Haemagglutinins of *Vibrio cholerae*: Molecular Characterization of the Mannose-Fucose Resistant Haemagglutinin (MFRHA).

Vicki L. Franzon.

Department of Microbiology and Immunology
University of Adelaide,
Adelaide, 5000
Australia

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For my Mother and Father, whom I love very much. Thank you both.

Abstract

The disease cholera is caused by *V. cholerae* of the O1 serotype. In contrast to organisms such as *Shigella* and *Salmonella*, *V. cholerae* is a non-invasive pathogen. It has been recognized that one of the most essential steps in the onset of cholera is the colonization of the small intestine. Hence considerable interest has been shown in identifying which factors may act as adhesins in the attachment of these organisms. Since 1961, when Bales and Lankford suggested that interaction between *V. cholerae* and red blood cells mimics that of the organism with the intestinal epithelium, a number of workers have become interested in the various haemagglutinins of *V. cholerae* and their properties. Hanne and Finkelstein (1982) have described four distinct haemagglutinins. One of these haemagglutinins is termed the mannose-fucose resistant haemagglutinin (MFRHA) and is found in all *V. cholerae* strains regardless of biotype. The general aim of this thesis is to report the first cloning, sequencing and detailed analysis of a gene encoding one of the *V. cholerae* haemagglutinins and to give some indication of whether the MFRHA protein may play a role in pathogenesis.

Chapter 3 describes the cloning and isolation of the MFRHA gene, characterization of its properties, localization of the coding region to within a 0.72 kb region and identification of the protein products using minicell analysis. The MFRHA gene was isolated from both biotypes and was shown to be identical. Chapter 4 analyzes the genetic organization of the MFRHA gene. This included sequencing of a 1,398 bp segment of *V. cholerae* DNA. Chapter 5 describes the construction of a deletion mutation in the MFRHA gene followed by insertion of an antibiotic marker and introduction of such a mutation into the *V .cholerae* chromosome.

Research of other Gram-negative pathogens suggests haemagglutinins are likely candidates for adhesins. Due to the number of *V. cholerae* haemagglutinins and the lack of characterization, one can only analyze their contribution by cloning the genes and introduction of specific mutations into the chromosome.

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This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of the my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to the thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Vicki Franzon

Abbreviations

A adenine

a.a. amino acid

Ab antibody

Ap ampicillin

BBA brush border adhesin

BHI brain heart infusion

bp base or nucleotide pair

BSA bovine serum albumin

C cytosine

CB colonization broth

CFA colonization factor agar

cha chicken erytrocyte haemagglutinin

Cm chloramphenicol

cpm counts per minute

CT cholera toxin

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dNTP deoxyribonucleoside triphosphate

ddNTP dideoxyribonucleoside triphosphate

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

EM electron microscopy

EtBr ethidium bromide

FAGLA furyacryloyl-gly-leu-NH_2 FSHA fucose sensitive haemagglutinin

G guanine

GM_1 Galactosyl-N-acetyl-galactosaminyl-Sialosyl-Lactosyl ceramide

Gm gentamycin

HA haemagglutinin

Hly haemolysin

Ig immunoglobulin

IM inner membrane

IPTG isopropyl-β-D-thiogalactopyranoside

kb kilobase pairs (s) or 1,000 bp

kDal kilodalton

KDO 2-Keto-3-deoxy octonic acid

Km kanamycin

KRT Krebs Ringer buffer

LB luria broth

LPS lipopolysaccharide

LT heat labile toxin

MFRHA mannose-fucose resistant haemagglutinin

mRNA messenger ribonucleic acid

MSHA mannose sensitive haemagglutinin

NA nutrient agar

NAG non-agglutinable

NB nutrient broth

NTG nitrosoguanidine

OD optical density

ORF open reading frame

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PEG polyethylene glycol-6000

perosamine 4-amino-4, 6-dideoxy-D-mannose

pmx polymyxin

quinovosamine 2-amino-2, 6-dideoxy-D-glucose

^{R} resistant

RBC red blood cell

RF replicative form

Rif rifampicin

RNA ribonucleic acid

rpm revolutions per minute

^{S} sensitive

SA slime agglutinin

SD Shine-Delgarno

SDS sodium dodecyl sulphate

SEM scanning electron microscope SHA soluble haemagglutinin

Sm streptomycin

Spc spectomycin

T thymine

Tc tetracycline

TEMED N,N,N′,N′-tetramethyl-ethylene-diamine

Tn transposon

Tris Tris (hydroxymethyl) aminomethane

ts temperature sensitive

TSB trypticase soy broth

U uracil

UV ultraviolet

v/v volume per volume

w/v weight per volume

X-gal N,N′-dimethyl formamide

Zincov 2-(N-hydroxycarboxamido)-4-methyl pentanoyl-L-ala-gly-NH_2

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Chapter 1

Review of the literature

1.1 Introduction

Diarrhoeal diseases constitute a significant health problem throughout the world and are responsible for several million fatalities each year (Aschcroft, 1964; Goodgame and Greenough, 1975; Levine and Edelman, 1979; Edelman and Levine, 1980; Black *et al.* 1981, 1982). In particular they are the major cause of mortality in young children and infants living in developing countries (Goodgame and Greenough, 1975; Mata, 1978; Levine, 1980; Black *et al.* 1982), as well as presenting a risk to the population of developed countries as travellers' diarrhoea (Shore *et al.* 1974; Gorbach *et al.* 1975; Dupont *et al.* 1976; Merson *et al.* 1976; Echevarria *et al.* 1981) and food-borne epidemics (Mata, 1978).

The major bacterial pathogens are:

- Enterotoxigenic *Escherichia coli* (ETEC)
- *Vibrio cholerae* 01
- Enteropathogenic *Escherichia coli* (EPEC)
- *Shigella* spp.
- *Campylobacter* spp.

- *Salmonella typhi*
- Nontyphoidal *Salmonella* spp.

(Friedman, 1978; Levine *et al.* 1983) These organisms can be grouped according to their degree of invasiveness.

ETEC and *V. cholerae* adhere to the gut mucosa but do not invade or destroy the brush border. They produce enterotoxins which bind to receptors initiating a cascade of events (Sprinz, 1969; Dupont *et al.* 1971; Carpenter, 1972; Norris, 1974; Levine, 1981; Levine, 1987).

EPEC organisms adhere to the mucosa but cause dissolution of the brush border (Ewing *et al.* 1957; Neter, 1959; Ulshen and Rollo, 1980; Rothbaum *et al.* 1982; Edelman and Levine, 1983; Levine, 1987).

Shigella species invade the mucosa and multiply within epithelial cells (Takeuchi *et al.* 1965; Takeuchi, 1967, 1971; Takeuchi *et al* 1968; Formal *et al.* 1971; Levine, 1982).

Some *Salmonella* species and *Campylobacter jejuni* cross the mucosa, invade the lamina propria and proliferate in the mesenteric lymph nodes (Takeuchi *et al.* 1965; Kent *et al.* 1966a, 1966b; Sprinz, 1966, 1969; Takeuchi, 1971).

Salmonella typhi invades the mucosa but, although it is ingested by macrophages, it is still capable of intracellular growth which results in a generalized infection (Edsall *et al.* 1960; Sprinz *et al.* 1966; Gaines *et al.* 1968).

V. cholerae 01 is responsible for endemic and epidemic cholera. The disease is spread by the faecal-oral route usually by ingestion of water which is contaminated with human faeces or by direct contact with infected individuals and food (Dutt *et al.* 1971; Levine and Nalin, 1976; Blake *et al.* 1977, 1980; Merson *et al.* 1977; McIntyre *et al.* 1979; Salmaso *et al.* 1980; Hughes *et al.* 1982). Although it seems that this highly contagious disease could be eradicated by improvement in sanitary conditions, there is little prospect of this happening in the near future in many highly populated parts of developing countries. Hence an effective cholera vaccine for the population of endemic areas is still a major goal of the World Health

Organisation (Friedman, 1978).

1.2 History of Cholera

Cholera has been endemic in India and the Ganges Basin for hundreds of years. Since the beginning of the nineteenth century, seven pandemics have occurred. The current seventh pandemic has spread from an initial focus, Indonesia in 1961 to The Far East, India, The Middle East, Africa and Europe (Cvjetanovic and Barua, 1972).

The aetiological agent of cholera was first described as *Vibrio cholerae* by Pacini, in 1854 (Hugh, 1964). However, his findings were viewed with some scepticism since a number of harmless vibrios could be isolated from a wide variety of environments (cited by Stephen and Pietrowski, 1983). In 1883, Robert Koch, whilst studying outbreaks in Egypt and India, managed to culture the organism and subsequently clearly demonstrated that *V. cholerae* was indeed the causative agent (Koch, 1883, 1884-cited by Pollitzer, 1959). In 1884, John Snow highlighted the role water plays in the spread of cholera (Snow, 1884-cited by Gangarosa, 1971). A safe water supply is still considered a requirement in the control of the disease.

Although the state of knowledge was less extensive than at present, the first six pandemics of cholera are thought to have been caused by *V. cholerae* of the Classical biotype. The first pandemic originated in the Ganges River Delta and spread to Asia and Africa. Subsequent pandemics occurred with transmission along the trade and travel routes from India to America, Africa and Europe. The current seventh pandemic is due to the El Tor biotype. The name El Tor originated from the site of first isolation by Gotschlich in 1905. He cultured the organisms at the quarantine camp at El Tor, on the Sinai Peninsula, from pilgrims returning from Mecca (Gotschlich, 1905, 1906-cited by Pollitzer 1959; Levine, 1980). At that time, El Tor vibrios differed from the Classical biotype and were believed to be incapable of causing cholera. This was purely a chance finding based on the observation that the pilgrims had shown no post-mortem evidence of cholera. This organism was

also found to be haemolytic (Cvjetanovic and Barua, 1972).

El Tor vibrios were thought to be non-pathogenic for man, until the discovery that the organism was responsible for a cholera outbreak on the islands of Sulawezi (Celebes) in Indonesia in 1938 (de Moor, 1938-cited by Kamal, 1974). The El Tor vibrios caused several localized outbreaks in the Indonesian archipelago during the 1940's and 1950's (Cvjetanovic and Barua, 1972), but it was thought that, although virulent, they lacked the potential to cause pandemics. However, in 1961, the seventh and current pandemic began and organisms of the El Tor biotype were responsible (Kamal, 1974). Besides spreading throughout Asia, Africa and the Middle East, this pandemic has also touched several developed countries, USSR, Italy, Portugal and the USA (Kamal, 1974; Stock, 1976; Blake, 1981).

It seems that between the early 1960's and 1982, the El Tor biotype was responsible for cholera virtually to the exclusion of the Classical biotype (Kamal, 1974; Goodgame and Greenough, 1975; Sundaram *et al.* 1980; Levine *et al.* 1983). However, in 1982 an investigation in Bangladesh showed the reappearance of the Classical biotype (Samadi *et al.* 1983). Whether the Classical biotype organism will go on to displace the El Tor biotype outside of the Ganges River Delta, remains to be seen.

1.3 The Aetiological Agent

The genus Vibrio belongs to the family *Vibrionaceae* (Bauman and Schubert, 1984). The family also includes the genera *Aeromonas, Plesiomonas, Photobacterium* and *Lucibacterium* (Shewan and Veron, 1975). As stated above, *V. cholerae* 01 is the causative agent of cholera. It is a Gram-negative single cell curved rod, usually 0.3-0.4 μm wide and 1.5-2.0 μm long having a single polar sheathed flagellum. Vibrios are aerobic or facultatively anaerobic, non-spore forming, indophenol, oxidase and catalase positive and capable of fermenting glucose without the formation of gas (Davis *et al.* 1980). *V. cholerae* grows profusely in media that is usually too alkaline (pH 9.0 to pH 9.6) for the growth of other bacteria, but is sensitive to acid.

1.4 Biotype Differentiation

Cholera vibrios belong to 0-subgroup 1, as defined by Gardner and Venkatraman (1935). *V. cholerae* 01 can further be divided into two biotypes, Classical and El Tor (Bauman *et al.* 1984).

Initially the biotypes were distinguished by the haemolytic capacity of the El Tor vibrios but there exists considerable variability in this property. A number of other more reliable differentiating characteristics are now used. The El Tor biotype is resistant to the antibiotic polymyxin B whereas Classical vibrios are sensitive (Gan and Tjia, 1963; Roy *et al.* 1965; Gangarosa *et al.* 1967). El Tor vibrios are also resistant to the Group IV phages of Mukerjee (Mukerjee, 1963), give a positive reaction to the Voges-Proskauer test at 37°C but negative at 22°C (or very weakly positive) (Sen, 1969) and when grown on a solid medium, are able to agglutinate chicken red blood cells (RBCs), in contrast to Classical organisms which cannot (Finkelstein and Mukerjee, 1963).

1.5 Serotype Differentiation

V. cholerae possess both H antigens and 0 somatic antigens. H antigens refer to determinants which are heat labile (100°C for 2 hours) and are associated with the flagellum. The heat labile H antigen is common amongst all strains of *V. cholerae* (Gardner and Venkatraman, 1935; Sakazaki *et al.* 1970; Bhattacharyya and Mukerjee, 1974; Bhattacharyya, 1975).

The term O-antigen refers to the heat stable polysaccharide fraction of the endotoxic lipopolysaccharide, which determines the antigenic specificity. *V. cholerae* 01 strains of both El Tor and Classical biotypes can be further subdivided into three subtypes of O-antigen; Inaba, Ogawa and Hikojima. Differentiation of these subtypes is based on the presence of three antigenic factors designated A, B and C. Factors A, B and a small amount of C are found in serotype Ogawa, whereas Inaba possesses antigens A and C (Burrows *et al.* 1946; Sakazaki and Tamura,

1971; Redmond *et al.* 1973; Redmond, 1979). The third, rare, serotype subclass, Hikojima expresses the three antigenic factors A, B and C (Burrows *et al.* 1946) but this serotype has been found to be unstable and suggestions have been made that Hikojima strains may be segregating diploids (Bhaskaran, 1959).

There exists evidence for serotype conversion. Serotype changes were detected in a *V. cholerae* strain being excreted by a confined patient (Gangarosa *et al.* 1967). This observation was supported by experiments using germ free mice where progressive changes in the *V. cholerae* serotypes during infection were noticed (Sack and Miller, 1969). Sakazaki and Tamura (1971) have isolated serotype convertants by treating cultures with antiserum. Ogg and co-workers (1978, 1979) have suggested that bacteriophage CP-T1 could be responsible for serotype changes by means of a lysogenic conversion. However CP-T1 itself uses the O-antigen of the LPS as a receptor and Southern hybridizations using cloned CP-T1 DNA fragments showed no evidence of CP-T1 existing as a prophage suggesting this conclusion could be incorrect (Guidolin and Manning, 1988).

1.6 Pathogenesis

V. cholerae gains entry into the host by the ingestion of contaminated water or food. Unlike organisms such as *Shigella* and *Salmonella*, *V. cholerae* does not normally produce detectable morphological or structural damage to the small bowel, invade the epithelium or cause histopathological lesions (Fresh *et al.* 1964; Sprinz, 1969; Ghosh, 1970; Carpenter, 1972; Norris, 1974; Levine, 1981). Once the organism is ingested, it must overcome gastric acidity (Hornick *et al.* 1971; Cash *et al.* 1974; Nalin *et al.* 1978; Levine *et al.* 1981). Approximately 10^8 organisms are usually needed for infection to occur in human volunteers, but, if stomach acidity is neutralized by sodium bicarbonate, the minimum infectious dose can be lowered to 10^4 organisms or less (Davis *et al.* 1980). Motility is then directed in response to certain chemotactic factors. Substances which can act as chemotactic attractants include a number of L-amino acids, monosaccharides and pepsin digested mucosal

extracts (Freter and O'Brien, 1981a; 1981b; Freter *et al.* 1981a; 1981b). The intestinal mucus can also act as a nutritional source with *V. cholerae* producing a mucinase and a DNase which are able to digest mucin and DNA contained in the mucus layer respectively.

For successful colonization, the vibrios must then adhere to the mucosal epithelium. The colonization process is important for a number of reasons:

- vibrios must compete with the normal flora found in the small intestine (Shinefield *et al.* 1972; Freter, 1974; Ofek and Beachey, 1980).
- they must be able to resist being flushed from the bowel by gut peristalsis (Florey, 1933; Dixon, 1960).
- the organism needs to multiply (Schrank and Verwey, 1976).
- close association with the gut facilitates the liberation of the toxin in close proximity to receptors and optimizes delivery (Peterson *et al.* 1972).

