers (OD_{600nm}) and with viable counts to determine the number of bacteria present in the culture. The cell numbers in the culture can lag more than the cell mass. A lag phase was noted for the four strains on transfer from a rich to poor medium (BHI to MM), as expected. Time is required for the cells to have a complete complement of enzymes required for the synthesis of the metabolites not present in that medium. All four strains grow equally well in the MM as seen in doubling time (Table 7.7) and in a growth rate curve (Figure 7.3). This excludes the likelihood that the P factor is having the effect of an auxtrophic marker on 569B and so decreasing its virulence.

Table 7.7

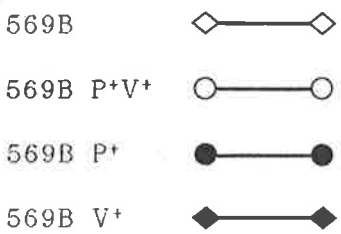
DOUBLING TIME OF THE 569B P/V SERIES IN MINIMAL MEDIUM

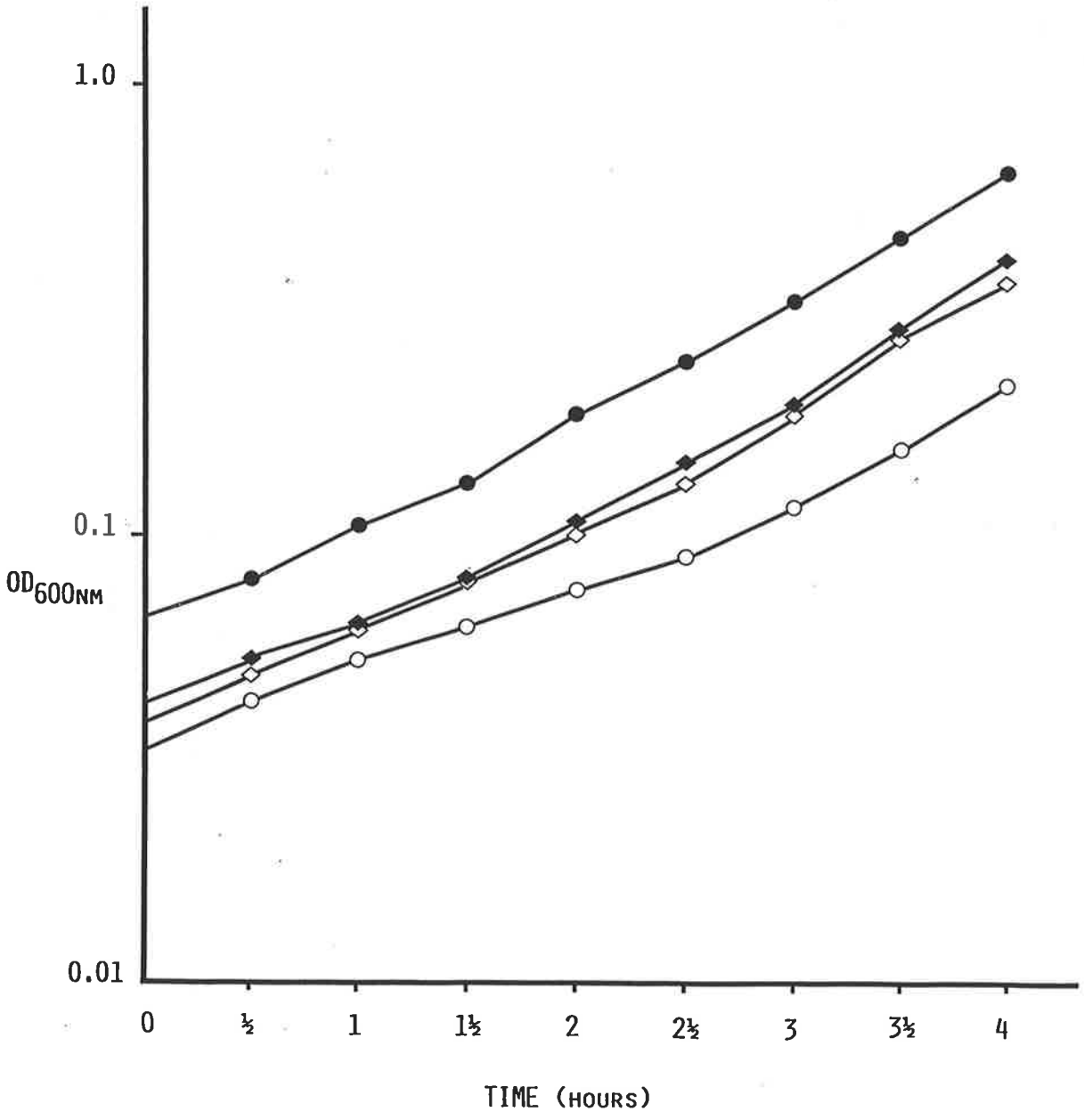
Strain/plasmid	Generation time (min)*
569B	39 ± 3
569B P^+V^+	41 ± 5
569B P^+	38 ± 4
569B V^+	38 ± 5

* Time for the population of cells to double in minimal medium The generation time was calculated from viable counts which were recorded on NA + Sm (100 µg/ml).

Figure 7.3.

Growth curve of 569B in the presence and absence of the P and V factors. The cells were grown in minimal medium and the OD monitored spectrophotometrically at 600nm. Viable counts were recorded on NA + Sm (100 μg/ml). The graph shown depicts the optical density of cells measured at 600 nm.




1.0
0.1
0.01
OD_{600NM}
0
½
1
1½
2
2½
3
3½
4
TIME (HOURS)

7.7.2 *In vitro* adherence assay.

The attenuating affect of the P factor on 569B by decreasing its ability to colonize the gut epithelium is probably not due to different rates of growth in limiting media (Table 7.7) but may conceivably be due to poor attachment. An *in vitro* adherence assay utilizing 5 cm intestinal strips of baby mice and determination of the mean recovery of firmly attached bacteria (Attridge and Rowley, 1983a) demonstrated that there was only a slight decrease (2 fold) in total adherence by the P^+ or P^+V^+ strains (Table 7.8). This difference was reflected in a P value of 0.05 for 569B P^+V^+ and 569B P^+, inferring that there is a difference in *in vitro* attachment. 569B V^+ adhered as well as 569B to the baby mouse intestinal strips.

7.7.3 Attenuating factor of P?

In a preliminary attempt to locate the attenuating principle of the P factor, survival was examined using 569B harbouring various tra^+/tra^- transposon mutants of P (Table 4.3) or one of the *Eco*RI fragments subcloned into pSUP401 (section 4.5.2). The motility of these 569B derivatives remained unaltered, as confirmed by the swarming in soft agar technique. A dose of 1×10^7 bacteria was fed to groups of 8 mice, since this dose would best distinguish between a 569B and 569B P^+ strain (Table 7.9), and survival measured at 48 hours. The distinction between a P^+ and P^- strain was very clear (Table 7.9), as previously observed. The subcloned *Eco*RI fragments in vector pSUP401 (*Eco*RI-2A, -4, -7, and -8; pPM882, pPM885, pPM888 and pPM889, respectively) did not alter the virulence of 569B, suggesting that these fragments do not contain the attenuating factor. All transposon mutants, regardless of their site of insertion in P restored virulence of 569B, suggesting that any manipulation of the P factor inactivates its attenuating ability.

Table 7.8

THE EFFECTS OF P AND V FACTOR ON THE *IN VITRO* ATTACHMENT OF *V. CHOLERAE* 569B

Strain/plasmid	mean ± SE $\log_{10}$[a]	total adherence (%)[b]
569B	6.07 ± 0.07	4.8
569B P^+V^+	5.83 ± 0.05	1.0
569B P^+	5.89 ± 0.10	2.7
569B V^+	6.07 ± 0.03	4.3

4 intestinal strips were prepared from baby mice for each strain.

a Raw data was converted to logarithmic values and represents the mean number of bacteria which adhered to individual intestinal strips (includes standard error (SE))

b total adherence (%) is the total number of bacteria recovered from the 4 intestinal strips divided by the total number of bacteria added.

Table 7.9

EFFECTS OF P::TN MUTANTS OR SUBCLONED FRAGMENTS OF THE P FACTOR ON PATHOGENICITY OF *V. CHOLERAE* 569B

569B + plasmid[a]	Survival[b]
-	0
P	8
P::Tn*1*	0
pSJ13	0
P::Tn*3*-1	0
P::Tn*5*-1	0
P::Tn*5*-7	1
P::Tn*5*-10	1
P::Tn*1732*-1	0
pSUP401	0
pPM882	0
pPM885	0
pPM888	0
pPM889	0

a Strains were grown in the presence of the appropriate antibiotic.
P::Tn mutants are described in Table 4.3 and plasmids pPM882, pPM885, pPM888 and pPM889 in Table 4.4.

b The survival of baby mice (groups of 8) 48 hours after oral administration of 1 - 2 x 10^7 organisms. This dose of bacteria best distinguished between a 569B and 569BP$^+$ strain.

7.8 Conclusions.

A role of the P factor in the suppression of virulence has been demonstrated. This decrease in virulence was reflected in much higher LD_{50} values of P^+ and P^+V^+ derivatives of 569B. No significant reduction in the amount of CT produced by *V. cholerae* 569B in the presence of the P or V factors was observed by ELISA quantitation of CT production. Neither P nor V showed any affect on expression from the *ctxAB* promoter in operon fusions using the CAT gene.

The effect exerted by the P factor on the strains is by decreasing its ability to colonize the gut epithelium as demonstrated *in vivo* in infant mice, however, *in vitro* adherence assays indicated that this was not purely due to an affect on adherence to baby mouse intestinal strips.

Chapter 8

Discussion

8.1 Introduction

The *V. cholerae* Classical Ogawa strain V58 originally isolated by Bhaskaran (1960) is known to harbour the P sex factor and although poorly studied, P has been used extensively in constructing a genetic map of the *V. cholerae* chromosome based upon its ability to mobilize chromosomal markers (Parker *et al.*, 1979). Numerous comparisons have been made between P and the F sex factor of *E. coli* K-12 because of the details which are available for F. The F and P factors appear to be similar in several respects; both have the ability to exist as autonomous replicons capable of derepressed self-transfer and also to mediate low-frequency transfer of bacterial chromosomal genes (Parker and Romig, 1972). However, the apparent failure of the P factor to form stable cointegrates with the chromosome (Parker *et al.*, 1979) is perhaps the most significant distinction between F^+ and P^+ donors.

This thesis has examined at a molecular level, P and two further plasmids detected in the *V. cholerae* 01 strain V58. Attempts have been made to assign functions to these plasmids and to characterize them at a molecular level.

8.2 Identification of three plasmids in *V. cholerae* strain V58 and relatedness to other plasmids.

Examination of plasmid DNA from strain V58 has revealed the presence of three plasmids, one of which is the P factor (Figure 3.1). The sizes of these three plasmids are; the P factor, 68 ± 1.6 kb, large cryptic plasmid (lcp) also shown to be identical to the V factor (see below), 34.1 ± 0.13 kb and the small cryptic plasmid (scp) 4.7 ± 0.13 kb.