Since cholera is still prevalent thoughout the world, particularly in developing countries, serious attention is being given by health organizations to the development of a vaccine. There are a number of approaches which can be taken to combat the disease. Since colonization of the intestinal epithelium and release and action on target sites of the enterotoxin are two important and essential steps for the manifestation of the disease, considerable effort has been given to understanding both of these processes.

1.7 Cholera Toxin (CT)

Initially, it was not readily accepted that an enterotoxin was responsible for the symptoms of cholera until De, in 1959, showed that injection of sterile culture filtrates into ligated segments of rabbit ileal loops, *in situ*, lead to rapid fluid accumulation in the lumen. The toxin molecule and its mode of action are now the best studied aspect of the disease.

CT has been purified and characterized (Finkelstein and Lospalluto, 1970; Finkelstein, 1972). This has enabled the role of the toxin in the pathogensis of the disease to be studied (Finkelstein, 1969, 1972; Pierce *et al.* 1971; Carpenter *et al.* 1974) and several reviews detail structure, function and biological activity (Field, 1979; Finkelstein, 1973; Van Heyningen, 1977; Holmgren, 1981). All pathogenic strains of *V. cholerae* 01 seem to produce immunologically identical toxins (Evans and Richardson, 1968; Finkelstein, 1969; Holmgren *et al.* 1971). The enterotoxin is a heat labile molecule with an appproximate size of 85 kDal. It is multimeric, being composed of two types of subunits; one A subunit which when cleaved, gives fragments A_1 (22 kDal) and A_2 (5 kDal),and five B subunits (11.6 kDal) (LoSpalluto and Finkelstein, 1972). This has been confirmed by the use of a lipid-layer crystallization technique for imaging B oligomers (Uzgiris and Kornberg, 1983). This revealed a ring of five protein densities with the central hole containing the A subunit (Ludwig *et al.* 1986).

1.7.1 Mode of Action of CT

The A_2 component of CT is thought to be involved in linking the A_1 subunit to the B subunits in the proper tertiary configuration. The B subunits mediate the binding of the toxin to the cell surface receptor, ganglioside GM_1 (Galactosyl-N-acetyl-galactosaminyl-Sialosyl-Lactosyl ceramide) (Cuatrecasas, 1973a, 1973b). As a result of binding, a confirmational change occurs allowing the A_1 fragment to enter which irreversibly activates the adenylate cyclase system (Kimberg *et al.* 1971; Bennet and Cuatrecasas, 1975; Moss and Vaughan, 1977, 1979; Cassel and Pfeuffer, 1978; Kassis *et al.* 1982). The mechansim of penetration of A_1 is not understood. One favoured hypothesis is that the B subunits unfold to create a hydrophilic channel through which the A subunit diffuses, followed by reduction to release the A_1 fragment (Gill, 1976).

The B subunits alone are non-toxic and contain no adenylate cyclase stimulating activity. The A subunit must be nicked to be enzymatically active and toxic (Mekalanos *et al.* 1979a; Pearson and Mekalanos, 1982). It is thought that the

soluble HA/protease may be an endogenous nickase providing *V. cholerae* with an in built mechanism for toxin activation (Booth *et al.* 1984).

Avian erythrocytes have been used in studying the stimulation of adenylate cyclase by cholera toxin. Cholera toxin has ADP-ribosyltransferase activity and catalyzes the reaction:

NAD^+ + acceptor protein $\longrightarrow$ ADP-ribose-acceptor protein + nicotinamide + H^+

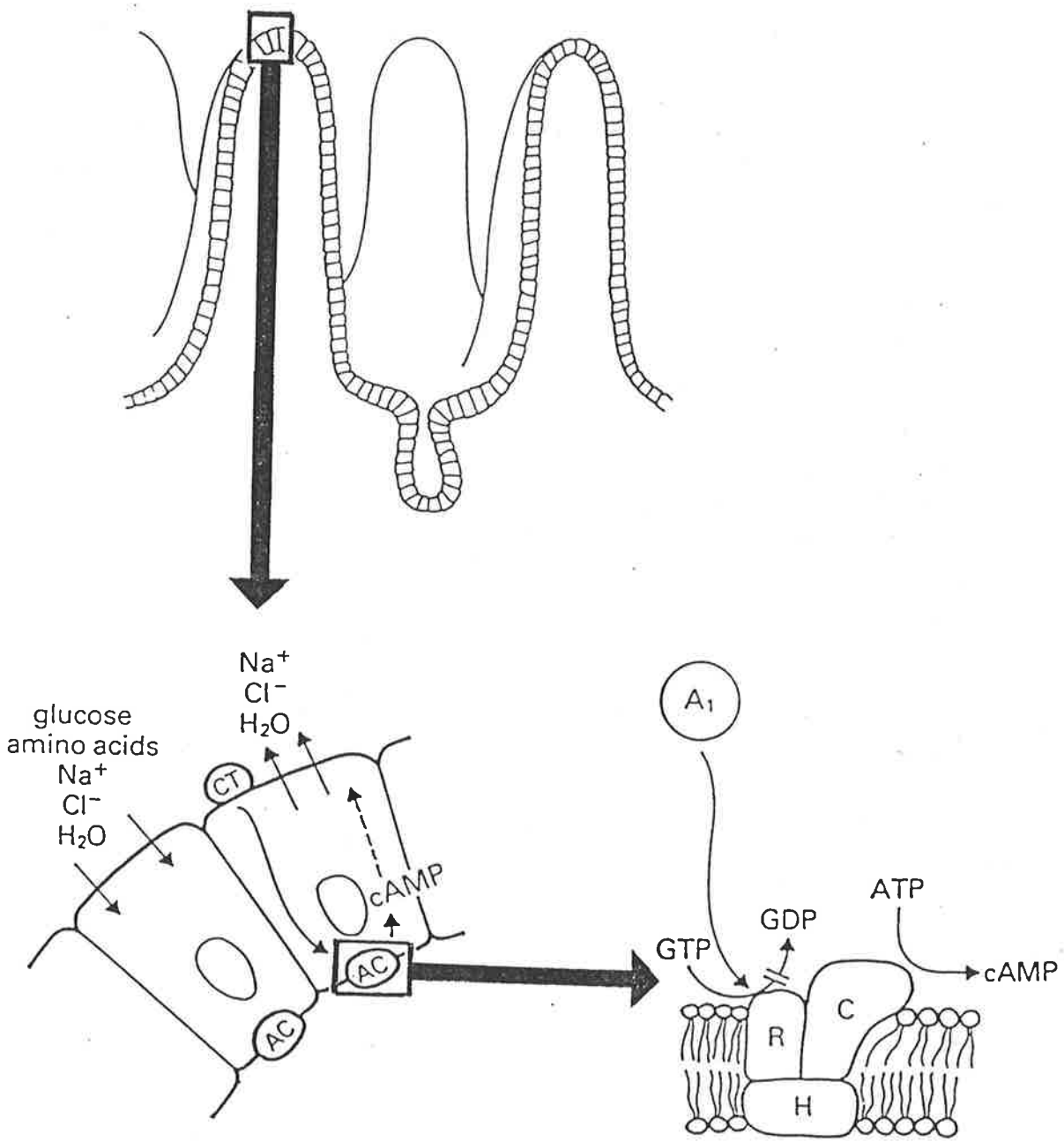
The A_1 fragment catalyzes the transfer of the ADP-ribosyl moiety from NAD^+ to the guanyl nucleotide (GTP) binding component of the adenylate cyclase complex (Cassel and Pfeuffer, 1978). The membrane bound proteins, which are ADP-ribosylated, have been shown in the pigeon erythrocyte system to have sizes of between 42-43 kDal. When ADP-ribose is transferred to the GTP binding protein, it locks the adenylate cyclase in an active form. This is due to inhibition of the regulatory feedback mechanism. The activation is normally turned off by hydrolysis of GTP to give the inactive adenylate-cyclase-GDP complex plus inorganic phospate. Cholera toxin prevents this GTPase turn-off reaction, thereby inhibiting deactivation of the stimulated adenylcyclase complex (Figure 1.2).

Thus intracellular levels of cAMP accumulate which, in turn, cause an efflux of HCO^-, Na^+, K^+ and Cl^- ions by the crypt cells and hence also water, as well as simultanteously decreasing absorption of coupled Na^+ and Cl^- ions by the villus cells. Hence diarrhoea results which, without appropriate therapy, can lead to rapid dehyrdation, renal shut down, shock and finally death. The loss of fluid via the gut may reach 10 to 15 litres per day.

1.7.2 Genetics of Cholera Toxin

Not only has the cholera toxin been extensively studied at a biological level but of all the studied *V. cholerae* genes, those relating to cholera toxin synthesis and

Figure 1.1 Mechanism by which cholera toxin causes diarrhoea. Binding of toxin to receptors on the lumen surface of ileal mucosal cells is followed by entry of fragment A_1, which interacts with the adenylate cyclase complex on the basal membrane inhibiting the GTPase-mediated turn-off cyclase (probably by ADP-ribosylation of the GTP-dependent regulator protein). Increased intracellular cyclic AMP levels cause, by some as yet unknown mechanism, efflux of Na^+ and Cl^- ions and hence also water (Stephen and Pietrowski, 1983).


glucose
amino acids
Na+
Cl−
H2O
Na+
Cl−
H2O
CT
cAMP
AC
AC
A1
GTP
GDP
ATP
cAMP
R
C
H

regulation have received the most attention. The *V. cholerae* enterotoxin genes have been cloned from a number of different strains in a number of laboratories (Kaper and Levine, 1981; Pearson and Mekalanos, 1982; Mekalanos, 1983, Mekalanos *et al.* 1983; Kaper *et al.* 1984a, 1984b; Kaper *et al.* 1985).

Cloning of the genes encoding CT was achieved by using hybridzation probes derived from the A and B subunit genes of the heat labile enterotoxin (LT) of *E. coli*, since there exists a high degree of homology between the DNA sequences of the CT and LT genes (Moseley and Falkow, 1980; Spicer *et al.* 1981; Spicer and Noble, 1982; Mekalanos *et al.* 1983). The nucleotide sequence of the CT genesy demonstrates that *ctxA* and *ctxB* form an operon (Lockman and Kaper, 1983; Mekalanos *et al.* 1983; Lockman *et al.* 1984).

Classical strains contain two widely separated copies of the *ctxAB* operon (Pearson and Mekalanos, 1982; Mekalanos, 1983). Originally, all El Tor strains were thought to contain only a single copy of this operon (Mosely and Falkow, 1980; Mekalanos, 1983), but it has subsequently been demonstrated that some El Tor strains carry multiple copies arranged on tandem repeats which are either 7 or 9.7 kb in length. The existence of a 2.7 kb repetitive sequence (*RS*1) was identified. *RS*1 is found at the junction of *ctxAB* tandem duplications (Mekalanos, 1983; Mekalanos, 1985). Differences in the copy number of the *RS*1 was shown to effect the size of the large tandem duplications and would also seem to be responsible for the amplication of *ctx* during intestinal passage in animals, giving rise to the hypertoxigenic phenotype of El Tor variants (Mekalanos, 1983). Duplication and amplification of *ctx* involves recombination events which are *recA*-dependent (Goldberg and Mekalanos, 1986a, 1986b). *RS*1 has been shown to mediate cointegrate formation and possibly transposition of *RS*1 may occur(Betley *et al.* 1986).

It was noted that the total amount of cholera toxin produced in *E.coli* was only 4% the amount made by *V. cholerae* (Pearson and Mekalanos, 1982). Consequently, it was thought *E.coli* may lack some positive control element produced by *V. cholerae* which promotes the high expression of the toxin genes (Mekalanos and Murphy, 1980). This positive regulatory gene called *toxR* has been cloned from

V. cholerae and has been found to increase expression of *ctx* in *E.coli* (Miller and Mekalanos, 1984). The nucleotide sequence of *toxR* has been determined and the product has been identified as a 32.527 kDal transmembrane regulatory protein (Miller *et al.* 1987). This protein binds to the sequence TTTTGAT (present as several tandem repeats) upstream from the *ctx* promoter and activates transcription.

Southern hybridization analysis has shown that the *toxR* gene is present in all *V. cholerae* isolates including those non-toxigenic strains lacking the *ctx* operon. This leads to the possibility of *toxR* regulating the expression of other genes. In fact, *toxR* is required for the expression of a pilus colonization factor which is involved in the colonization of the intestine (Taylor *et al.* 1987a, 1987b). It also affects expression of the *ompU* gene which encodes a 38 kDal outer membrane protein (Miller, 1985-cited by Taylor *et al.* 1987a). In fact, recent analysis has shown that approximately 12 different transcriptional units are *toxR* regulated (Taylor *et al.* 1988b).

A second regulatory gene designated *toxS* has been identified (Taylor *et al.* 1988a). This gene encodes a periplasmic protein which interacts with the carboxy-terminus of the membrane inserted ToxR protein. This interaction is thought to lead to dimerization of ToxR which enables it to then bind to the DNA regions it regulates. It has been suggested that the combination of ToxR and ToxS, acts as an enviromental sensor and then exerts the appropriate changes in virulence gene expression (Figure 1.3).

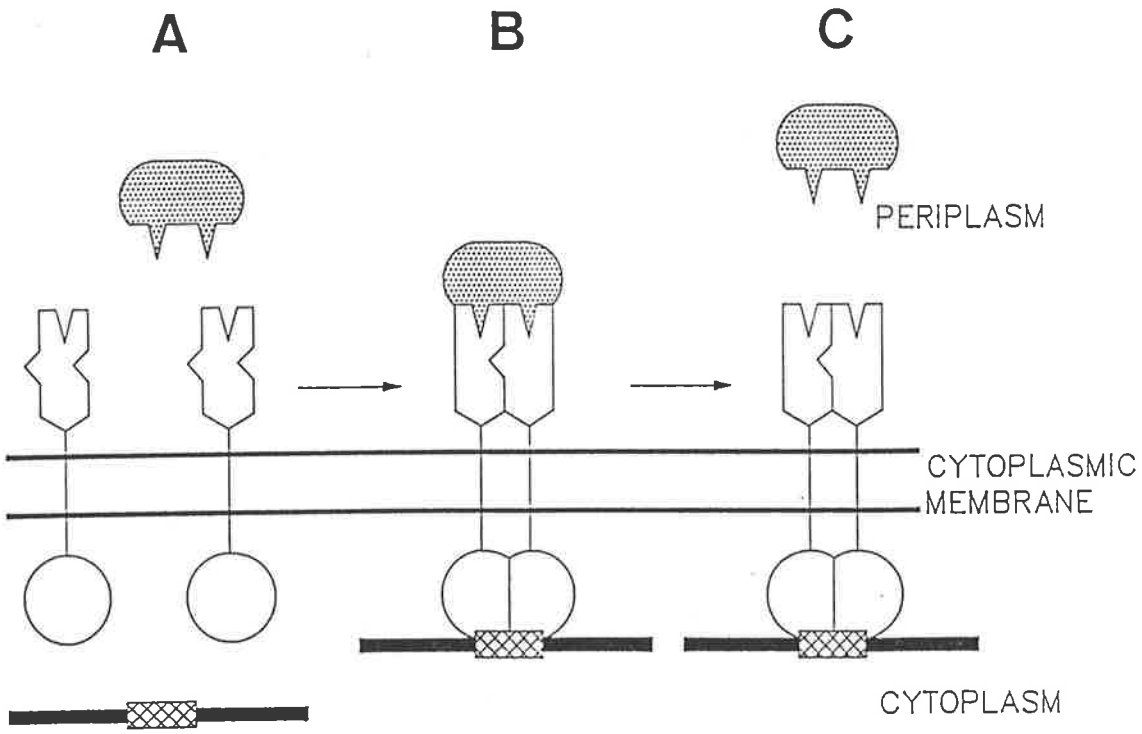
The *ctx* operon of the El Tor biotype has been mapped and localized to between the *nal* and *his* genes on the *V. cholerae* chromosome (Newland *et al.* 1984; Sporecke *et al.* 1984). Since all Classical strains have two copies, mapping has proven more difficult. One copy has been mapped to the same position as the El Tor *ctxAB* operon, but the location of the second copy is unknown. The *toxR* gene can be mapped to the *his* region of the chromosome but on the opposite side to *ctxAB* (Vasil *et al.* 1975; Baine *et al.* 1978).