Previous reports of the size of the P factor have varied considerably between 52 and 120 kb (Datta *et al.*, 1973; Newland *et al.*, 1984a). Datta *et al.* (1973) calculated the molecular weight of the P factor from their strain, RV69, to be 78 MDal (116 kb) by sedimentation velocity experiments and EM measurements. The size of the P factor obtained here was consistent with sizes calculated for P and P::Tn derivatives imported from other laboratories (Johnson and Romig, 1979b; Khan *et al.*, 1985). Johnson and Romig (1979b) constructed a P::Tn*1* derivative (pSJ5) and used sedimentation rates and EM contour lengths and calculated P to be 47.5 MDal (equivalent to 70 kb) and the P::Tn*1* to be 51 MDal (equivalent to 76.9 kb), the difference would be due to the Tn*1* (5 kb). Johnson and Romig (1979b) also detected the presence of two cryptic plasmids, termed I and II. From sedimentation rates and contour length measurements they determined the sizes of these DNA species to be; cryptic I, 3.2 MDal (4.7 kb, scp) and cryptic II, 24.7 MDal (36 kb, lcp). These workers also noted that these plasmids were present in both P^- and P^+ strains, although the cryptic I plasmid was present in small numbers and was not detected in the centrifugation experiments. The plasmid sizes for the scp and lcp determined during this study confirm the findings of

Johnson and Romig (1979b) and the observation that the scp is often difficult to visualize in ethidium bromide stained agarose gels. Datta *et al.* (1973) in their investigations of the P factor also reported the presence of a second smaller plasmid which was not consistently isolated from the strains but was present in both P^+ and P^- strains. Although they calculated this plasmid to be 37 Mdal (56 kb), it probably corresponds to the 34 kb plasmid detected in this study, since their estimate of the size of P was also discrepant. In addition, Datta *et al.* (1973) failed to identify a plasmid corresponding to the scp of V58.

8.2.1 The cryptic plasmids

Cook *et al.* (1984) described the consistent isolation of two plasmids from Classical strains from the sixth pandemic which appear to correspond to lcp and scp of strain V58.

Both plasmids were present as very weak bands on agarose gels which may reflect a low copy number or a loss of plasmid during the DNA isolation procedure. The scp is usually difficult to visualise in agarose gels and the lcp tends to comigrate with or just slightly higher than the chromosomal DNA making its presence often difficult to detect. It seems that these plasmids are commonly identified in both old (Cook *et al.*, 1984) and recent isolates (section 6.) of Classical strains.

8.2.2 The V factor as a plasmid.

The V factor described by Bhaskaran and Sinha (1971) was identified as a factor which could cause the production of lacunae and was able to mediate transfer chromosomal markers at a very low frequency, however, it was not classified as a plasmid.

In this study plasmid DNA was isolated from strain KB9 harbouring the V factor and was shown to be identical to the lcp by restriction

endonuclease cleavage and by Southern hybridization using the subcloned fragments of the lcp (Figures 6.8 and 6.9). The lcp also possesses all other properties attributed to V and has an identical *Hin*dIII cleavage pattern to the 31.5kb (21 MDal) plasmid of Cook *et al.* (1984) indicating the widespread nature of this plasmid. No homology was detected between the lcp (V factor) and the P factor (Figure 6.8, 6.9).

The P and V factors can stably coexist indicating that they belong to different incompatibility groups. This is not an unexpected observation, since the lcp and the P factor have been isolated from the same strain, inferring compatibility between the plasmids.

Neither P nor V exerts a transfer inhibition effect on the other. This is in contrast to some plasmids which do repress the transfer of another plasmid, such as with the F factor which is repressed for transfer in the presence of R100, however the reverse is not the case (Finnegan and Willetts, 1973).

Bhaskaran and Sinha (1971) proposed that the presence of the V factor in a cell which has newly acquired the P factor does not repress the P factor, however, after a period of time the P factor is repressed for transfer. This was demonstrated by frequencies of chromosomal marker transfer and distinction on the size of lacunae formation which would be dependent on factors such as the number of donor cells present on the plate. Established strains which are P^+V^+ should be used in examining the effect of transfer inhibition rather than cells which have newly acquired a plasmid, as a period of time may be required for full expression of plasmid encoded genes (Finnegan and Willetts, 1972). The use of transposon-tagged P or V factors enables the plasmids to be accurately followed from different host cells. Strains harbouring both plasmids have enabled the observation that

transfer of either plasmid is not inhibited by the presence of the other plasmid. Both the P and V factors are derepressed for transfer and could be defective in the same *fin* (fertility inhibition) function. It would be of interest to examine transfer inhibition in the presence of other conjugative plasmids so that a more detailed analysis of the P and V *fin* genes could begin.

8.3 Restriction analysis of the three plasmids in *V. cholerae* strain V58.

DNA of the three plasmids from strain V58 was subjected to digestion with a large number of restriction endonucleases facilitating the construction of restriction maps for the P factor (68 kb), the lcp (34 kb) and the scp (4.7 kb) (Figures 4.3, 5.2 and 5.8, respectively). Such maps are a prerequisite for mapping functions to different regions of the plasmids.

8.4 Possible roles of the P factor.

Previous characterization of the P factor has been limited. It has been identified in *V. cholerae*-01 and shown to promote chromosomal mobilization (Bhaskaran, 1960; Parker and Romig, 1972). Further analysis by Datta *et al.* (1973), demonstrated a copy number of one, that it was capable of self transfer at a high frequency and that it had a G + C composition of 42%. The relatedness of P to the *V. cholerae* chromosome and its similarity to the F factor of *E. coli* K-12 has also been discussed (Wohlhieter *et al.*, 1975).

P is one of the few conjugative plasmids reported to be stably maintained in *V. cholerae*. In this study an investigation of properties of the P factor other than those previously described was undertaken.

A property commonly associated with large conjugative plasmids is the resistance to metal toxicity (Trevors *et al.*, 1985). It is not a ubiquitous property among enteric bacteria with conjugative plasmids, and thus the lack of any demonstratable resistance mediated by the P factor to any of the metals tested is not unusual.

A number of conjugative plasmids have been shown to confer resistance to killing by normal serum. This includes the F factor of *E.coli* K-12, a number of other F-like plasmids (Moll *et al.*, 1980; Hansen *et al.*, 1982), and a 70kb virulence plasmid of *Yersinia enterocolitica* (Balligand *et al.*, 1983). However, serum resistance in *Y. pestis* and *Y. pseudotuberculosis* is not plasmid encoded (Perry and Brubaker, 1983). Serum resistance mediated by F, R100-1, R6-5 and other F-like plasmids is encoded by the *traT* gene which is also involved in surface exclusion (Achtman *et al.*, 1980). Unlike these other plasmids, the P factor does not confer serum resistance, even though it mediates surface exclusion.