Figure 1.2 Model of ToxR and ToxS interactions (from Manning, 1988, based on Taylor *et al.* 1988b).

A A molecule of the periplasmic ToxS protein (shaded) is able to interact with the periplasmic exposed side of the cytoplasmic membrane associated ToxR protein.

B This leads to dimerization of ToxR which enables the cytoplasmic exposed side of ToxR to recognize its DNA binding site (cross-hatched region) resulting in activation (or suppression?) of the appropriate genes.

C Once dimerization of ToxR has occurred, the complex becomes ToxS independent.


A
B
C
PERIPLASM
CYTOPLASMIC
MEMBRANE
CYTOPLASM

1.7.3 Antitoxic Immunity

Cholera toxin is highly immunogenic with nearly all the antibodies directed against the B subunit (Peterson *et al.* 1979; Svennerholm, 1980; Holmgren, 1981). Ninety percent of North American volunteers developed rises in their serum IgG antitoxin after an experimental cholera infection. Serum IgG antitoxin levels are elevated for a long duration, detectable at least two years post immunization (Levine *et al.* 1977; Robins-Brown *et al.* 1980; Young *et al.* 1980). Hence a number of workers have attempted to produce *V. cholerae* strains which have altered toxin production (Howard, 1971; Finkelstein *et al.* 1974; Holmes *et al.* 1975; Mekalanos *et al.* 1978; Ruch *et al.* 1978; Baselski *et al.* 1979; Honda and Finkelstein, 1979 ; Mekalanos *et al.* 1979b; Mekalanos and Murphy, 1980; Mekalanos *et al.* 1983; Kaper *et al.* 1984a, 1985b).

Honda and Finkelstein (1979) isolated by NTG mutagenesis a mutant which was defective in the production of A but still produced the B subunit. This A^- B^+ mutant was designated Texas Star-SR and offered substantial hope for an attenuated cholera vaccine, since it lacks the A subunit which is toxic and retains the B subunit which is highly immunogenic but alone has no toxic effect. However, when this strain was orally administered to stimulate a specific secretory IgA response, it was found to have a number of associated disadvantages. Due to the method of isolation, repeated NTG mutagenesis, multiple mutations exist which are ill-defined. The precise location of the gene or genes which are reponsible for the avirulence of Texas Star-SR is not known. Thus, theoretically, reversion to virulence could occur. In studies using Texas Star-SR, it was shown that mild diarrhoea was experienced in a significant proportion of volunteers. This has been suggested to be due to a shiga-like toxin which is still being produced (Kaper *et al.* 1985; O'Brien and Holmes, 1987).

In the rabbit infant model, results with cholera toxin deleted *V. cholerae* mutants have implied that cholera toxin production may, in fact, stimulate the *V. cholerae* colonization process of the intestine (Mekalanos *et al.* 1985; Pierce *et al.* 1985).

1.8 Adhesion

To inhibit adherence and subsequent colonization as a means of preventing the disease, it is first necessary to identify the "adhesins" involved. Once indentified, antibodies to these molecules can be assessed for their protective ability. One potential use for this information is that the genes encoding for the adhesins may be able to be cloned and expressed in carrier strains, such as the live oral typhoid vaccine strain, *Salmonella typhi* Ty21a (Germanier and Fürer, 1975; Wahdan *et al.* 1980). A number of workers have shown this approach to be feasible for a variety of antigens (Formal *et al.* 1981; Clements *et al.* 1984; Tramont *et al.* 1984; Stevenson and Manning *et al.* 1985; Dougan *et al.* 1986; Maskell *et al.* 1986). Formal *et al.* (1981) have introduced the *Shigella sonnei* Form 1 antigen genes into the *galE S.typhi* strain Ty21a, therefore creating a potential bivalent vaccine strain. Stevenson and Manning (1985) have introduced the cloned *K88*ab gene cluster from an enterotoxigenic *E.coli* into the avirulent *S. typhimurium galE* strain G30 and shown it to function as an efficient oral immunogen. *S. typhimurium aroA* mutants have also been used for delivery of heterologous antigens such as the *E.coli* heat labile enterotoxin B subunit (Maskell *et al.* 1987).

A variety of antigens have been suggested to play a role in *V. cholerae* adhesion. These include major outer membrane proteins (Kabir, 1980; Kelley and Parker, 1981; Kabir and Showkat, 1983), lipopolysaccharide (LPS) (Holmgren and Svennerholm, 1977; Chitnis *et al.* 1982), flagellar sheath proteins (Eubanks *et al.* 1977; Hranitzky *et al.* 1980; Attridge and Rowley 1983a, 1983b), fimbriae (Al-Kaissi and Mostratos, 1985; Ehara *et al.* 1986, 1987) and haemagglutinins (Jones *et al.* 1976; Jones and Freter, 1976; Chaicumpa and Atthasisiha, 1977; Bhattacharjee and Srivastava, 1978; Faris *et al.* 1982; Hanne and Finkelstein, 1982; Finkelstein *et al.* 1983; Holmgren *et al.* 1983; Kabir and Showkat, 1983).

The most extensive studies of adhesion by *V. cholerae* have been performed by Jones and Freter (Freter and Jones, 1976; Jones and Freter, 1976; Jones *et al.* 1976). Three *in vitro* systems were used; isolated rabbit brush border membranes,

slices of rabbit ileal intestinal mucosa and direct haemagglutination of erythrocytes.

Since 1961, when Bales and Lankford suggested that the interaction between *V. cholerae* and RBCs may mimic that of the organism with the intestinal epithelium, haemagglutination tests have been employed as a potentially simple means of studying adhesive properties. However, the relevance of the model is questionable due to a number of disadvantages, the simple cell surface of the erythrocytes is unlikely to reflect the more complex receptors present on the intestinal epithelial cells. Haemagglutinating activity depends greatly on the cultural conditions, such as growth media, temperature and the species of erythrocytes. The presence of several haemagglutinins can be overlooked by the masking of one major potent haemagglutinin. There are restrictions using *in vitro* models and the studies using the above three systems have often given conflicting results.

1.8.1 Studies with brush border membranes and RBC's

Jones *et al* (1976) studied the adhesion of *V. cholerae* Classical strain P using isolated brush border membranes obtained from rabbit small intestinal epithelial cells and human type O erythrocytes, as an indication of adhesive capactiy.

V. cholerae seems to penetrate the mucous layer and travel along tracks which offer least resistance to movement. After chance collision, the vibrios were shown to adhere to the microvillus surface but this adhesion was temperature dependent. In particular, adherence was unstable at 37°C. Maximum adherence was obtained after 15 minutes and then dissociation occurred, so that the number of vibrios remaining after 45 minutes of incubation were few. It is possible that the surface adhesin is turned over, since vibrios incubated in buffer for 45 minutes had a reduced capacity to adhere to fresh brush borders whereas vibrios in broth cultures retained adhesiveness at 37°C for up to 48 hours. Adhesion was greatest with hardly any dissociation at 22°C. Little or no dissociation occurred at 0-4°C. Haemagglutination had a similar temperature dependence as adhesion, giving maximum titres at 22°C (titre of 1:64), less activity at 37°C (titre of 1:16) and lowest titres at 4°C (titre of 1:4 or less).

These authors noticed that bacteria grown in broth cultures adhered to and haemagglutinated human RBCs whereas RBCs from rabbit, guinea pig, horse, chicken, sheep and cow gave minimal or negative reactions. Classical vibrios grown on agar medium, however, were non-adhesive and non-haemagglutinating. Both adhesion and haemagglutination seem to require the presence of calcium ions. Strontium ions could partially replace the calcium requirement for adhesion but had no effect on haemagglutination (Jones *et al.* 1976). Another study (Jones and Freter, 1976), again using the Classical strain P, shows adherence to brush borders to be inhibited by L-fucose and to a lesser extent, D-mannose but this inhibition was incomplete. The two sugars did not have an additive effect and so it is possible that D-mannose could form part of a L-fucose receptor was also revealed that L-fucose residues linked to an insoluble carrier such as agar beads, bound vibrios and this reaction was inhibited by L-fucose but not D-fucose, indicating some specificity of attachment. Vibrios did not adhere to untreated agar beads. Haemagglutination was also inhibited by L-fucose but not by D-mannose.

1.8.2 Studies with Intact Rabbit Intestinal Mucosa

The adhesion of *V. cholerae* to intact mucosal surfaces has been examined (Freter and Jones, 1976) and differs in a number of respects from the brush border membrane system. The most significant differences were that adhesion to intact tissue was not prevented by fucose or mannose, calcium ions were not required and adhesion to brush borders was transient, whereas adhesion to intestinal slices remained identical after 20 or 60 minute incubation. It was also found, perhaps surprisingly, that the soluble material from peptic digests of mucosal material inhibited vibrio attachment to both ileal slices and brush borders, as well as haemagglutination of erythrocytes. Only adherence to intestinal slices was prevented by antibodies to the somatic O-antigen of *V. cholerae* lipopolysaccharide. This occurred at subagglutinating bacterial concentrations, indicating that the antibodies are having a direct effect on adhesion, probably by masking attachment sites on the vibrios. It was also found that agar grown vibrios could still adhere to mucosal slices but had lost

their ability to adhere to brush borders.

Therefore, these workers suggest the possibility that there exists at least two specific mucosal receptors, one which is L-fucose sensitive and situated on the brush border epithelium, the other is a fucose resistant receptor whose location on the intact mucosa is unknown. The comparative adhesive properties of *V. cholerae* in the three *in vitro* attachment model systems are summarized in Table 1.1.

1.9 Slime envelope or Slime Agglutinin (SA)

A number of workers have observed that some *V. cholerae* produce a substance referred to as a slime envelope or slime agglutinin (SA). Bales and Lankford (1961) first demonstrated the existence of a slime envelope by staining with Indian ink, but were unsure whether this was responsible for the haemagglutinating activity of the cultures in use. Chulasamaya and Lankford (1970) extended this observation and showed staining of the slime envelope with Leifson's flagella stain but not by African blue or periodate Schiff stains. Electron microscopy of formalin fixed vibrios stained with phosphotungstic acid revealed irregular stained areas surrounding the vibrios of both biotypes. Tweedy *et al.* (1968) have examined the slime envelope of non-cholera vibrios and found it to be a network of strands unlike fimbriae. Chulasamaya and Lankford (1970) demonstrated that removal of the slime envelope gave a simultaneous loss of a thermostable haemagglutinin.

More recently, Attridge and Rowley (1983a) suggested that the properties of the brush border adhesin are similar to those which they described for the SA. It was found that both the SA and brush border adhesin (BBA) are not produced by organisms grown on agar plates. Both exhibited haemagglutination, as well as being denatured at 37°C. The SA was expressed by vibrios grown in nutrient broth at 37°C but not when grown in nutrient broth at 25°C or trypticase soy broth (TSB) at either 25°C or 37°C. Attachment has been studied using a number of substrates with both mouse mucosal surfaces as well as mouse serosal surfaces.

In addition, a variety of inert particles such as glass Ballotini beads, polyvinyl

chloride Pevikon beads, polystryrene Dowex particles and guinea pig erythrocytes were included. These substrates were used to study the specificity of binding. Also included in the study were motile Fla^+ and non-motile Fla^- variants. The Fla^+ strains of both Classical and El Tor strains adhered to the mouse intestinal mucosal surface. Whereas only the El Tor vibrios attached to the serosal surfaces (Classical vibrios adhered weakly).

From their results, it was postulated that the SA is the same as the BBA. The SA/BBA mediates haemagglutination and non-specific binding to a number of various surfaces (Table 1.1).

Attridge and Rowley (1983a) also studied the role the flagellar adhesin might play using motile and non-motile variants. The flagellar adhesin is suggested to be solely responsible for adherence to rabbit ileal slices and mouse mucosal surfaces since conditions which denature the SA do not affect attachment to these substances.

However, it is apparent that varying culture conditions, the SA and the specificity or non-specificity of adhesion under different situations, presents a very complex problem. This can perhaps best be solved by identifying and selectively eliminating the various individual "adhesins" by specific mutation. The relationship between the adhesin and cell surface receptor also needs to be examined.

1.10 Lipopolysaccharide (LPS)

1.10.1 Structure

Lipopolysaccharides of many Gram-negative bacteria including *V. cholerae* conform to a common structural principle. They consist of a heteropolysaccharide (which in turn is divided into the core region and the O-specific sidechains) which is covalently bound to a hydrophobic lipid portion, the lipid A (Lüderitz *et al.* 1966; Lüderitz *et al.* 1971). Lipid A is the endotoxic component (Gmeiner *et al.* 1971; Galanos *et al.* 1972; Lüderitz *et al.* 1973) whereas the polysaccharide moiety is responsible for serological specificity (Lüderitz *et al.* 1971). Numerous studies on the composition

Table 1.1 Comparitive adhesive and haemagglutinating properties of *Vibrio cholerae* in three *in vitro* attachment model systems (adapted from Freter and Jones, 1976).

Characteristic	Adhesion to Rabbit Intestinal Brush Borders	Haemagglutination of Human Erythrocytes	Attachment to Rabbit Ileal Intestinal Mucosa
Bacterial phenotype/genotype:			
agar-grown (motile)	−	−	+
broth culture (motile)	+	+	+
non-motile mutants (flagella$^-$)	−	−	−
motile revertants	+	+	+
Spontaneous elution of attached broth culture vibrios	+	+	−
Attachment in the absence of added calcium ions	−	−	+
Inhibition of attachment:			
by L-fucose	+	+	−
by D-mannose	±	−	−
by anti-vibrio O-antibody	−	−	+
by intestinal mucosal extract (pepsin digest) after incubation in buffer at 37^{0}C	+ −	+ ±	+ +

\+ activity present;

− activity absent or inhibited;

± partial inhibition of activity

of the LPS of *V. cholerae* have been published (Jann *et al.* 1973; Raziuddin, 1977; Redmond, 1978, 1979; Hisatune and Kondo, 1980; Raziuddin, 1980; Kabir, 1982; Brade, 1985), however, the exact structure is unknown.

Lipid A is normally linked to the polysaccharide moiety via a ketosidic bond from 2-Keto-3-deoxy octonic acid (KDO) (Lüderitz *et al.* 1966, 1971). The outstanding difference between the LPS of *V. cholerae* and the LPS of most Gram-negative organisms is the lack of the usual dissacharide of KDO. (Jann *et al.* 1973; Redmond *et al.* 1973; Westphal, 1975; Brade, 1985). The single phosphorylated KDO residue present is much more resistant to acid hydrolysis (Brade, 1985). It was thought that, in *V. cholerae*, fructose may have replaced the second KDO acid unit and consequently, the link between lipid A and the core may be a fructo-furanoside residue (Jann *et al.* 1973). However, more recently Kaca *et al* (1986) showed that, upon periodate oxidation, fructose was destroyed indicating that fructose does not link the core and lipid A regions. These workers postulate that it maybe a KDO-phosphate which is the link.

Characteristically, *V. cholerae* LPS contains several unusual sugars, including 2-amino-2, 6-dideoxy-D-glucose (quinovosamine) (Jann *et al.* 1973) 4-amino-4, 6-dideoxy-D-mannose (perosamine) (Redmond, 1975) and 4-amino-4-deoxy-L-arabinose (Redmond, 1978). LPS from both the Ogawa and Inaba serotypes contain quinovosamine and perosamine, however, Ogawa LPS contains the additional sugar, 4-amino-4-deoxy-L-arabinose (Redmond, 1978). Structural studies revealed that the O-specific side chain is a linear homopolymer of D-perosamine of approximately sixty repeating units (Redmond, 1979; Kenne *et al.* 1982) in which the terminal amino group is acylated with quinovosamine (Kenne *et al.* 1979). However, it has been reported that the side chain consists of only eighteen repeat units (K. Hisatsune, personal communication). This structure appears to be common to both the Ogawa and Inaba serotypes. It has therefore been suggested that this backbone structure of the LPS represents the antigenic determinant A. It was discovered that mutation from the smooth (possesses O-antigenic side chain) of *V. cholerae* to the rough form (lost all or part of the O-antigenic side chain) involved

total elimination of the amino sugars D-quinovosamine and D-perosamine (Hisatsune and Kondo, 1980). This elimination resulted in the loss of O specificity of smooth form LPS and concominant appearance of strong cross-reactivity among rough forms regardless of the serotype of their smooth parent strains (Hisatsune and Kondo, 1980). The presence of 4-amino-4-deoxy-L-arabinose in Ogawa but not Inaba may indicate the role in determining antigenic factor B specificity. Isolation of monoclonal antibodies directed against cholera LPS (Gustafsson *et al.* 1982) should provide valuable information about the chemical nature of O-antigenic specificity.