Plasmid incidence in *V. cholerae* is low and those isolated from *V. cholerae* have almost all been R factors belonging to the incompatibility groups IncC (Davey and Pittard, 1975; Hedges and Jacob, 1975) or IncJ (Yokota and Kuwahara, 1977; Kuwahara, 1979). The nineteen Inc test plasmids which have been used to determine the incompatibiblity group of conjugative plasmids in enteric bacteria were introduced into *V. cholerae* as it was demonstrated that the P factor cannot be transferred to *E. coli* K-12 (section 3.4). Introduction of these test Inc plasmids also permitted examination of the stability of these plasmids in *V. cholerae*. One third of the test Inc plasmids could not be transferred by liquid or filter matings into *V. cholerae* (Table 3.1). Plasmids R1-16, R446b, TP114 and R447b were unstable in *V. cholerae*.

The instability of these R factors makes them potentially useful vectors for introducing transposons into *V. cholerae* for mutagenesis.

Plasmids from different incompatibility groups vary in their host range among Gram-negative bacteria. Some Inc groups exhibit a very narrow host range, such as plasmids from the IncJ group whereas plasmids of incompatibility groups N, P and W have a broad host range (Tardif and Grant, 1980; Ward and Grinsted, 1982). The low incidence of R factors may be explained in part by the limited number of Inc groups able to be maintained in *V. cholerae*. It was not possible to determine the Inc group of P, however, P does not belong to Inc groups C, D, I, M, N, P, T, U or X. It seems likely that P probably belongs to a new incompatibility group and is unstable outside *Vibrio* or closely related species.

Probing the test Inc plasmids with the P factor showed no homology, similar to the observation by Ingram (1973) who demonstrated that conjugative plasmids from different incompatibility groups as a rule do not show extensive homology. However, Golub and Low (1986) have demonstrated that some unrelated conjugative plasmids show homology to the F factor leading region and with the *ssb* gene (single-stranded DNA-binding protein) (Golub and Low, 1985). Once a similar region has been identified on the P factor, homology to this region and the *ssb* gene could be examined and characterized. The F fragment (*Eco*RI fragment 3; Kolodkin *et al.*, 1983) which encodes the *ssb* gene (at 59.2-59.6kb, map position on F) could be used to probe the P factor to determine whether a homologous region is contained on P and whether it is already cloned.

Conjugation experiments with a P::Tn*3*-1 donor and a variety of species demonstrated that the P::Tn*3*-1 will transfer to *V. cholerae*-01 and non-01 strains equally well and to other *Vibrio* species at a 100-

1000 fold lower frequency. *Aeromonas* species were either 100-1000 fold poorer recipients than *V. cholerae*-01 or did not either accept or maintain the P::Tn*3*-1 at all. Transfer to *Aeromonas* species is not unexpected as this genus also belongs to the *Vibrionaceae* family.

Transfer of P::Tn*3*-1 into *E. coli* K-12 was not observed either by liquid or filter matings even when a restrictionless host was employed, however, *S. typhimurium* exhibited a low recipient ability for P::Tn*3*1 but the plasmid was not maintained and those transconjugants detected appear to have Tn*3* but not P. Thus P is probably unable to replicate or be stably maintained in species such as *E. coli* K-12 and *S. typhimurium* but is maintained/replicated in *Vibrio*-related species.

8.4.1 Roles of the P and V factors in virulence.

The major virulence determinants of *V. cholerae*-01 are motility, adherence, colonization and the production of cholera toxin (CT). Each of these factors is important in inducing the final manifestations of the disease.

A series of derivatives of strain 569B were constructed to examine the effect of P and V on the virulence of *V. cholerae*-01. Neither of the plasmids affected motility (section 7.2.1) and CT production nor was fluid accumulation (FA) in ligated rabbit ileal loops significantly different from the parent strain (Table 7.1). The lack of an effect on CT was also demonstrated using *ctx* operon fusions. However, it was clear that P was having an effect on suppressing virulence.

Since neither motility nor the production of CT was influenced by the presence of P (or V), colonization was investigated utilizing the infant mouse virulence model for *V. cholerae* (Ujiiye *et al.*, 1968). It was demonstrated that the LD_{50} (48 hours) was dramatically increased in the presence of the P factor and further analysis revealed that persistence of 569B is being repressed by P (section 7.7). This

confirms a suggestion by Hamood *et al.* (1986) that the P factor could be influencing colonization rather than CT production (Khan *et al.*, 1985).

The roles postulated for P in CT biosynthesis by Sinha and Srivastava (1978) and Khan *et al.* (1985) have not been confirmed by these studies. This agrees with Newland *et al.* (1984a) who have shown that neither pSJ5 (P::Tn*1*) nor a variety of other plasmids exhibited an effect on CT production. The Tn*1* in pSJ5 has been mapped in the *Eco*RI-1 fragment of the P factor (Figure 4.5) which could possibly result in the inactivation of a potential regulatory region acting on CT biosynthesis. However, this seems unlikely since it has been shown that the whole P factor itself is not affecting CT production, and that almost any manipulation of p can eliminate its affects on suppression of virulence.

Growth of the 569B derivatives were examined in minimal medium but no affect attributable to P was observed.

The only affect of P was demonstrated by an *in vitro* adherence assay in which a 2 fold (1-2% compared with 4% by the parent) decrease in initial attachment to the gut was found in P^+ strains. This 2 fold decrease in adherence exhibited by a P^+ strain may be significant enough to put the strain at a disadvantage when competing with the natural flora of the gut. However, it seems unlikely that this is solely responsible for the marked affects on colonization (Table 7.8).

The P factor has possible interest as an attenuating marker in live oral vaccines since it reduces the virulence of a strain harbouring it. It is able to bind to the gut, however at a reduced capacity *in vitro*. This could be even more dramatic *in vivo* when other extracellular enzymes/proteins produced by *V. cholerae* are present. Further investigations would be required to identify the attenuating factor on

P and whether P can affect the immune response. It would be of interest to determine whether P strains are still as immunogenic. This would involve orally immunizing with P^+ and P^- strains in animal models or human volunteers and then analysing the degree of antibody production and/or subsequently challenging with a P^- strain to assess the level of protection.