Lipid A of *V. cholerae* like that of *Enterobacteriaceae* is made up of a central backbone of (1-6)-linked D-glucosamine dissacharide units substituted at positions 4′ and 1′ by pyrophosphate residues. The hydroxyl groups are substituted by long fatty acids and the amino groups by β–hydroxy-myristic-acid (Broady *et al.* 1981). The fatty acid composition of *V. cholerae* strains has been looked at and revealed the presence of considerable amounts of odd numbered fatty acids (C15:0 and C17:0). The presence of fatty acids within lipid A seems to be essential for endotoxic activity (Nowotny, 1969; Shands, 1971) since alkaline digestion of lipid A, which cleaves the ester linked fatty acids, leads to complete loss of toxicity.

1.10.2 LPS genetics

Bhaskaran in 1964 using a Classical *V. cholerae* strain derived a crude linkage map of the chromosome, mapping the genes for O antigen (*oag*), now referred to as *rfb* (Ward *et al.* 1987) between markers *ilv* and *arg*.

—*str*—*pur-1*—*ilv-1*—oag—*arg-1*—*leu-1*—*his-1*—

This location has been confirmed by Parker and co-workers (Parker *et al.* 1979).

The genes determining the biosynthesis of the Inaba and Ogawa serotypes have been cloned and expressed in *E.coli* K-12 (Manning *et al.* 1986) resulting in the production of an heterologous LPS in which the *V.cholerae* O-antigen is

substituted onto the *E.coli* core leading to the production of a smooth LPS having the appropriate serotype specificity (Manning *et al.* 1986; Ward *et al.* 1987).

Restriction analysis of these clones and determination of the chromosomal organization in both Inaba and Ogawa strains by Southern hybridization suggests that only minor changes, which must lie in this region, are associated with serotype conversion (Ward *et al.* 1987). The genes for O-antigen biosynthesis are present in a cluster of about 16 to 19 kb in length. The nucleotide sequence is currently being determined (P.A. Manning, personal communication). This will provide information on the genetic organization and number of genes present and enable the basis of serotype conversion to be defined.

1.10.3 Anti-LPS immunity

V. cholerae LPS has attracted a great deal of attention since it is highly immunogenic and antibodies to the LPS seem to be protective, also LPS itself may play a role in mucosal adhesion. Further proof of the immunogenicity of LPS was shown when following a cholera infection, serum vibriocidal antibody detected was mainly, but not completely, against LPS. These levels rose substantially in more than 90% of North American volunteers who had been challenged with cholera (Clements *et al.* 1982).

Convincing evidence for this comes from Manning *et al.* (1986) who used *E.coli* K-12 strains harbouring plasmids expressing *V. cholerae* LPS to immunize rabbits. The antisera produced was analyzed for their protective activity in the infant mouse animal model system and found to be highly protective against challenge with *V. cholerae* organisms. These antibodies were as protective as antibodies raised against heat killed *V. cholerae* organisms.

The studies of Chitnis *et al.* (1982) on *in vitro* adhesion of *V. cholerae* in isolated adult rabbit ileal loops have also implicated LPS in adherence. Inaba purified LPS inhibited attachment of Inaba *V. cholerae* whereas LPS from *E.coli* had no effect. Antiserum to Inaba LPS also inhibited adhesion of both Inaba and Ogawa organisms. It is possible that the effects of the anti-LPS serum could be

due to steric hindrance. However, Booth *et al.* (1985) found they could prevent adherence by the use of monclonal antibodies directed against specific determinants on the O-antigen.

1.11 Flagellum, Flagellar Sheath and Proteins

Electron microscopy of *V. cholerae* shows a single polar flagellum which consists of a core with an average diameter of 125-165 $\mathring{A}$ surrounded by a sheath (Follet and Gordon, 1963; Ogasawara and Kuno, 1964). The total flagellum diameter being 270-350 $\mathring{A}$ (Follet and Gordon, 1963). The sheath seems to be continuous with outer membrane as demonstrated using both ferritin and fluorescein labelled antibodies (Das and Chatterjee, 1966; Bhattacharyya, 1975; Hranitzky *et al.* 1980) and most recently by protein A-colloidal gold labelling (Fuerst and Perry, 1987). The basal complex has four typical rings as well as concentric membrane rings. The L ring is associated with the lipoplysaccharide layer of the outer membrane and the P ring with the peptidoglycan. Both the S and M rings are in the plasma membrane (Ferris *et al.* 1984).

As mentioned previously, the heat labile antigens are associated with the flagellum and all *V. cholerae* 01 strains have a common H antigen (Gardner and Venkatraman 1935; Sakazaki 1970; Bhattacharyya and Mukerjee 1974; Bhattacharyya, 1975). Hranitzky *et al.* (1980) characterized the flagellar sheath and showed that antibodies to a sheath protein reacted with a number of strains in labelling studies suggesting that the sheath protein is the common H antigen amongst vibrios. Eubanks *et al.* (1977) described a non-LPS antigen associated with the flagellum and that antibodies to this protein are protective against cholera in the one animal model tested. A number of workers have suggested therefore that the flagellar sheath protein may be an adhesin involved in attachment of the organism to the intestinal epithelium (Eubanks *et al.* 1977; Hranitzky *et al.* 1980; Attridge and Rowley, 1983a, 1983b).

Various studies have suggested that motility is an important factor in viru-

lence and that non-motile variants have decreased virulence (Guentzel and Berry, 1975; Schrank and Verwey, 1976; Guentzel *et al.* 1977; Guentzel *et al.* 1981; Attridge and Rowley, 1983). For example, Guentzel and Berry (1975) compared the ability of motile strains and non-motile derivatives to kill suckling mice. They found that a loss of motility results in reduced virulence possibly due to a diminished chance of the vibrios to associate with the mucosa. Close contact may permit more efficient toxin delivery. However, Classical strain 569B is hypertoxinogenic and only weakly motile and has been demonstrated to have reduced virulence in some animal models (Guentzel and Berry, 1975), but to be extremely virulent in the infant rabbit model and in human infection (Woodward *et al.* 1976). It may be possible that toxin overproduction may compensate for reduced motility. Hence the association of motility and virulence at that stage still seem to be unclear. Jones and Freter (Freter and Jones, 1976; Jones and Freter, 1976; Jones *et al.* 1976) using rabbit intestinal brush border membranes and ileal slices *in vitro* reported that non-motile variants of *V. cholerae* did not attach even when they were centrifuged onto the membrane allowing contact to occur. However, in their studies, they suggested that their non-motile variants differed from their motile parent strain by the absence of a specific adhesin.

Attridge and Rowley (1983) decided to look at the question of whether the flagellum structure merely served as an agent of motility or was itself directly involved in adhesion by possibly carrying the actual adhesin. Using a motile variant of strain 569B, they incubated the organism with antibodies to non-LPS somatic determinants and demonstrated that motility was not diminished but the adherence potential was. When anti-LPS serum was used at low levels, the bacteria were immobilized and adherence unaffected. This indicates that motility is unnecessary for attachment. Hence they suggested that motility and binding capacities of the flagellum were independent. Electron microscopy studies, (Nelson *et al.* 1976; Nelson *et al.* 1977) have shown that vibrios align parallel to the intestinal epithelium and do not attach by their flagella. It could be that the initial contact is made by the flagellum and the organism then adheres horizontally to allow greater surface

to surface contact.

Recently three proteins associated with the flagellum have been identified as having sizes within the range 33 to 37 kDal. The 37 kDal protein has been cloned and localized to within a 2.2 kb DNA fragment (Srivastava *et al.* 1987).

1.12 Fimbriae (pili)

Fimbriae (pili) are thin filamentous appendages which extend from the organism into the surrounding environment, are variable in number and dimensions and may be arranged in a polar or peritrichous fashion (Sokatch,1979). Fimbriae have been implicated as adherence organelles in many organisms and in some, have actually been demonstrated to mediate attachment. The best studied examples are the fimbriae produced by enterotoxigenic *E.coli* (ETEC) (Gaastra and De Graaf, 1982; Knutton *et al.* 1984; Levine *et al.* 1986; Levine, 1987). It has been naturally assumed that fimbriae may also have a role in attachment for *V. cholerae*. Consequently, a number of studies have been concerned with the characterization of pili present on *V. cholerae*.

By electron microscopy, Barua and Chatterjee (1964) managed to identify a small number of fimbriae with diameters ranging from 6 to 8 μm on El Tor organisms. However, because of a slime layer surrounding the organisms, observation of the fimbriae was made difficult and these authors concluded that fimbriae could not be positively demonstrated. The studies of Tweedy *et al.* (1968) involved culturing the organisms at 37°C in tryptone water. Under these conditions, 10% of Classical strains had fimbriae with a maximum of 9 per cell whereas 50% El Tor strains had up to 50 per cell. Tweedy estimated the diameters to be between 6 and 10 μm, similar to the observations of Barua and Chatterjee. These fimbriae have been compared with type 1 fimbriae (Brinton, 1965; Duguid, 1966) and F pili (Brinton, 1965; Lawn, 1966) and several conclusions can be drawn; type 1 fimbriae are more numerous per organsim and more rigid. F pili are present less frequently than those detected on the El Tor vibrios and are longer. Bhaskaran *et al.* (1969)

have demonstrated fertility in vibrios due to sex pili. These sex pili are similar in diameter and numbers per cell though slightly shorter. This leads to the possibility that the fimbriae observed by Tweedy *et al.* (1968) may function as sex pili and not in adherence at all. Faris *et al.* (1982) have also reported the presence of fimbriae but, according to Booth *et al.* (1986), "the fimbriated organisms do not look like cholera vibrios".

A number of workers have been unable to demonstrate the presence of fimbriae (Finkelstein and Mukherjee, 1963; Lankford and Legsomburana, 1965; Nelson *et al.* 1976; Booth *et al.* 1986). The differences in various reports on fimbriae may be due to the ease with which fimbriae are lost from the surface or may depend on the *in vitro* culture conditions being used.

Recently, Ehara and co-workers (1986) have reported the existence of fimbriae 5-7 nm in width on *V. cholerae* during colonization of the rabbit small intestine. This is an interesting finding since Booth *et al.* (1986) could not detect fimbriae during *in vivo* infections of rabbit intestinal tissue. These authors (Ehara *et al.* 1986) have also developed a medium which gives reported reproducible expression of fimbriae enabling the purification of fimbriae from both Classical and El Tor strains (Ehara *et al.* 1987). The purified fimbriae were examined by electron microscopy and SDS-PAGE gels. The structural subunit is a 16 kDal protein which is antigenically indistinguishable regardless of biotype and serotype. Fimbriae from an El Tor strain gave high HA titres with human RBCs and was inhibited by the addition of D-mannose and L-fucose. Al-Kaissi and Mostratos (1985) have looked for the presence of fimbriae using three tests as an index: haemagglutination, pellicle formation and electron microscopy. They found maximum production of fimbriae in stationary phase after culturing in liquid media plus glucose and that growth on solid agar inhibited formation of fimbriae. Twenty percent of El Tor strains were fimbriated with 4-6 fimbriae per cell, whereas 10% of Classical strains were fimbriated with 2-4 per cell. Strains which possessed the most fimbriae gave higher HA titres under all conditions, indicating that the two properties may be related.

Taylor and co-workers (1987) utilized transposon Tn*phoA* to obtain fusions

between *phoA*, the gene for *E.coli* alkaline phosphatase and *V. cholerae* genes. They isolated a *phoA* mutant which had a marked decrease in the intestinal colonization of suckling mice (Herrington *et al.* 1987; Shaw *et al.* 1987; Taylor *et al.* 1987a). This mutation is in the structural gene *tcpA* which encodes a 20.5 kDal protein. This is the major subunit of a *V. cholerae* pilus which is co-regulated with the cholera toxin. Therefore the regulatory gene *toxR* also controls transcription of *tcpA*. A possible organization for the 8 genes so far identified which are involved in the production of the Tcp pilus has been postulated (Taylor *et al* 1988b).

Southern hybridization analysis with *tcpA* probes have shown that *tcpA* sequences are highly conserved in El Tor and Classical clinical isolates but that most environmental and NAG strains do not contain such sequences (Taylor *et al.* 1987, 1988). Expression of the Tcp pilus seems to be under the control of the transcriptional activator encoded by the *toxR* gene.

1.13 Outer Membrane Proteins

The cell envelope of *V. cholerae* is typical for a Gram-negative organism with an inner membrane (IM), a layer of peptidoglycan and an outer membrane (OM) (Hisatsune *et al.* 1972). In addition to LPS, the OM has a few very abundant proteins. Since outer membrane proteins have been demonstrated to be involved in the pathogenesis of a number of bacteria they may be protective antigens (Craven and Frasch, 1979; Gulig *et al.* 1982; Svenson *et al.* 1979; Buchanan *et al.* 1980; Loeb and Smith, 1980; Fernandes *et al.* 1981; Swanson, 1981). The outer membrane of *V. cholerae* has also been the subject of investigation (Kabir, 1980; Kelley and Parker, 1981; Manning *et al.* 1982). Immunochemical studies demonstrate that strains of *V. cholerae* belonging to both the major serotypes (Inaba and Ogawa) and both biotypes have cross reacting protein antigens located in their outer membrane (Kabir, 1983; Manning and Haynes, 1984).

In an infection where cell surface interactions are so important, it is only natural to suggest that OM proteins may have a role in the *V. cholerae* infectious

process. Evidence also suggests that they are immunogenic in humans (Kabir, 1983). *V. cholerae* has a high degree of surface hydrophobicity which may be important to overcome the negative surface charge of the gut epithelial cell, so that molecules on each cell surface may interact (Kabir and Ali, 1983). Antibodies to some undefined cell envelope proteins have been shown to be protective in the infant mouse model (Neoh and Rowley, 1970; Neoh and Rowley, 1972; Attridge and Rowley, 1983; Sharma *et al.* 1987). Sears *et al.* (1984) have evaluated the immune response in humans to OM proteins after an experimental cholera infection. It was found that 50% of infected patients gave a measurable immune response to OM proteins. This response was similar regardless of whether the OM antigens used in the ELISA assays were obtained from either biotype or serotype.

V. cholerae has a number of major outer membrane proteins. These include a group with sizes 44 kDal to 47 kDal, a heat modifiable protein of 35 kDal and another of 26 kDal (Kabir, 1980; Kelley and Parker, 1981; Manning *et al.* 1982; Richardson and Parker, 1985a; 1985b). The outer membrane protein profile is influenced by growth medium and cultural conditions (Kabir, 1980), as has been observed with other bacteria (Schnaitman, 1974; Lugtenberg *et al.* 1976). The 44 kDal to 47 kDal proteins, probably represent the major cell porins. The 35 kDal protein has been shown to share properties with the OmpA protein of *E.coli* K-12 and other *Enterobacteriaceae* (Alm *et al.* 1986). It is heat-modifiable and trypsin sensitive and antibodies to the 35 kDal protein cross react with the usually conserved carboxy terminus of the OmpA protein. Interestingly, a DNA probe to the carboxy terminus of *Serratia marcescens ompA*, which in turn is related to that of *E.coli* K-12, can be used to detect homologous DNA sequences in *V. cholerae* (Alm *et al.* 1986). Richardson and Parker (1985) identified and characterized *V. cholerae* surface proteins by radioiodination. Nine radiolabelled proteins were found in preparations obtained in lithium chloride-lithium acetate extraction and they corresponded with outer membrane proteins as defined by sucrose density centrifugation and triton X-100 insolubility.

Using antiserum prepared against live *V. cholerae*, Manning and co-workers

(1985) cloned in *E.coli* K-12 the gene for a 22 kDal exposed outer membrane protein of *V. cholerae*. This protein is a minor protein in *V. cholerae* but is produced in large amounts on the cell surface of *E.coli*, possibly due to the high gene dosage afforded by the cloning vector or to the absence of normal regulation. By Southern DNA hybridization the DNA encoding the protein was shown to be conserved between both El Tor and Classical strains. The immunological importance of this protein is currently being established and appears to be similar to a protein that is readily detected with convalescent phase human antisera (P.A. Manning, personal communication).