Further investigations to explore which aspect of colonization is being affected by the P factor could involve EM studies to assess whether fewer pili are present on the surface of the bacterium or possibly immuno-gold studies using anti-*tcp* to demonstrate an effect on this pilus which has been implicated in colonization (Miller *et al.*, 1987).

8.5 Possible roles of the scp and lcp.

V. cholerae-01 strain V58 has been shown to harbour two plasmids in addition to the P factor, however, no role has been assigned to either plasmid.

8.5.1 The large cryptic plasmid.

The V factor (Bhaskaran and Sinha, 1971), originally described in a *V. cholerae* non-01 strain has been identified as a plasmid (Chapter 6) and shown to be indistinguishable from the lcp from strain V58 and a plasmid found in isolates of the sixth pandemic.

Like P, lcp does not encode resistance to metal ions (Trevors *et al.*, 1985) nor does this plasmid play a role in the virulence of *V. cholerae* (refer to section 8.4.1).

A plasmid of similar size to lcp but isolated from a *V.cholerae* non-01 strain contains homologous sequences to the *tdh* (thermostable direct haemolysin) gene from *V. parahaemolyticus* (Nishibuchi *et al.*, 1985). Southern hybridization using a 415bp probe to the *tdh* gene has

not revealed homology to any either V (lcp) or the P factor (Figure 3.5) despite the similar origins of V and this plasmid.

At only 34kb, (sections 3.2 and 5.2) the lcp (V) is particularly small for a conjugative plasmid, perhaps even the smallest yet to be reported in Gram negative bacteria. Those conjugative plasmids which have been looked at in any detail are at least 39kb in size (Thatte *et al.*, 1985). The whole lcp is about the same size as the Tra region of F, but other functions such as replication are also necessary.

This suggests that the lcp is less complex in terms of *tra* functions than other conjugative plasmids and thus examination of the *tra* region of lcp would be of interest. Perhaps a reason for the lack of functions on the lcp is that the coding capacity of the plasmid is mainly taken up with essential functions such as replication and transfer regions and that it provides a replication/transfer module for generating R-factors.

8.5.2 The small cryptic plasmid.

The possibility that the scp encodes a restriction/modification system was examined utilizing a bacteriophage test system for proficiency in host-DNA restriction based on the original observation of Arber (1974) of a differential efficiency of plating of bacteriophage lambda on restriction-proficient and restriction-deficient strains of *E. coli* K-12 similar to that used previously (Imbesi and Manning, 1982). Bacteriophage CP-T1, VcIV and ET1 were used in determining the eop in the presence and absence of the scp (Chapter 3), however, no difference was observed with scp^+ compared to scp^- strains.

It seems unlikely that the scp encodes a restriction/modification system. It is not known whether the bacteriophage used (VcIV, CP-T1 and ET1) contain unusual bases in their DNA, such as the T4 bacteriophage of *E. coli* K-12 (Erikson and Szybalski, 1964; Mathews

and Allen, 1983). However, extensive restriction data on bacteriophage ϕ149 (a Classical group IV bacteriophage) (Sengupta *et al.*, 1985) and CP-T1 (Guidolin *et al.*, 1984) suggests that this is unlikely, since their DNA is readily cleaved by most restriction endonucleases. ET1 bacteriophage is poorly characterized and it is not known whether its DNA is modified. The T-even bacteriophages contain an unusual base in their DNA (5-hydroxy-methylcytosine (HMC) instead of cytosine) and this has been well characterized for bacteriophage T4 of *E. coli* K-12 (Mathews and Allen, 1983). Further glycosylation of the HMC results in the T4 DNA being virtually cloaked with these molecules and this has the consequence that purified T4 DNA is virtually resistant to a variety of restriction endonucleases. Restriction/modification systems have been described in *V. cholerae*-01 (Imbesi and Manning, 1982) and VcIV and ET1 can differentiate the two biotypes. Analysis of this system has not been investigated into a division of the *hsd* (host specficity of DNA) into the *hsdS* (specificity polypeptide), *hsdM* (modification methylase) or *hsdR* (restriction endonuclease) nor has there been an examination into the relatedness to other systems as done by Daniel *et al.* (1988) for *hsd* genes in *E. coli* and other enteric bacteria.

If the scp does encode a restriction system, then perhaps the bacteriophage examined simply lack the sites for it. However, further analysis of the scp is required including the isolation of isogenic scp^- and scp^+ strains. Curing of the scp may not be that simple, since attempts to cure small plasmids from other *Vibrio* species, like *V. vulnificus* have been unsuccessful (Davidson and Oliver, 1986).

8.6 Physical analysis of the P factor.

Restriction analysis of a plasmid facilitates its examination at a molecular and physical level. The initial stage of examining genes encoded by a large replicon involves the subcloning of the various regions as a series of overlapping restriction fragments to minimize the possibility of interupting coding sequences. Utilizing these subcloned fragments it is then possible to embark on a more detailed analysis at a physical level as well as providing probes for particular genes.

Analysis of the *tra* region of the P factor was of interest as it was expected that the organization may not necessarily be similar to that of the F factor of *E. coli* K-12. Previous heteroduplex analysis has shown that there is little or no homology between F and P (Wohlhieter *et al.*, 1975). However, heteroduplexing is an insensitive method and so may merely reflect a lack of homology at the DNA level which may not be representative of the similarities exhibited at an organizational or physical level.

Site directed mutagenesis of the P factor using transposons Tn*3*, Tn*5* and Tn*10* has been used to determine the physical location of genes associated with transfer and surface exclusion functions. Twenty seven independent transposon mutants were tested for transfer (Tra) and surface exclusion (Sfx) properties (Table 4.3) and their sites of insertion mapped on the P factor. Tra$^-$ and Sfx$^-$ P::Tn derivatives span 16 kb of the contiguous fragments *Eco*RI-1 and *Eco*RI-2B contained within the *Xba*I-1 fragment (Figure 4.5). However, there are extensive regions of P in which no insertion was isolated and so the transfer region could be significantly larger than this and in fact multiple *tra* regions.

The Tra locus in plasmids from different incompatibility groups may vary from 15kb (pKM101/IncN (Langer *et al.*, 1981)) to 40kb (ColIb-

P9/IncI$_1$ (Rees *et al.*, 1987)). The Tra region need not encompass a single contiguous region as for F (Willetts and Wilkins, 1984) but, may be 2 or 3 regions such as with R27/IncHI (Taylor *et al.*, 1985) and ColIb-P9/IncI$_1$ (Rees *et al.*, 1987), respectively. The transposon mutants isolated in the P factor, map in clusters (Figure 4.5), suggesting that several regions are involved. However, this clustering could reflect hot-spotting by the transposons. The size of the putative Tra region of P factor lies within the range observed with other conjugative plasmids examined from a variety of Inc groups.

The three fragments, *Eco*RI-1, *Eco*RI-2B and *Xba*I-1 which appear to encode transfer functions have not been cloned even using low copy number vectors. One possible explanation is that cloning of these fragments would result in very large recombinant plasmids. Large plasmids are poorly transformable and often unstable. Analysis of the Tra proteins of the F factor has demonstrated that many are membrane proteins (Willetts and Skurray, 1987). Overproduction of outer membrane proteins or lethal proteins by cloning of DNA in high copy number vectors or under the control of strong promoters on plasmids contributes to host instability and /or poor transformability (Manning *et al.*, 1982). O'Connor and Timmis (1987) have recently described the construction of a plasmid vector which contains a highly repressible expression system that allows the cloning of potentially deleterious genes, such as outer membrane proteins. This vector, pDOC55 exploits an antisense RNA system to facilitate the cloning of possible overexpressed genes. Attempts to subclone the *Eco*RI-1, *Eco*RI-2B or *Xba*I-1 fragments of the P factor into this vector may help to overcome this problem.

Another explanation for the unsuccessful attempts to clone this region is that partitioning (*par*) genes may be contained within these

fragments. Partitioning is necessary for the correct distribution of plasmids into daughter cells (Austin and Abeles, 1983) and has been analysed for F (Mori *et al.*, 1986), P1 (Abeles *et al.*, 1985) and R1 (Nordström *et al.*, 1980). The *par* region of F lies adjacent to the *tra* region and encoding two plasmid-encoded proteins, the products of *sopA* and *sopB*, and a *cis*-acting site, *sopC*, all of which are required for stable maintenance (Austin and Wierzbicki, 1983; Ogura and Hiraga, 1983). If the P *par* region is located within the *Eco*RI-1 or *Xba*I-1 fragments, it could well present problems in their cloning, since plasmid loss in the presence of a cloned *par* gene in the absence of the other is greatly increased (Novick, 1987). Funnell (1988) has shown that when *parB* of P1 is subcloned, excess of the ParB protein destabilizes the plasmid. If an equivalent *parB* region is contained on P, it would very likely result in the recombinant plasmid being unstable.

The replication of ColE1 and P15A derived replicons are dependent upon DNA polymerase I (*polA*) of the host cell (Staudenbauer, 1976; Chang and Cohen, 1978). Introduction of recombinant plasmids with these replicons into an *E. coli* K-12 *polA* strain would require the presence of a replication origin not dependent upon *polA*, such as the cloning of the P replication origin region. In an initial attempt to localize the replication region of P, the various subclones were transformed into a *polA* strain. None of the recombinant plasmids could be transformed into this strain, suggesting that they do not contain a replication origin or that such an origin may not function in *E. coli* K-12. Further support of this latter notion comes from the observation that P::Tn derivatives could neither be conjugated or transformed into *E. coli* K-12. Thus, it is not possible to define whether or not *polA* is required for P replication, as it is with the IncP2 conjugative plasmid pMG2 (Prince and Barlam, 1985). The

replication of F is dependent on a number of host-encode proteins which include *dnaB*, *dnaG* and *polC* (Willetts and Skurray, 1987), however, it is not dependent upon *polA* (Lane, 1981), hence P may not necessarily be dependent upon the host *polA*.

The genes involved in the biosynthesis and assembly of P pilin into the sex pilus could be expected to lie within the Tra region as the pilus is essential for conjugation. This has been seen with the F factor (Helmuth and Achtman, 1975), IncF$_o$*lac* plasmid EDP208 (Finlay et al., 1983), IncI$_1$ plasmid ColIb-P9 (Rees *et al.*, 1987) and IncN plasmid pCU1 (Thatte *et al.*, 1985).

By analogy with the F factor, *traA-H(G)* which code for the biosynthesis of F pili (Ippen-Ihler and Minkley, 1986), could be contained within the 16kb region, the putative P *tra* region of the *Eco*RI-1 fragment as mapped by transposon mutagenesis (section 4.3). Within this region of DNA, transposon mutants extend which are no longer transfer proficient (transfer frequency $< 10^{-7}$, Table 4.3) which infers that essential transfer functions such as for P pilus biosynthesis have been interupted. The F pillus biosynthesis region *traA-H(G)* is approximately 17kb in length and a similar sized region (16.5kb) is found on the IncF$_o$*lac* plasmid EDP208 (Finlay *et al.*, 1983). The P::Tn cluster comprising of Tn*10*-2 to Tn*10*-5 could well have inactivated an equivalent *traA* region (F pilin gene) since *traA* mutants are likely to be totally Tra$^-$. The cluster of P::Tn mutants located at the 30kb position (Tn*5*-8 through to Tn*5*-18) may represent an analogous region to the *traG* (donor stability) and *traN* (pair formation) (transfer frequencies of 2.1 and 1 x 10^{-4}, respectively; Manning et al, 1981) and these may correspond to P::Tn mutants Tn*5*13 to Tn*5*-14 and Tn*5*-8 to Tn*5*-11, respectively. The distance between these two regions is approximately 12kb which would be similar to that seen with F. The

pilus genes for the thin and thick pili of IncI$_1$ plasmid lie within similar sized regions, the Tra1 and Tra2 regions, respectively (Rees *et al.*, 1987). If subcloning of the Tra region proves to continue to give problems then another approach could be taken to identify the extent of the T ra region. The isolation of bacteriophage specific for the P pilus, like the male specific DNA and RNA phages which use other sex pili (Crawford and Gestsland, 1964; Caro and Schnös, 1966) could provide a powerful selection for Tn insertion mutants in the pili biosynthesis genes of P. Mapping of these insertions would enable accurate localization of a region on the P factor analagous to that containing *traA-H(G)* of F.