Stevenson *et al.* (1985) have purified the 26 kDal (OmpV) outer membrane protein of *V. cholerae* by ion-exchange elution on hydroxyapatite followed by gel filtration in the presence of SDS. Antiserum to purified protein was then generated in rabbits and by screening gene banks constructed in *E.coli* K-12, a clone expressing the OmpV protein was isolated (Manning *et al.* 1985; Stevenson *et al.* 1985; Manning *et al.* 1986). However, unlike the 22 kDal protein, the expression of this 26 kDal major outer membrane protein is very poor in *E.coli* K-12 despite being the major OM protein of *V. cholerae*. A possible explanation for this poor expression comes from analysis of the DNA sequence (Pohlner *et al.* 1986a). Prior to the initiation codon is an excellent Shine-Dalgarno sequence, however this sequence falls within a region capable of forming a stem loop structure encompassing the entire 5′ end of the mRNA. This could reduce translation by inhibiting the binding of the mRNA to the 16S ribosomal subunit. Operon fusion studies have confirmed the notion of translational control (A. Barker, personal communication) and it has been proposed that a positive regulatory element is involved which is present in *V. cholerae* but absent from *E.coli* K-12. Further analysis of the DNA sequence and determination of the amino acid sequence of the mature protein demonstrated that OmpV is synthesized in a precursor form with a 19 amino acid NH_2-terminal extension or signal peptide sequence (Pohlner *et al.* 1986a). The derived amino acid sequence has marked hydrophilic regions, several of which correspond to the antigenic determinants on the native and denatured forms of the protein (Pohlner

et al. 1986a, 1986b).

The immunogencity of OmpV has been investigated. OmpV is present in all *V. cholerae* strains, irrespective of their biotype or serotype but it is not present in other vibrios such as *V. mimicus* and *V. fluvialis* (Manning and Haynes, 1984). A transposon insertion mutant in *ompV* has been constructed and shown to be equally competitive with the wild-type in an *in vivo* model assay, suggesting that OmpV is not involved in adhesion or colonization (Taylor *et al.* 1987).

1.14 Soluble Proteins

In addition to CT, *V. cholerae* elucidates a wealth of other extracellular proteins which may contribute to colonization. These include proteases, a neuraminidase, haemolysins and DNases. Some of these potential colonization factors or toxins have been studied in some detail.

1.14.1 Haemolysins (Hly)

The production of a soluble haemolysin was originally one of the criteria for differentiating between vibrios of the Classical and El Tor biotypes. However, this has since become an unreliable test since weakly haemolytic El Tor strains have been isolated (Roy and Mukerjee, 1962) as well as strains that produce no haemolysin but have other biochemical properties characteristic of the El Tor biotype (deMoor, 1963). It has also been reported (Richardson *et al.* 1986) that Classical strains exhibit haemolytic activity when assayed using chicken and rabbit RBCs.

The El Tor haemolysin has been purified (Chaicumpa and Attasishtha, 1979; Yamamoto *et al.* 1984) and shown to be a 61 kDal protein. The gene encoding this protein has been cloned by two independent groups (Goldberg and Murphy, 1984; Manning *et al.* 1984). Manning and co-workers have identified three proteins designated HlyA, HlyB and HlyC with sizes 80 kDal, 70 kDal and 22 kDal respectively. HlyA appears to be the haemolysin with HlyB and HlyC possibly being involved in production.

Interestingly, the product of the cloned structural gene seen in *E.coli* K-12 is 80 kDal in contrast to the purified protein from *V. cholerae*, which is 61 kDal. It was also observed that, although the haemolysin is actively excreted in *V. cholerae*, it remains in the periplasm of *E.coli* K-12 and is not released into the growth medium (Manning *et al.* 1984; Mercurio and Manning, 1985). Therefore, presumably the major cleavage of the protein occurs upon release from the organism. Such a large difference in size of the intra- and extracellular forms of another haemolysin produced by some *E.coli* has previously been reported (Goebel and Hedgpeth, 1982; Coleman *et al.* 1983; Kehoe *et al.* 1983).

However recently Alm and co-workers (1988) have found that if culture supernatants of a haemolytic El Tor strain were treated with trichloroacetic acid, then upon staining with Coomassie Brillant Blue the 80 kDal form was observed instead of the 61 kDal protein. Hence these workers postulate that the haemolysin is exported in the 80 kDal form but specific cleavage occurs which gives two products of approximately 65 kDal and 15 kDal. (1983)

Mercurio and Manning have analyzed cellular fractions for haemolytic activity and demonstrated that, in *E.coli* K-12, the haemolysin transverses the cytoplasmic but not the outer membrane and remained localized within the periplasmic space. If the genes are introduced into *E. coli* K-12 harbouring either *tolA* or *tolB* mutations, the activity is released giving a zone of haemolysis very similar to that observed in *V. cholerae* (Mercurio and Manning, 1985). Mutants in *tolA* and *tolB* are known to have defective outer membranes resulting in leakage of periplasmic proteins (Bernstein *et al.* 1972; Anderson *et al.* 1979). Hence, although *E.coli* K-12 can secrete these proteins into the periplasm, it apparently lacks a specific excretion mechanism present in *V. cholerae* for the release of soluble proteins into the growth medium.

Southern DNA hybridizations showed that the DNA encoding the haemolysin gene is present in all *V. cholerae* strains regardless of biotype suggesting that,in non-haemolytic strains, the genes are present but are not expressed (Goldberg and Murphy, 1984; Brown and Manning, 1985). The presence of homologous DNA was

detected in non-01 strains indicating a close relationship between the non-01 and 01 haemolysins. This has also been demonstrated by Yamamoto *et al.* (1984) who have purified the haemolysin from a non-01 strain and found it to be immunologically identical to the protein from 01 strains.

Goldberg and Murphy (1985) have examined DNA from cloned *hly* loci from a haemolytic El Tor (RV79 hly^+), a non-haemolytic El Tor derivative (RV79 hly^-) and the Classical strain 569B. Restriction endonuclease analysis was used to detect differences between the cloned genes. They found that the genes from a haemolytic and non-haemolytic El Tor differed by the addition of a 10-15 bp insertion in the haemolytic strain. Also a 20 bp deletion was detected in the cloned *hly* locus of the Classical 569B strain with respect to the El Tor *hly* gene. Whether this represents a change in a regulatory region or in the structural gene is as yet unknown. Alm *et al.* (1988) have sequenced the *hlyA* genes from both a Classical and El Tor strain which has enable direct comparison of changes in the nucleotide sequence which could account for concominant loss of haemolytic activity. The sequence of the *hlyA* gene of *V. cholerae* El Tor strain O17 (Hly^+) reveals a 738 amino acid sequence which gives a predicted protein 82.246 kDal. Analysis of the sequence of *hlyA* from a Classical strain 569B (Hly^-), shows a 11 bp deletion which results in a stop codon generating a truncated HlyA protein of size 26.936 kDal. This deletion has been shown to be specific and always present in Classical strains, when some 150 isolates were screened with a specific oligonucleotide (R. Alm, personal communication)

Richardson and co-workers (1986) have isolated two distinct haemolytic clones from the Classical strain 395. One clone is identical to the cloned structural gene of El Tor. This has been designated haemolysin I. The second clone did not hybridize to the El Tor haemolysin and has a unique restriction enzyme digestion pattern. This has been designated haemolysin II. This unique haemolysin causes the *E.coli* cells to have a slight green colouration when grown on blood agar.

It has been proposed that a locus, designated *hlyR*, is responsible for the regulation of haemolysin I production (von Mechow *et al.* 1985). This gene is

located in the *his-tox* region of the chromosome. Hly^- and $ToxR^-$ phenotypes were isolated and appeared separate, therefore suggesting that the gene controlling haemolysin I is different from *toxR*. However, this conclusion rests on the study of a single recombinant colony from a conjugation experiment. Sequencing data has shown there to be no *toxR* binding sites before *hlyA* (Alm *et al.* 1988). Therefore, whether *hlyR* is identical or a separate identity from *toxR* (or *toxS*) requires closer examination.

Green and co-workers (1983) have mapped the chromosomal locus associated with the El Tor biotype specificity, namely production of haemolysin (*hly*), the chicken erythrocyte haemagglutinin (*cha*) and resistance to polymyxin B (*pmx*). They found that all three markers are closely linked and were located between the *pyrA*-201 and *his*-2 alleles on the *V. cholerae* genetic map. Goldberg and Murphy (1984) used mutator vibriophage VcA-3 insertions in the *hly* locus to promote high-frequency transfer. These workers mapped the *hly* locus between *ilv* and *arg* on the El Tor chromosome. This contradicts the linkage data of Green *et al.* (1983) which shows no significant linkage between *hly* and *arg*.

The exact function of the *V. cholerae* haemolysin has yet to be established. *V. parahaemolyticus* pathogenic strains produce a thermostable haemolysin which has cytotoxic and cardiotoxic effects (Miyamoto *et al.* 1980). Ichinose *et al.* (1987) have shown that the haemolysin of non-O1 strains is the enterotoxic factor responsible for gastro-enteritis. As mentioned previously, constructed cholera toxin gene deletion strains still produce residual diarrhoea. It has been speculated that the *V. cholerae* haemolysin may be the second diarrhoeagenic factor involved (Mekalanos *et al.* 1983; Ichinose *et al.* 1987).

1.14.2 DNase

To date, no suitable and reproducible transformation or transfection system has been developed in *V. cholerae* possibly due to the production of a potent extracellular DNase by the organism. When compared with other enteric organisms, *V. cholerae* has a low incidence of R plasmids (Prescott *et al.* 1968; Hedges *et al.*

1977) and the limited ability of *V. cholerae* strains to accept and maintain foreign plasmids may also be related to the production of a DNase (Focareta and Manning, 1987). *Serratia marcescens* also produces an extracellular DNase which has been implicated as the reason for poor transformability and low yields in obtaining plasmid DNA and these effects were overome by introducing mutations in the DNase (Timmis and Winkler, 1973).

Structural genes encoding for extracellular DNase production have been cloned by two groups (Newland *et al.* 1985; Focareta and Manning, 1987). Newland *et al* (1985) have designated their gene *xds* and narrowed the limits of the coding region to within 3.5 kb, with mini-cell data suggesting a protein of 100 kDal.

Focareta and Manning (1987) have also cloned a gene encoding a *V. cholerae* DNase. They found that, although the DNase is excreted into the external medium by *V. cholerae*, cell fractionations showed it to be localized in *E.coli* K-12 to the periplasm. The gene has been localized to a 1,200 bp fragment and the nucleotide sequence has been determined giving a single open reading frame of 690 bp which corresponds to a protein of 26.389 kDal. It has a typical NH_2-terminal signal sequence of 18 amino acids which, when cleaved, would give a mature protein of 24.163 kDal. This agrees with the size observed on SDS-PAGE under non-denaturing conditions where it was possible to detect DNase activity. Hence the protein sizes of the DNases cloned by the two groups are different. This discrepancy appears to have been resolved by Southern hybridization studies which imply that the two DNases are also genetically different (T. Focareta, personal communication).

By transposon-facilitated recombination, Newland and co-workers (1985) have mapped the position of *xds* between the *pro*-1 and *ile*-201 markers on the El Tor chromosome. The locus of the other DNase gene (Focareta and Manning, 1987) has not been mapped.

Recently T. Focareta (personal communication) has introduced *in vivo* deletions into the *V.cholerae* chromosome in the genes encoding both of the DNases. He constructed *V.cholerae* strains which contained deletions in each DNase as well as a strain that harboured mutations in both. When the transformability of these

strains were tested it was found that each strain was now able to be transformed, however, the strain which had both mutations gave a 10-fold increase in the number of transformants compared to strains possessing singular deletions. This indicated that the inability of *V. cholerae* strains to be transformed was due to the presence of two DNases.

1.14.3 Neuraminidase

The neuraminidase gene *nanH* of *Vibrio cholerae* has been cloned (Galen *et al.* 1987). The structural gene has been sequenced indicating a mature protein of 75.9 kDal.

1.14.4 Soluble Haemagglutinin (SHA)

Finkelstein and co-workers (1978) have described the isolation and purification of what was originally thought to be an adhesive factor from *V. cholerae*. This haemagglutinating factor was originally termed "cholera lectin" but was subsequently designated soluble HA (SHA) since it could be detected in cell-free supernatants in contrast to the cell-associated HAs (Finkelstein and Hanne, 1982; Hanne and Finkelstein, 1982). More recently, it has been referred to as the SHA/protease due to the proteolytic activity exhibited (Booth *et al.* 1983, 1984; Finkelstein *et al.* 1983). This protein is found in all strains regardless of biotype or serotype and is the best studied of the haemagglutinins.

The SHA is found in late exponential phase cultures and the haemagglutination titre is dependent on the species of erythrocytes used in the assay and is also variable within a species. For example, chicken RBCs which gave high titres were termed responders, chicken RBCs which gave low titres were termed non-responders (Finkelstein *et al.* 1978; Hanne and Finkelstein, 1982). Maximum titres were obtained with mouse RBCs and chicken responder RBCs, whereas rabbit and human RBCs gave minimal reaction. Calcium ions are required in the assay buffer if maximum haemagglutination titres were to be obtained. Haemagglutination is

not inhibited by any of the sugars so far tested (Hanne and Finkelstein, 1982).

The SHA has been purified (Finkelstein *et al.* 1978; Chaicumpa *et al.* 1982; Finkelstein and Hanne,1982; Svennerholm *et.al.* 1983). There are apparently three distinct pI isotypes of the SHA which exist as non-covalently associated polymers of 32 kDal subunits (Finkelstein and Hanne, 1982). It was noted that haemagglutination activity was heat labile since recovery of activity during purification was extremely low (<0.2%). This suggested the possible presence of proteolytic activity. It was shown that both activities resided in a single molecule, since specific antibodies to the SHA inhibited protease activity. Electron microscopy of purified SHA showed that the material formed long filaments and also binds firmly to the hydrophobic matrix phenylsepharose, therefore showing hydrophobic properties.

Schneider and Parker (1978) have purified and characterized a mucinase of *V. cholerae* which has several features in common with the SHA/protease. The partially purified mucinase gave a precipitin reaction with antiserum to the SHA/protease, indicating identity. Protease deficient mutants isolated by NTG treatment show a loss of virulence in the infant mouse model, although toxin was still produced (Schneider and Parker, 1978). However, it must be remembered that such mutants probably contained multiple genetic lesions.

Young and Broadbent (1982) could detect three types of extracellular proteases. The activity of Type I is inhibited by phenylmethyl-sulfonyl-fluoride and lima bean trypsin inhibitor. Type II activity was inhibited by metalloprotease and serine protease inhibitors and digested mucin. Type III was inhibited by EDTA. The Type II protease, described by Young and Broadbent (1982), could be identical to the SHA/protease. Thus, not only is there a SHA/protease which has a number of biological activities, but a number of other proteases exist (Freter, 1955; Hsieh and Liu, 1970; Kusama and Craig, 1970; Dahle and Sandvik, 1971; Sandvik and Dahle, 1971; Schneider *et al.* 1981; Young and Broadbent, 1982). The role played by these enzymes in pathogenesis has yet to be elucidated.

When partially purified SHA was administered intra-intestinally into infant rabbits prior to innoculation of cholera vibrios, the attachment of the vibrios was

markedly inhibited (Finkelstein *et al.* 1978). It was also shown that Fab fragments from anti-SHA serum significantly inhibited attachment of vibrios compared with Fab fragments from pre-immune serum. However, efforts to determine whether antibodies directed towards the SHA/protease are protective have proved disappointing (Booth *et al.* 1985). Experiments in which infant rabbits were administered with antibodies to SHA/protease prior and following inoculation with vibrios gave negative results. The infant rabbits developed cholera in the same time course as those controls given normal serum. This conclusion is supported by the data of Svennerholm *et al.* (1984) who have developed an enzyme linked immunosorbent assay for the detection of specific SHA antibodies. Acute and convalescent serum samples were taken from Bangladeshi patients and from North American volunteers and examined. Only two of the ten Bangladeshi patients and one of the seventeen North American volunteers gave a detectable serum Ab response to the SHA after infection with cholera. None of the ten North American volunteers gave an IgA gut mucosal response but four of eight Bangladeshi patients did. This data suggests that *in vivo* the SHA is poorly immunogenic.

Activity of the SHA has been assayed on a number of subtrates using [^{125}I]-labelled proteins (Finkelstein *et al.* 1983). It was capable of hydrolyzing fibronectin, mucin and could cleave lactoferrin. It has been suggested that this activity may aid the organism to digest host proteins which are specifically or non-specifically associated with the host defence systems to prevent colonization by the organism. Lactoferrin may act by withholding iron and reducing the availability to microbes in mucosal secretions (Bullen, 1981) and so hydrolysis of lactoferrin may increase iron levels. The ability for the SHA to hydrolyze mucin may facilitate penetration through the layer of mucous which covers the epithelial binding sites (Nelson *et al.* 1976). In fact, in 1947, Burnet reported the existence of a *V. cholerae* mucinase which is probably identical to the SHA (Burnet, 1947, 1948, 1949; Finkelstein *et al.* 1983). Fibronectin is a high molecular weight glycoprotein present on the surface of mammalian cells (Yamada and Olden, 1978; Mosher, 1980) and has been reported to interfere with the adherence of *Pseudomonas aeruginosa* to local epithelial cells

(Woods *et al.* 1981). Booth *et al.* (1984) have suggested that the SHA/protease may be an endogenous enzyme of *V. cholerae* responsible for the nicking of cholera toxin resulting in activation. Un-nicked cholera enterotoxin was purified and incubated with SHA/protease for 90 minutes at 37°C. The un-nicked toxin was converted to the nicked form with corresponding increased biological activity as measured in the Y1 adrenal cell assay. If the SHA was inhibited, then nicking was prevented. Trypsin also was found to activate the un-nicked enterotoxins of *V. cholerae* and *E.coli* (LT). It is not known whether host proteins such as trypsin have a role in toxin activation in the small bowel or whether the SHA/protease is the endogenous *V. cholerae* nickase. It would seem reasonable that the organism elucidates a nickase since special precautions must be taken so that cholera toxin can be isolated in the un-nicked form. This contrasts with the closely related but non-excreted *E.coli* LT enterotoxin which is easily isolated in the un-nicked form.

The SHA/protease is a zinc metalloendopeptidase (Booth *et al.* 1983). Zincov [2-(N-hydroxycarboxamido)-4-methyl pentanoyl-l-ala-gly-NH_2] an inhibitor designed for Zn containing metalloproteases (Nishino and Powers, 1979) inhibited both haemagglutinating and proteolytic activities. Purified SHA was incubated with erythrocytes in the presence or absence of Zincov. If Zincov was added before the erythrocytes, no haemagglutination occurred. When Zincov was added 30 minutes after the RBCs, haemagglutination still occurred. Thus haemagglutination may depend on a proteolytic event since the addition of Zincov before incubation of RBCs with SHA prevents haemagglutination. The SHA/protease is a calcium stabilized zinc containing enzyme similar to the group of zinc metalloendoproteases (Morihara, 1974; Holmquist, 1977). The hydrolysis of FAGLA (furylacryloyl-gly-leu-NH_2) indicates the enzyme can cleave a gly-leu bond (Feder and Schuck, 1970).

The fact that the SHA/protease is excreted would seem to negate the posiblility of functioning as an adhesin, unless perhaps the role is in the detachment of the vibrio, enabling it to be freed to seek new hosts and re-infect (Booth *et al.* 1985). However, Finkelstein and Hanne (1982) suggest that the presence of the SHA in supernatants represents an artefact of *in vitro* growth and that the SHA

is only detected in late log phase cultures, when cell lysis and the concominant release of membrane fragments may be occurring (Hoekstra *et al.* 1976). It has been proposed that *in vivo* conditions may be completely contradictory and that the haemagglutinin is soluble *in vitro*, but cell-associated *in vivo*.

1.15 Cell-associated Haemagglutinins

In 1961, Bales and Lankford (1961) reported that *V. cholerae* was capable of direct haemagglutination of RBCs from several mammalian species. Finkelstein and Mukerjee (1963) observed that vibrios of the El Tor biotype grown on solid medium could agglutinate chicken RBCs whereas strains of the Classical biotype did not. Since then, a number of workers have described cell-associated HAs of *V. cholerae* (Jones *et al.* 1976; Chaicumpa and Atthasishtha, 1977; Hanne and Finkelstein, 1982; Holmgren *et al.* 1983; Yamamoto *et al.* 1987). Hanne and Finkelstein (1982) have described four distinct HAs, three of which are cell-associated. These are the D-mannose, D-fructose sensitive HA expressed by El Tor strains, the L-fucose sensitive HA expressed by Classical strains and the L-fucose, D-mannose resistant HA expressed by both El Tor and Classical strains. Each will be discussed in turn

1.15.1 D-mannose, D-fructose sensitive haemagglutinin

This is the major cell-associated HA (MSHA) of El Tor vibrios and is the one which is used to distinguish between the biotypes (Finkelstein and Mukerjee, 1963). Haemagglutination is sensitive to D-mannose at 4 μg/ml and is also inhibited by D-fructose. It is expressed by vibrios grown both on solid media or in broth cultures. Further characterization has shown that calcium ions need not be included in the assay buffer for maximum HA titres. This is in contrast to the SHA. The MSHA has activity with all human (type A, B and O) RBCs as well as those from responder and nonresponder chickens.

Using transposon facilitated recombination, Green *et al.* (1983) have mapped the locus determining the El Tor biotype characteristics, including the MSHA which

they termed chicken erythrocyte haemagglutinin (Cha). All El Tor biotype determinants were closely linked to each other and to the *leu*-2 locus. The gene order of *cha* and *pmx* could not be determined since they were unable to isolate polymyxin sensitive, but still haemagglutinating recombinants. Srivistava and Srivistava (1980) have analyzed the role of the MSHA in adherence using isolated rabbit intestine discs. Upon addition of D-mannose, both haemagglutination and adherence due to the wild-type adherent El Tor strain KB207 were inhibited. CD11 and CD12, derivatives of KB207 isolated after NTG mutagenesis, only exhibit mannose resistant haemagglutination and adhere poorly.

The only purification of a cell-associated HA to date has been by Chaicumpa and Atthasishtha (1979) who have partially purified a HA from *V. cholerae* El Tor strain 017. Rabbits were injected intramuscularly with purified HA and the resulting antiserum found to contain protective antibodies using the infant mouse model. Sim and Chaicumpa (1981) then asked whether these Abs protected by agglutination of the vibrios or by masking the HA sites. IgG from the anti-HA serum was cleaved with pepsin and papain to give $F(ab)_2^1$ and Fab fragments. The monovalent Fab fragments were able to reduce adherence to *in vitro* isolated intestinal epithelium cells, indicating that masking of the HA sites should be sufficient to confer protection. However, Fab fragments gave a very low degree of protection whereas IgG and $F(ab)_2^1$ preparations were able to confer almost the same level of protection.

Using Tn*5* mutagenesis, Finn *et al.* (1987) derived a MSHA-negative mutant SB001 of a cholera enterotoxin-negative strain JBK70. They compared the ability of both the mutant SB001 and the wild-type parent JBK70 to colonize the rabbit ileum. The haemagglutinin negative mutant strain exhibited a 4 $\log_{10}$ decrease in the ability to colonize. Rabbits were orally immunized with SB001 and subsequently challenged with the wild-type strain N16961 and the toxin negative derivative JBK70. Such rabbits were protected equally as those rabbits which were either immunized with N16961 or JBK70. Therefore the mutation does not decrease the use of SB001 as an immunizing strain despite a decreased ability to colonize the intestine.

1.15.2 Fucose-sensitive haemagglutinin

Strains of the Classical biotype express a cell-associated HA (FSHA) is specifically inhibited by L-fucose. This HA gives maximum titres with human erythrocytes and minimal or no reaction with rabbit, guinea pig, horse, chicken, sheep or bovine RBCs (Hanne and Finkelstein, 1982). Hanne and Finkelstein (1982) monitored the HA production during growth using *V. cholerae* Classical strain CA401 and observed that the FSHA was expressed transiently very early in exponential phase growth. In contrast, other workers (Freter and Jones, 1976; Jones and Freter, 1976; Jones *et al.* 1976) have detected FSHA activity for up to 18 hours. The studies of Holmgren *et al.* (1983), show that the haemagglutination of Classical strains was inhibited by glycoproteins and oligosaccharides in human milk and many of these are fucosyl or fucosyl-sialyl-derivatives. Thus, these glycocompounds are possibly very closely related to the cell bound receptor for the bacterial adhesins.

It was mentioned that the FSHA is the major cell-associated HA of Classical vibrios but it can also be detected in El Tor strains by using MSHA negative mutants (Hanne and Finkelstein, 1982). Spontaneous MSHA negative variants of an El Tor strain have been isolated by incubating vibrios with chicken RBCs. The RBCs were then sedimented resulting in removal of HA positive vibrios from the mixture. The isolation of isogenic MSHA negative mutants of an El Tor parent is very useful since other haemagglutinins can be overlooked. This is especially the case in the presence of a major HA which is produced under particular growth conditions and is also dependent on the nature of the erythrocyte species used. In this way, it was discovered that El Tor strains also produce the FSHA but that it is usually masked by the potent MSHA.

1.15.3 Mannose-Fucose resistant haemagglutinin

This cell-associated HA (MFRHA) whose haemagglutinating activity, unlike the previous two, is not inhibited by either mannose or fucose nor any of a number of sugars which have been tested. It is active upon RBCs from both responder

and non-responder chickens (Hanne and Finkelstein, 1982). Whilst monitoring the haemagglutination pattern of MSHA negative mutants, it was observed that, in late logarithmic to stationary phase, a cell-associated MFRHA is produced. It is present in both Classical and El Tor vibrios. This HA has not been purified or characterized beyond this and what role it plays in adhesion and pathogenesis has yet to be determined.

1.16 Aims of this Study

Adhesion and colonization of the small intestine is an essential step in the infectious process of *V. cholerae* and a definite prerequiste for the manifestation of the disease. The so called "adhesins" involved in attachment are as yet unknown. Many molecules have been postulated to play a role but data so far are inconclusive.

V. cholerae possesses a number of haemagglutinins which could act as adhesive factors. However at present, research on the haemagglutinins of *V. cholerae* at the genetic level is scarce. The objective of this study was to characterize the mannose-fucose resistant cell-associated haemagglutinin at the DNA level with the aim of contributing to the genetics of *V. cholerae* as well as enabling the introduction of specific defined mutations into the wild-type gene. Such a mutation would enable exact studies on the role such a haemagglutinin may play in the colonization process.

Chapter 2

Materials and Methods

2.1 Growth 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) was the general growth medium for *E. coli* K-12 strains. *V. cholerae* strains were grown in Brain Heart Infusion (BHI) (Difco) prepared as directed by the manufacturers. Luria broth (LB) and 2 × TY medium was prepared as described by Miller (1972). Minimal A medium (M13 minimal media) was also prepared as described by Miller (1972) and supplemented prior to use with $MgSO_4$, glucose and thiamine-HCl to concentrations of 0.2 mg/ml, 2 mg/ml and 50 μg/ml respectively.

NA is nutrient agar, which is blood base agar (Difco) prepared without the addition of blood. Soft agar contains equal volumes of NB and NA. H agar consisted of bacto-tryptone (16 g/litre) (Difco), NaCl (8 g/litre) and bacto-agar (12 g/litre) (Difco). H top agar was like H agar but also contained 8 g/litre bacto-agar.

Antibiotics were added to broth and solid media at the following final concentrations: ampicillin (Ap) 25 μg/ml; chloramphenicol (Cm) 25 μg/ml; kanamycin (Km) 50 μg/ml; tetracycline (Tc) 10 μg/ml for *E. coli* and 4 μg/ml for *V. cholerae* strains.

Incubations were at 37°C unless otherwise specified. Normally, liquid cul-

tures were grown in 20 ml McCartney bottles or 125 ml side-arm flasks. Optical densities (OD) were measured at 650 nm using a Unicam Instruments spectrophotometer which had been adapted to read side-arm flasks.

2.2 Chemicals and reagents

Chemicals were Analar grade. Phenol, polyethylene glycol-6000 (PEG), sodium dodecyl sulphate (SDS) and sucrose were from BDH Chemicals. Tris was Trizma base from Sigma. Caesium chloride (Cabot) was technical grade, ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA) was Analar analytical grade.

Antibiotics were purchased from Sigma (ampicillin, kanamycin sulphate), and Calbiochem (tetracycline, chloramphenicol). All other anti–microbial agents (dyes, detergents and antibiotics) were purchased from Sigma Chemical Co., BDH Chemicals Ltd., Glaxo, or Calbiochem.

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N′–methylene bisacrylamide and urea were from BRL.

The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and their corresponding dideoxyribonucleotide triphosphate homologues (ddATP, ddCTP, ddGTP and ddTTP), were obtained from Boehringer-Mannheim. Adenosine-5′–triphosphate, sodium salt (ATP) and dithiothreitol (DTT) were obtained from Sigma. The substrate 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Boehringer-Mannheim.

M13 sequencing primer and [^{32}]P-dCTP, at a specific activity of 1,700 Ci/mmole were obtained from BRESA (Adelaide). [^{35}]S-methionine (1,270 Ci/mmole) was purchased from Amersham. Phosphorylated *Cla*I, *Eco*RI and *Pst*I linkers (8-mer) were purchased from New England Biolabs, Inc., Beverley, Mass. *Bgl*II linkers were obtained from BRESA. Linkers were obtained in a lypholized form and resuspended in 0.1 ml of TE buffer, pH 8.0 and stored frozen at -20^{o}C.

2.3 Enzymes

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

Restriction endonucleases *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *EcoRI*, *Hin*dIII, *Kpn*I, *Mlu*I, *Nde*I, *Nru*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sau*3A, *Sma*I, *Taq*I, *Xba*I and *Xho*I were purchased from Boehringer-Mannheim, Sydney, Australia. Nuclease *Bal*31 was purchased from New England Biolabs.

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

2.4 Maintenance of bacterial strains

All strains were maintained as lyophilized cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to a 10 ml bottle of NB and grown with shaking at 37^{0}C for 16 h. The other half was streaked onto two nutrient agar plates and incubated at 37^{0}C for 16 h. Antibiotics were added to the media when appropriate. If the colony form was uniform, single colonies were selected and picked off plates for subsequent storage or use. Short–term storage of strains in routine use was acheived by suspension of freshly grown bacteria in glycerol (32% (v/v)) and peptone (0.6% (w/v)) at -70^{0}C. Fresh cultures from glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar plate (with of without anitbiotic) followed by incubation at 37^{0}C for 16h just prior to use.

Bacterial strains were prepared for long–term storage by suspension of several colonies in a small volume of sterile skimmed milk. Approximately 0.2 ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25 in.×4 in. freeze

drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilised in a freeze drier. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constricton was made just above the level of the plug. The ampoules were evacuated to a partial pressure of 30 microns and then sealed at the constriction without releasing the vacuum. Finally the ampoules were labelled and stored at 4^0C.

2.5 Bacterial strains

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 *E. coli* K-12 and any other strains used in this study.

2.6 Plasmids

R-factors, plasmids and cloning vectors which were used in this study are listed in Table 2.3.

2.7 Sources and preparation of red blood cells

The red blood cells routinely used for the haemagglutination assay were obtained from BALB/c mice. The blood samples were collected in 3.8%(w/v) sodium citrate, washed three times in modified Krebs-Ringer buffer (KRT) (Freter and Jones, 1976) and resuspended to 1%(v/v) before use in haemagglutination experiments.

Human RBCs of blood groups O, A and B were provided by volunteers in our laboratory.

2.8 Haemagglutination assay

Techniques for quantitation of haemagglutination and haemagglutination inhibition with sugars were adapted from Jones *et al.* (1976). Cultures were shaken overnight (16 hours) at 37°C. Bacterial cells were sedimented by centrifugation at 5,000 x g for 10 min. The pellet was then resuspended in 1 ml of modified Krebs-Ringer buffer. Bacteria were twofold serially diluted in round-bottomed microtitre plates (Catalog no. 1-221-24; Dynatech Laboratories, Inc., Alexandria, Va.) in 50 μl of modified Krebs-Ringer buffer. A 50 μl sample of 1% washed RBCs was then added, the tray was tapped and the RBCs were allowed to settle at room temperature for 60 min. The titre was defined as the reciprocal of the highest dilution at which haemagglutination was visible.