As expected, the transfer ability of the surface exclusion mutant, Tn*5*-1 (Figure 4.5) is not affected, as has been observed with other plasmids (Helmuth and Achtman, 1975; Achtman *et al.*, 1980).

Introduction of recombinant plasmids into minicells to analyse the plasmid encoded proteins is a means of identifying how many genes are present. However, few proteins were detected when plasmids containing the various cloned fragments were analysed in this manner. One plasmid, pPM804 (*Eco*RI-4 fragment in pACYC184) expressed proteins of 24 kDal and 23 kDal, which are readily detectable in both whole cell extracts and in minicells (Figures 4.9A and 4.10A respectively). These proteins are, however, not evident in cellular extracts of *V. cholerae* harbouring the P factor. The lack of detection of proteins by direct analysis of whole cell extracts (with the exception of the two proteins encoded on the *Eco*RI-4 fragment) is not totally unexpected. A similar observation has been made with many of the F Tra region products (Ippen-Ihler and Minkley, 1986).

Only about a third of the coding capacity of the P DNA can be accounted for (Table 4.4). Even long exposures of the autoradiographs

did not reveal many additional bands. Kennedy *et al.* (1977) found that many proteins of the F Tra region were difficult to detect by autoradiography and proposed that the intensity of the band reflected the nett rate of synthesis. The inability to detect proteins with [^{35}S]-methionine could be due to the proteins having a very short half life or a slow rate of synthesis resulting in bands of lower intensity. Alternatively, the proteins synthesized have only a few or no methionine residues. These problems could be overcome in several possible ways; labelling the plasmid encoded proteins with [^{14}C]-amino acids or instead of minicells utilizing an *in vitro* DNA dependent transcription/translation system (Zubay, 1973) where little processing of the proteins occurs. Another possibility is that during the cloning process the genes have been removed from the regulatory elements responsible for their expression. This can be overcome by cloning the DNA adjacent to a powerful promoter which can be regulated such as the lambda P_L promoter (Bernard *et al.*, 1979). The subcloning of contiguous *Eco*RI fragments may result in promoters or regulatory elements being contained within the region. However, the recombinant plasmid pPM814 which contains a contiguous region of approximately 10 kb does not exhibit a greater number of plasmid encoded proteins than the individual fragments, inferring that this region may be internal within an operon or that *trans* acting proteins may also be required for expression.

Interpretation of polypeptides synthesized in minicells from recombinant plasmids may be complicated by the production of fusion products, in which a segment of a gene from the inserted DNA is joined in frame with a segment of a gene from the vector. This fusion product may be expressed from either a vector or a cloned promoter depending on how it is constructed. The *Eco*RI-9 fragment has been

cloned into the *cat* gene of pACYC184 (pPM809) and this produces two proteins of 39 kDal and 18 kDal which exceed the coding capacity of the cloned fragment. The 39 kDal protein could possibly be due to the production of a fusion protein from the *cat* gene and a P factor gene. Reversal of the orientation of the cloned fragment or inactivation of possible promoters may identify fusion products. The *Eco*RI-9 fragment has also been cloned into the *cat* gene of pSUP401 (pPM885) and in this case it does not produce a 39 kDal protein, however, the 18 kDal protein is still present, confirming that the 39 kDal polypeptide may be a fusion product. The *Eco*RI site is approximately 400bp from the beginning of the *cat* gene (Chang and Cohen, 1978) and would account for approximately 15 kDal of the fusion product. The *Eco*RI site is approximately 300bp from the end of the cat gene. A protein using a cloned promoter and the N-terminus of the *cat* gene would have a maximum size of 11 kDal, however, no such protein has been detected.

The two proteins (24 kDal and 23 kDal) encoded in the *Eco*RI-4 fragment of the P factor have been localized to the outer membrane fraction of the cell envelope. It seems that the natural promoter(s) for expression of these proteins has also been cloned as expression occurs in both orientations of the fragment with respect to the vector. Using transposon mutagenesis and deletions of the *Eco*RI-4 fragment it has been possible to determine that the direction of transcription is from the *Eco*RI site towards the *Xba*I site. The coding region has been localized to 1.7kb from the *Eco*RI site. The exact location of the genes for the 23 and 24 kDal proteins has not been determined. The gene for the 24 kDal protein precedes the gene for the 23 kDal protein from the observation that the deletion of 0.2kb (*Eco*RI-*Eco*RV; pPM847) of the *Eco*RI-4 fragment results in a very low amount of the 23 kDal being produced, however, no 24 kDal is produced, inferring that this

fragment is required for its expression. These two proteins are likely to have a signal sequence, since they are located in the membrane of the cell. All membrane proteins which have so far been examined have a signal sequence (Randall and Hardy, 1984). From the transposon insertions and deletions it was not possible to distinguish the possible configurations of the two genes. The two genes may be adjacent to each other in the same orientation or possibly the second (for the 23 kDal) is in the opposite orientation. If this is the case, it could also be possible that the 24 kDal is affecting the regulation of the 23 kDal and when under the influence of the vector promoter (P5) both proteins are produced in greater amounts (pPM843, Figure 4.14). When both genes are in the same orientation a similar effect by the P5 promoter would be observed. Another explanation could be that the genes are overlapping, however, in a different frame. Expression of both proteins would be affected by the transposon insertions if this were the configuration of the genes. Alternatively, the 23 kDal protein may be the result of a restart of the 24 kDal protein. Following the beginning of the 24 kDal protein there may possibly be another ATG codon with a signal sequence which would result in the 23 kDal protein being synthesized. Again, transposon insertions into this coding region would affect both proteins. The roles of the 23 kDal and 24 kDal or other proteins detected in minicells play in relation to the P factor remain unknown.

8.7 Physical analysis of the cryptic plasmids.

All of the scp and 60% of the lcp have been subcloned in an initial step towards examining the genes encoded on these plasmids.

8.7.1 The large cryptic plasmid (lcp).

Examination of plasmid encoded proteins in whole cell extracts and in minicells has shown that only a small proportion of the potential coding capacity is being expressed by the subcloned fragments of the lcp. As seen with the P factor this may be because the genes have been removed from their natural promoters during the subcloning.

Subcloning individual fragments has its uses, however, larger fragments such as contiguous *Eco*RI fragments or with other restriction endonucleases are eventually more useful for examination for plasmid encoded proteins as it is more likely that regulatory regions are also contained within these cloned regions. In this study only the *Eco*RI fragments (of lcp) were cloned resulting in a small proportion of the maximal coding capacity being analysed.

The lcp (V) is of considerable interest because of its small size for a conjugative plasmid and likely that most of the coding capacity will be utilized for essential conjugative and replicative functions (refer to 8.5.1). Investigation into complementation between the *tra* region of lcp and P as to whether the lcp (V) *tra* region can act in *trans* to complement P *tra* mutants could be analysed further.

8.7.2 The small cryptic plasmid (scp).

Examination of cloned fragments when introduced into the minicell producing strain of *E. coli* K-12 of the scp has resulted in the identification of three proteins (28, 25 and 13.5 kDal). The two *Sph*I subclones each encode one of these proteins; the 28 kDal on pPM851 and the 13.5 kDal protein on pPM852 (Figure 5.10). Longer exposure of the autoradiograph reveals a 25 kDal protein which would map across the single *Cla*I site. Together these three proteins represent about half of the coding capacity of scp.

The replication region would require approximately 1kb, leaving only a small amount of DNA to encode any other functions. Plasmid pPM853 containing the whole scp cloned at the *Cla*I site could provide a basis for developing host specific/or shuttle cloning vectors since scp is very stable in *V. cholerae*. The low copy number of scp would be advantageous for such a shuttle cloning vector when analysing P factor encoded proteins.

8.8 Epidemiology of the three plasmids of strain V58.

The subcloning of the three plasmids P, lcp and scp from strain V58 (sections 4.4, 5.3, 5.6, respectively) has provided a set of probes which enabled an epidemiological survey of a collection of 76 *Vibrio* strains including laboratory, clinical and environmental isolates to be carried out. When this collection was first examined for the incidence of plasmids, a low rate (12%; 5/39) of plasmid carriage in the 01 strains was observed consistent with previous reports (Hedges and Jacob, 1975; Prescott *et al.*, 1968; Hedges *et al.*, 1977). However, 47% (9/19) of environmental isolates of *V. cholerae* non-01 strains harboured plasmids, mostly within the range of 5 to 20 kb. Newland *et al.* (1984b) noted that both clinical and environmental isolates of *V. cholerae* 01 had a much lower frequency of plasmid carriage (2/112 = 2%) than clinical and environmental *V. cholerae* non-01 isolates (47/187 = 25%). The plasmids isolated by Newland *et al.* (1984b) from the non-01 strains were all small (3-6kb) as seen with the Queensland *V. cholerae* non-01 strains examined in this study (Chapter 6).

The isolation of plasmids from other *Vibrio* species has been reported; *V. parahaemolyticus* (Guerry and Colwell, 1977), *V. anguillarum* (Crosa, 1980) and *V. vulnificus* (Davidson and Oliver,

1986). Generally, the phenotypic traits that these plasmids encode have not been determined, although one plasmid, pJM1 has been shown to mediate an iron sequestering system in *V. anguillarum* (Crosa, 1980). It appears that the scp remains cryptic like other small plasmids isolated in different species: *Neisseria gonorrhoreae* (Korch *et al.*, 1985) and *Bacteriodes spp.* (Callihan *et al.*, 1983).

The scp (4.7 kb) was detected by colony hybridization in five clinical isolates whereas the lcp was not detected in any of the strains (Chapter 6). The P factor was only detected by Southern hybridization in one clinical isolate of *V. cholerae* 01 (AA14041, a Classical strain) and could be detected by the lacunae assay. None of the other plasmids detected in this strain collection showed any homology to the P factor. Large molecular weight plasmids are not often reported to have been isolated from *Vibrio* species, however, Davidson and Oliver (1986) detected plasmids up to 150 MDal (225kb) in some unidentified lactose-fermenting *Vibrio spp.*.

8.9 Concluding remarks.

This study has reported a detailed analysis of the plasmids of *V. cholerae* 01 strain V58. Previous studies of the P factor have been limited only to a functional use as a conjugative plasmid capable of transferring chromosomal markers, facilitating the construction of genetic maps of both biotypes.

A more detailed analysis in this study has demonstrated the presence of an exclusion system and possibly a single region involved in transfer functions. An additional function of the P factor, that of suppression of virulence, has been confirmed and shown to be due to an inability to colonize the small intestine, in part due to poorer adherence by P^+ strains.

Investigation of roles played by the other two plasmids have been unsuccessful, however, many commonly encoded plasmid functions have been eliminated.

Further examination of the *tra* region of P and comparisons with those of other plasmids as well as the identification of the region involved in the suppression of virulence of *V. cholerae* are areas which should continue to be analysed.

The development of a shuttle cloning vector using the scp would have the advantage of being able to introduce various genes into *V. cholerae* on a low copy number stable plasmid.

Continued analysis of the lcp (V) would be of interest, in view that it seems to be a particularly compact conjugative plasmid, suggesting a less complex conjugational system.

APPENDIX

Publications:

Material contained in this thesis has already been published in the following papers:

Bartowsky, E.J., Morelli, G., Kamke, M. and Manning, P.A. (1987) Characterization and restriction analysis of the P sex factor and the cryptic plasmid of *Vibrio cholerae* strain V58. *Plasmid* 18: 1-7.

Bartowsky, E.J. and Manning, P.A. (1988) Molecular cloning of the plasmids of *Vibrio cholerae* 01 and the incidence of related plasmids in clinical isolates and other *Vibrio* species. *FEMS Microbiol. Lett.* 50: 183-190.

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