2.9 Haemagglutination inhibition assay

To test for inhibition of haemagglutination, a suspension of bacteria adjusted to contain equivalent to twice the haemagglutination titre dose (2 haemagglutination units) was used. Sugars (10 mg/ml in modified Krebs-Ringer buffer) were twofold serially diluted in microtitre plates. The haemagglutinin suspension was then added and allowed to act for 30 min at room temperature. RBCs were then added and incubated for a further 60 min, after which the trays were read. Sugars tested include D-mannose, D-fructose, D-glucose, D-galactose and L-fucose.

2.10 Assay for chemotaxis

To test for chemotaxis, the capillary test described by Freter and O'Brien (1981a) was used.

2.11 Antisera

2.11.1 Antisera production

Rabbit antisera to the purified soluble haemagglutinin (SHA) were generously provided by both R.A. Finkelstein (Department of Microbiology, Columbia, Missouri) and A.–M. Svennerholm (Department of Medical Microbiology, Göteberg, Sweden).

Antisera against both the native and denatured forms of the mannose–fucose resistant haemagglutinin were prepared as follows:

(a) Antiserum to the native form was prepared by giving rabbits three intraveneous injections at fortnightly intervals of live bacteria (i.e. *E. coli* cells harbouring the cloned haemagglutinin).

(b) Antiserum to the denatured form of the protein was raised to bands extracted from polyacrylamide gels. This was accomplished by electrophoresing cell envelope preparations (10 mg/ml) on a SDS polyacrylamide gel, staining a strip with Coomassie Brilliant Blue to identify the location of the desired band, then aligning this strip with the gel and excising the corresponding location. The strip was washed in distilled water to remove SDS and then homogenized and injected with Freund's incomplete adjuvant. Rabbits were immunized subcutaneously, by three repeated injections at fortnightly intervals.

2.11.2 Selective absorption of antiserum by intact cells

The antisera were absorbed by mixing 2 ml of antiserum with 10^9 *E. coli* K-12 cells, incubating at 37°C for 60 min and removing the cells by centrifugation (5,000 rev/min, 10 min). This process was repeated a second time with incubation at 4°C overnight. The serum was then filter sterilized, using a 0.22 μm pore Millipore filter (Millipore Corp., Bedford Mass.).

2.12 Transformation procedure

Transformation was performed essentially by the method described by 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 BHI and incubated with shaking until the culture reached an OD of 0.6 (4 x 10^8 cells/ml). 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 $CaCl_2$. This was allowed to stand for 60 min on ice before addition of DNA. Competent cells (0.2 ml) were then mixed with DNA (volume made to 0.1 ml with TE buffer: 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 and then 3 ml BHI was added followed by incubation with shaking at 37°C for 1-2 hours. The culture was plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

2.13 DNA extraction procedures

2.13.1 Plasmid DNA isolation

Plasmid DNA was isolated by one of the three following procedures:

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 shaking overnight culture were pelleted (this yielded about 1 × 10^9 cells) in an Eppendorf 5414 centrifuge for 30 secs, resuspended in 50 μl STET buffer (5%(w/v) sucrose, 5%(v/v) Triton X-100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0). Lysozyme (5 mg/ml; 5 μl) was added and the suspension left at room temperature for several min. Samples were then placed in boiling water for 35 secs and immediately spun for 10 min in an Eppendorf centrifuge. The chromosomal pellet was removed and plasmid DNA 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 centrifuge for 10 min, washed once with 1 ml 70%(w/v) ethanol, dried *in vacuo* and dissolved in 20 μl TE buffer. This method was also scaled up for use with 10 ml cultures.

Method 2: Triton X-100 cleared lysates were prepared from 10 ml overnight cultures by a modification of the procedure of Clewell and Helinski (1969, 1970). Cells were resuspended in 0.4 ml 25%(w/v) sucrose in 50 mM Tris-HCl, pH 8.0. Lysozyme (50 μl, 10 mg/ml freshly prepared in H_2O) and 50 μl of 0.25 M EDTA, pH 8.0 were added to cells in Eppendorf tubes and left to stand on ice for 15 min. 0.5 ml 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, SS34, Sorvall). The supernatant was extracted twice with TE saturated phenol (pH 7.5) and twice with diethyl-ether. Plasmid DNA was precipitated by the addition of an equal weight of propan-2-ol and allowed to stand at $-70°$ C for 30 min. The precipitate was collected (10 min, Eppendorf 5414), washed once with 1 ml 70%(v/v) ethanol, dried and resuspended in 50 μl TE buffer.

Method 3: large scale plasmid purification was performed by the three step alkali lysis method (Garger *et al.* 1983). Cells from a litre culture were harvested (6,000 rpm, 15 min, 4°C, GS-3, Sorvall) and resuspended in 24 ml 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 M NaOH, 1%(w/v) SDS), followed by 5 min incubation on ice resulted in total lysis of the cells. After the addition of 28 ml 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, GSA, Sorvall). 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 aqueous phase was precipitated with 0.6 volume of 100%(v/v) propan-2-ol at room temperature for 10 min and collected by centrifugation (10,000 rpm at 4°C, 35 min, GSA, Sorvall). After washing in 70%(v/v) ethanol, the pellet was dried *in vacuo* 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 19 gauge needle attached to a 1 ml syringe. The ethidium bromide was extracted using CsCl saturated isopropanol. CsCl was then removed by dialysis overnight against three changes of 2 litres TE at 4°C. DNA was stored at 4°C.

2.13.2 Preparation of *V.cholerae* genomic DNA

V. cholerae genomic DNA was prepared according to Manning *et al* (1986). Cells from a 20 ml shaking 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 20 min. TE buffer (0.75 ml) and 0.25 ml lysis solution (5%(w/v) sarkosyl, 50 mM Tris-HCl, 0.25 M EDTA, pH 8.0) were 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%(v/v) ethanol. The precipitate was washed twice with 70%(v/v) ethanol, dried *in vacuo* for 60 min and dissoloved in 1 ml TE buffer. This was achieved by heating to 56°C for several min. This generally yielded high molecular weight DNA at concentrations ranging from 0.5–1.0 mg/ml.

2.14 Analysis and manipulation of DNA

2.14.1 DNA quantitation

The DNA concentration 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).

2.14.2 Restriction endonuclease digestion of DNA

Cleavage reactions of the restriction enzymes *Hin*dIII, *Bam*HI, *Eco*RI, *Pst*I, *Cla*I and *Xba*I were performed using SPK buffer (10 ×: 200 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 5 mM dithioerythritol, 1 mM EDTA, 500 mM KCl and 50% glycerol). The remaining restriction digests were carried out as described by Davis *et al.* (1980). 0.1-0.5 μg of DNA or purified restriction fragments were incubated with 2 units 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%(w/v) Ficoll, 0.1%(w/v) bromophenol blue) was added.

2.14.3 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%(w/v) agarose gels (Seakem HGT), 13 cm long, 13 cm wide and 0.7 cm thick. Gels were run at 100 V for 4-5 hours 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 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%(w/v) was used for separation of restriction fragments, which were recovered by one of the following methods.

Method 1: DNA bands were excised and the agarose melted at 65°C. Five volumes of 20 mM Tris-HCl, 1 mM EDTA, pH 8.0 buffer was added and the agarose extracted with phenol:water and then phenol:chloroform (both 1 g/ml). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one tenth volume of 3 M sodium acetate, pH 5.0.

Method 2: After separation of fragments had occurred, the gel was lightly stained with ethidium bromide and the bands visualized by long-wave UV light. The agarose in front of the desired restriction fragment was removed and dialysis tubing was placed, such that the DNA moved into this well by electrophoresis and could subsequently be collected by a pasteur pipette. The ethidium bromide was extracted using isoamyl alcohol, followed by dialysis overnight against TE at 4°C, with at least three changes. DNA was stored at 4°C.

2.14.4 Isolation of DNA fragments less than 1,000bp

Digested DNA was end-labelled using α-[^{32}P]-dCTP. Prior to loading onto the gel. a one-tenth volume of tracking dye (10× : 1%(w/v) bromophenol blue, 50%(v/v) glycerol, 37.5 mM EDTA) was added. The sample was then loaded onto a 30% polyacrylamide gel. The gel was electrophoresed at 400 V until the tracking dye reached the bottom of the gel after which the glass plates were separated and the gel placed on film for 30 min. The gel slices which contained the labelled DNA fragments were located by super-imposing the autoradiograph over the gel. The DNA band was excised and the DNA was eluted by soaking the gel slices overnight in 400 μl of gel elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1%(w/v) SDS, pH 7.6) and the supernatant was ethanol precipitated.

2.14.5 Calculation of restriction fragment size

The size of restriction enzyme fragments were calculated by comparing their relative mobility with that of *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA.

The calculated sizes of the SPP1 *Eco*RI standard fragments used which differed from those published (Ratcliff *et al.* 1979), were calculated using bacteriophage lambda and plasmid pBR322 as standards and using the program DNAFRAG (Rood and Gawthorne, 1984). The sizes (kilobases, kb) used were: 8.0; 7.1; 6.0; 4.78; 3.44; 2.77; 1.93; 1.88; 1.55; 1.43; 1.2; 1.03; 0.7; 0.48.

2.14.6 *In vitro* cloning

DNA to be subcloned (3 μg) was cleaved in either single or double restriction enzyme digests. This was combined with 1 μg of similarly cleaved vector DNA, then ligated with 2 units of T4 DNA 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 reaction was stopped by heat inactivation of the T4 DNA ligase at 65°C for 10 min. The ligated DNA was then used directly for transformation of strain LE392. Transformants were screened for insertional inactivation of the appropriate drug resistance (Ap or Tc), wherever possible, prior to plasmid DNA isolation.

2.14.7 Generation of deletions using nuclease *Bal*31

DNA (200 μl) was digested with the appropriate restriction endonuclease and heat inactivated at 65°C for 10 min. The linearized DNA was then digested with *Bal*31 (the digest contained 225 μl linearized DNA, 30 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 12 mM $CaCl_2$, 0.25 mM NaCl, 3 units *Bal*31) and samples were taken from the digest at various times (min intervals) and the reaction stopped by the addition of 10 μl GEBS (GEBS: 20%(v/v) glycerol, 50 mM EDTA, 0.05%(w/v) bromophenol blue, 0.5%(w/v) sarkosyl). The samples were desalted in 1 ml Sepharose CL-6B columns, dried *in vacuo* and resuspended in 20 μl of water. The ends were filled in with Klenow by adding 2.5 μl of 10 × nick translation buffer [10 ×: 0.5 M Tris-HCl, pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 μg/ml BSA (Pentax Fraction V), and 1 μl each of dNTP's (2 mM)] and 5 units/μl of Klenow and incubating at room temperature for 30 min. The reaction was stopped with 5 μl GEBS and heated at

65°C for 10 min. The DNA was desalted and dried down as before and the linkers were ligated to the DNA. Linker ligation was performed by adding 10 μl of 1 × linker kinase buffer (10 ×: 0.66 M Tris-HCl, pH 7.6, 10 mM ATP, 10 mM spermidine, 0.1 M $MgCl_2$, 150 mM dithiothreitol, 2 mg/ml BSA), 3 μl of phosphorylated linkers and 2 units of T4 DNA ligase and incubated overnight at 4°C. The reaction was stopped with 30 μl of 1/4 GEBS and heated at 65°C for 10 min. The DNA was desalted and digested with the appropriate restriction endonuclease to cleave the linkers, heated at 65°C for 10 min and then ligated overnight at 4°C. The ligated DNA was transformed into *E. coli* K-12 strain DH1 and the sizes of the deletions were determined by isolating plasmid DNA, digesting with appropriate restriction endonucleases and analyzing the digests on a 0.8%(w/v) agarose gel.

2.14.8 Dephosphorylation of DNA using alkaline phosphatase

0.1-0.5 μg of digested plasmid DNA was incubated with 1 unit of molecular biology grade alkaline phosphatase for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to give a final concentration of 3 mM followed by heating at 65°C for 10 min. The reaction mix was then extracted twice with hot TE saturated phenol and twice with diethyl ether. DNA was precipitated overnight at −20°C with two volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, pH 8.0. The precipitate was collected by centrifugation (15 min, Eppendorf 5414), washed once with 1 ml 70%(v/v) ethanol, dried *in vacuo* and dissolved in TE buffer.

2.14.9 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E. coli* DNA polymerase I. Typically, 1 μg of digested DNA, 2 μl of 10 × nick-translation buffer (Maniatis *et al.*, 1982), 1 μl of each dNTP (2 mM) and 1 unit Klenow fragment were mixed and incubated for 30 min.

The reaction was stopped by heating at 65°C for 10 min, followed by removal of unincorporated dNTPs and enzyme by centrifugation through a Sepharose CL-6B column.

2.14.10 End-filling with T4 DNA polymerase

Plasmid DNA was cleaved and cohesive ends 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 (2 mM) and 1 μl of 10 × T4 DNA polymerase buffer (Maniatis *et al.* 1982). After a 5 min incubation at 37°C, the reaction was stopped by heating at 65°C for 10 min. Salt, unincorporated nucleotides and enzyme were removed by passage through a Sepharose CL-6B column.

2.14.11 Ligation of Linkers to blunt DNA ends

Phosphorylated linkers were ligated to blunt ends generated by T4 DNA polymerase by overnight incubation of 1 μg plasmid DNA with approximately 3 μl linkers and 4 units T4 DNA ligase in a final volume of 10 μl of 1 × linker-kinase buffer (Maniatis *et al.* 1982).

2.14.12 Construction of gene banks

In vector pBR322

Whole genomic DNA from *V. cholerae* strain 569B was partially digested using restriction endonuclease *Bam*HI. 10 μl of chromosomal DNA (3 μg) was digested for up to 60 min at 37°C with 10 units of *Bam*HI in a final volume of 20 μl. The extent of digestion was checked by electrophoresis. 15 μl of the partially digested DNA was added to 1 μl (0.3 μg/ul) of pBR322 (cut with *Bam*HI and treated with alkaline phosphatase) and 3 μl of 10 × modified ligation buffer (10 μl 1 M Tris-HCl, pH 7.5, 5 μl 1 M MgCl, 5 μl 1 M DTT, 3 μl 0.1 M ATP and 27 μl H_2O). T4 DNA ligase was then added and the mixture was made to 30 μl with H_2O. After incubation at 10°C for 3 hours, the reaction was diluted by addition of 60 μl of 1 ×

ligation buffer and then further incubated overnight at 4°C. Ligation was stopped by heating for 10 min at 65°C and the ligated DNA (90 μl) was used to transform 200 μl of competent cells (*E. coli* K-12 strain LE392). More than 2,000 transformants were obtained, of which at least 95% were Ap^R Tc^S.

In cosmid vector pHC79

Genomic fragments of approximately 40 kilobases (kb) were obtained by controlled partial digestion with the restriction endonuclease *Sau*3A (Maniatis *et al.* 1982). The cosmid vector used was pHC79 (Hohn and Collins, 1980) which was restricted with *Bam*HI and treated with alkaline phosphatase to prevent self-ligation. The two DNAs were mixed, ligated overnight and packaged *in vitro* into bacteriophage λ. The packaged phage were then used to infect *E. coli* K-12 strain DH1. Cells harbouring cosmid clones were detected by plating onto nutrient media containing Ap. At least 90% of colonies were Tc^S.

2.14.13 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 (1,700 Ci/mmole in ethanol) was dried *in vacuo* in an Eppendorf tube, resuspended with 80 μl water, 10 μl of 10 × 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, dTTP. DNA (1 μg) was added and incubated at 37°C for 10 min. DNA polymerase I (5 units) was added to the mix and allowed to incubate at 16°C for 2 hours. [^{32}P]-labelled DNA was separated from unincorporated label by centrifugation though a mini-column of Sepharose CL-6B.

2.14.14 Southern transfer and hybridization

Bidirectional transfers of DNA from agarose gels to nitrocellulose paper (Schleicher and Schüll) were performed as described by Southern (1975) and modified by

Maniatis *et al.* (1982).

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

Filters were washed twice with shaking at 37°C for 30 min in 2 x SSC, containing 0.1%(w/v) SDS. This was followed by two further washes in 0.1 x SSC plus 0.1%(w/v) SDS at 65°C. After drying in air (15 min, room temperature), the filters were covered in plastic wrap and placed on film for autoradiography at −70°C with intensifying screens.

2.14.15 Colony hybridization

Colonies containing DNA hybridizing with radioactively labelled DNA fragments were detected by the procedure of Grunstein and Hogness (1975) an outline of which follows. Patched colonies were grown for 5 hours at 37°C. Nitrocellulose discs were placed on top of the colonies and allowed to absorb. The filters were then passed through four different solutions at intervals of five min: i) 10%(w/v) SDS; ii) 0.5 M NaOH, 1.5 M NaCl; iii) 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0; iv) 2 × SSPE (20 ×: 3.6 M NaCl, 200 mM NaH_2PO_4, pH 7.4, 20 mM EDTA, pH7.4). Filters were then placed colony side up on Whatman paper, air dried and baked at 80°C for 2 hours *in vacuo*.

2.15 Transposition with Tn*1725*

Tn*1725* (Cm) transposition to plasmid DNA was performed in the following manner: Plasmid pRU669 (R_{ts}1:Tn*1725*) (Ubben and Schmitt, 1986) was transferred

by conjugation, into an *E. coli* K-12 derivative harbouring the target plasmid. This was achieved by mating for 3 hours at 30°C in a standing culture which consisted of 0.1 ml of an overnight culture of C600 [$R_{ts}1$:Tn*1725*] with 0.9 ml V271 and 1 ml BHI broth. Following plating of 0.1 ml of mating mix on NA containing Cm and Ap, independent exconjugants were purified and used for growing up an overnight cultures at 37°C in NB containing both antibiotics to select for the transposon (Cm) and the plasmid (Ap). Triton X-100 lysates (10 ml) prepared from these cultures were used to transform C600, again selecting for both the plasmid and the transposon. Following overnight incubation at 37°C, transformants were randomly chosen for analysis of their plasmid DNA.

2.16 Protein analysis

2.16.1 Minicell procedures

Minicells were purified and the plasmid-encoded proteins labelled with [^{35}S]-methionine as described by Kennedy *et al.* (1977) and modified by Achtman *et al* (1979). This involved separation of minicells from whole cells (500 ml overnight culture in LB medium) by centrifugation through two successive sucrose gradients, pre-incubating to degrade long lived mRNAs, then pulse labelling with [^{35}S]-methionine in the presence of methionine assay medium. Minicells were subsequently solubilized by heating at 100°C in 100 μl of 1 × sample buffer (Lugtenberg *et al.* 1975).

2.16.2 SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 11-20% gradients for proteins using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100°C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 5 hours (11-20% gradient gels). Protein staining was achieved by incubation, with gentle agitation overnight at room temperature in 0.06%(w/v) Coomassie Brilliant Blue

G250 (dissolved in 5%(v/v) perchloric acid). Destaining was accomplished with several changes of 5%(v/v) acetic acid, with gentle agitation for 24 hours.

Size markers (Bio-Rad) were phosphorylase B (92.5 kDal), bovine serum albumin (66.2 kDal), ovalbumin (45 kDal), carbonic anhydrase (31 kDal), soybean trypsin inhibitor (21.5 kDal) and lysozyme (14.4 kDal).

2.16.3 Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 hours on a Bio-Rad gel drier. [^{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]-phosphate labelled DNA, the gels were exposed to film for 6-72 hours at $-70°$C, using intensifying screens.

2.16.4 Small scale cell envelope isolation

Whole membrane material was isolated from 10 ml mid-exponential phase cultures by the method of Manning *et al.* (1982). The cultures were harvested by centrifugation (10 min) at 2,000g and the cells were washed with 10 ml 30 mM Tris-HCl, pH 8.1. The cells were pelleted, by centrifugation, resuspended in 0.2 ml 20%(w/v) sucrose, 30 mM Tris-HCl, pH 8.1 and transferred to SM24 tubes (Sorvall) on ice. After addition of 20 μl lysozyme (1 mg/ml in 0.1 M EDTA, pH 7.3) incubation was continued on ice for a further 30 min. 3 ml of 3 mM EDTA, pH 7.3 was added and the cells were sonicated in a Branson sonicator (four, 15 secs pulses on 50% cycle). Membrane material was pelleted by centrifugation (20,000 rpm, 60 min, 4°C, Sorvall) and resuspended in 100 μl 1 $\times$ sample buffer (Lugtenberg *et al.* 1975). Samples were stored at $-20°$C. 10-15 μl amounts were loaded onto SDS-polyacrylamide gels.

2.16.5 Whole cell preparation

1 ml of an overnight culture (1×10^9 cells) was placed in a microfuge tube and the cells were collected by centrifugation (30 secs, Eppendorf 5414). The cell pellet was

resuspended in 100 μl of 1 $\times$ sample buffer (Lugtenberg *et al.* 1975) and heated at 100°C for 3 min prior to analysis by SDS-PAGE gel electrophoresis. The remainder of the sample was stored at -20^0C for future use.

2.16.6 Western transfer and protein blotting

The procedure used was a modification of that described by Towbin *et al.* (1979). Samples were subjected to SDS-PAGE (11-20% gradient gels) and transferred to nitrocellulose (Schleicher and Schüll) at 200 mA for 2 hours in a Trans-Blot Cell (Biorad). The transfer buffer used was 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 5%(v/v) methanol. After transfer, the nitrocellulose sheet was incubated for 30 min in 5%(w/v) skim milk powder in TTBS (0.05%(v/v) Tween 20, 20 mM Tris-HCl, 0.9%(w/v) NaCl) to block non-specific protein binding sites.

Antiserum was diluted 1/1000 in TTBS, 0.02%(w/v) skim milk powder (unless stated otherwise) and incubated with gentle agitation at room temperature for 2-16 hours. The antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. Detection of bound antibody was achieved by incubating for 2-16 hours (gentle agitation) with goat anti-rabbit IgG coupled with horseradish peroxidase (Nordic Immunology) at a dilution of 1/5000 in TTBS plus 0.2%(w/v) skim milk powder. The nitrocellulose sheet was then washed four times (5 min intervals) with TTBS, followed by two 5 min washes in TBS (20 mM Tris-HCl, 0.9%(w/v) NaCl).

To detect the presence of the antigen-antibody complexes peroxidase substrate (9.9 mg 4-chloro-1-napthol dissolved in 3.3 ml -20°C methanol added to 16.5 ml TBS containing 15μl hydrogen peroxide) was then added and allowed to incubate 10-15 min with shaking, as described by Hawkes *et al.* (1982).

2.16.7 Colony transfer and blotting with antiserum

A nitrocellulose disc (9 cm diameter) was placed onto agar plates containing the colonies to be screened. Once the colonies had adhered to the disc (3 min), it was

removed and placed, colony side up, on a piece of Whatman 3 MM paper moistened with 0.5 M HCl to lyse and fix the colonies, then allowed to stand in the dark for 30 min. The cell debris were removed from the nitrocellulose with a jet of saline (0.9%(w/v) NaCl). The method used for antigen detection was the same as that for western blotting.

2.17 M13 cloning and sequencing procedures

2.17.1 Preparation of M13 replicative form (RF) DNA

Fresh 2 × TY broth (10 ml) was inoculated with 10 μl of an overnight culture of JM101 (in M13 minimal medium). A single plaque of M13mp18 or M13mp19 picked from an H agar plate with a sterile toothpick was added to this bottle. The culture was grown at 37°C with vigorous shaking for 6 hours. Bacterial cells were removed by centrifugation (5,000 rpm, 10 min, bench centrifuge) and the supernatant added to 1l NB containing 10 ml of a shaken overnight culture of JM101. Following incubation for 14 hour at 37°C with shaking, replicative form DNA was subsequently prepared as described above for plasmid DNA purification.

2.17.2 Cloning with M13mp18 and M13mp19

The M13 vectors, M13mp18 and M13mp19 (Messing and Vieira, 1982) were used for selective cloning of restriction enzyme generated DNA fragments. Stocks of M13 vectors cleaved with various enzyme combinations were stored at 4°C, after heat inactivation of enzymes. Plasmid DNA was cut with the appropriate enzyme combinations for subcloning into the M13 vectors.

The ligation conditions used for blunt ends and cohesive ends were identical. The reaction mixtures consisted of the DNA to be cloned (100 ng) and the DNA vector (20 ng) in a final volume of 10 μl of ligation buffer. Ligation with T4 DNA ligase was carried out overnight at 4°C.

2.17.3 Transfection of JM101

Strain JM101 was made competent for transformation as described in section 2.9. Competent cells (0.2 ml) were added directly to the ligation mixes and incubated on ice for 30 min. This was followed by a 2 min heat shock at 42°C. Cells were then transferred to sterile test tubes to which was added a mixture of JM101 indicator cells (200 μl), 100 mM IPTG (40 μl) and 2%(w/v) X–gal in N, N'–dimethyl formamide (40 μl) and finally 4 ml H top agar. The mixture was poured as an overlay onto an H agar plate and incubated overnight at 37°C.

2.17.4 Screening M13 vectors for inserts

White plaques were picked from X-gal, IPTG plates with sterile toothpicks and added to 1 ml 2 × TY broth in microfuge tubes containing a 1:100 dilution of an overnight culture of JM101. These tubes were incubated for 5 hours at 37°C. The cells were pelleted by centrifugation (30 secs, Eppendorf) and 0.1 ml of supernatant was used to innoculate 10 ml NB containing 0.1 ml JM101 (overnight culture). This mixture was incubated with shaking at 37°C overnight. RF DNA, suitable for restriction analysis,was prepared by the Triton X-100 cleared lysate method (section 2.10.1). After restriction enzyme digestion, DNA was examined on 1%(w/v) agarose gels.

2.17.5 Purification of single-stranded template DNA

M13 RF DNA containing appropriate inserts were reintroduced into JM101 and single white plaques from this transfection picked with sterile tooth picks to innoculate 2 ml 2 x TY broth containing 20 μl of an overnight culture of JM101. After vigorous shaking at 37°C for 6 hours, the culture was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was transferred to clean tubes and recentrifuged for 5 min. A 1 ml aliquot of the supernatant from each tube was withdrawn and mixed in a fresh tube with 0.27 ml 20%(w/v) PEG, 2.5 M NaCl. These tubes were then incubated at room temperature for 15 min. The phage were pelleted

by centrifugation for 5 min in an Eppendorf 5414 centrifuge and the supernatant discarded. Following another short spin (10 sec), the remainder of the PEG/NaCl supernatant was removed with a drawn out pasteur pipette. The pellets were re-suspended in 0.2 ml TE buffer. Redistilled TE saturated phenol (0.1 ml) was then added to the phage suspension and the tubes were briefly vortexed. After standing for 15 min at room temperature, the tubes were centrifuged for 2 min and 0.15 ml of the top phase transferred to clean tubes. To the aqueous phase was added 6 μl of 3 M sodium acetate, pH 5.0 and 400 μl absolute ethanol. Single-stranded DNA was precipitated at $-20°$C overnight, followed by centrifugation for 15 min in an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70%(v/v) ethanol followed by centrifugation. After drying *in vacuo* the pellets were resuspended in 25 μl TE buffer and stored at $-20°$C until required.

2.17.6 Dideoxy sequencing protocol

The method is based on that described by Sanger *et al.* (1977,1980). Stock solutions of the four dNTPS and ddNTPs were 10 mM in TE buffer and stored frozen at $-20°$C. Working stocks of the dNTPs were made by diluting to 0.5 mM with TE. Working stocks of the ddNTPs were diluted to the following concentrations in TE : ddATP (0.1 mM), ddCTP (0.1 mM), ddGTP (0.3 mM) and ddTTP (0.5 mM).

The deoxynucleotide mixes (A, C, G, T) were made for each of the four sequencing reactions, with [^{32}P]-dCTP, as follows:

Components	Mixes			
	A°	C°	G°	T°
0.5 mM dATP	4*	40	40	40
0.5 mM dCTP	–	5	–	–
0.5 mM dGTP	40	40	6	40
0.5 mM dTTP	40	40	40	6
10 × TE buffer	10	10	10	10

*figures indicate volume in μl

Mixes of N° and working solutions of ddNTPs were made by the addition of the following combination of components:

Components	mixes			
	A°+ddA	C°+ddC	G°+ddG	T°+ddT
N°	7*	7	7	7
ddNTP	14	14	14	14

*figures indicate volume in μl

These were stored at $-20°$C until required for later use in sequencing reactions.

The annealing of synthetic primer to template was achieved by incubating 6 μl template, 1 μl M13 primer, 1 μl 10 x TM buffer (100 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$) and 2 μl water. The mixture was heated at 65°C for 60 min and then allowed to cool at room temperature. Rows of four microfuge tubes (one tube for each sequencing reaction) were prepared containing 2 μl of annealed DNA. 5 μCi of [^{32}P]-dCTP was dispensed into each of four tubes marked A, C, G and T and dried. The solution of appropriate N°/ddN mix was used to resuspend the dried label. The N°/ddN label mix (2 μl) was aliquoted into each of four tubes (one for each

sequencing reaction) containing 2 μl of annealed DNA. To the side of each tube was added 2 μl Klenow fragment (0.125 units/μl TM buffer). These components were simultaneously brought together by a brief spin in an Eppendorf 5414 centrifuge and the reaction mixes incubated at 37°C for 13 min. Chase solution (2 μl), consisting of 0.25 mM of each dNTP and 0.025 units Klenow/μl, was added to the side of each tube and the chase reaction started by another brief spin. After 15 min at 37°C, 4 μl formamide dye mix (95%(w/v) formamide, 0.1%(w/v) xylene cyanol, 0.1%(w/v) bromophenol blue, 10 mM EDTA, pH 8.0) was added to stop the reaction. Reaction mixes were heated in a 100°C heating block for 2.5 min and immediately 0.5-1.0 μl loaded onto 6% polyacrylamide denaturing gels (see below). For re-running, these samples were boiled for 60 secs prior to loading.

2.17.7 DNA sequencing gels

Polyacrylamide gels for DNA sequencing were prepared using glass plates 33 × 39.4 cm and 33 × 42 cm. Spacers and combs were high density polystyrene (0.25 mm thick). The gel mix contained 70 ml acrylamide stock (5.7%(w/v) acrylamide, 0.3%(w/v) bis-acrylamide, 8 M urea in 1 × TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA, pH 8.3), 420 μl 25% ammonium persuflate and 110 μl TEMED (N,N,N',N'-tetramethyl-ethylene-diamine, Sigma). After thorough mixing this gel mix was poured into a clean gel sandwich and the comb inserted. Polymerization took place for 60 min at 37°C, with the gel in a horizontal position.

The gel was mounted onto the sequencing apparatus and a waterjacket was attached to the outside plate of the gel. This consisted of a plastic bag wedged between two 0.3 cm thick spacers and a third plate. The plastic bag was filled with 1 × TBE buffer and this was sufficient to evenly distribute heat throughout the gel. Gels were pre-electrophoresed at 700 V for 30 min. After the samples had been loaded the gel was electrophoresed using a constant voltage (700 V) for 15 min, which was increased to 1200 V (33 mA). After 4 hours the samples were reloaded into a second set of wells on the same gel. The gel was further electrophoresed, initially at 700 V, then 1200 V for 2.5 hour by which time the bromophenol blue dye front

from the second loading, had reached the bottom of the gel. Plates were separated by heating one side of the gel with hot running water. Generally the polyacrylamide gel bound to the cold plate. Tissue paper was used along the borders of the gel to hold it to the plate during the fixation procedure which involved slowly washing the gel using 1.5 litres of 10%(v/v) acetic acid, 20%(v/v) ethanol in a 60 ml syringe. The gel was then dried at 100°C for 20 min. Plastic wrap was used to cover the gel before placing on film for autoradiography. Autoradiography was performed at room temperature, without the use of intensifying screens, for 16-24 hours.

2.17.8 Analysis of DNA sequences

Sequencing data was subject to analysis using the computer program Nucleic Acids Analysis System, version 1.7, the IBI Pustell Sequence Analysis Program version 4.0 and the LKB DNA and protein analysis programs, DNASIS and PROSIS.

2.18 Animal experiments

2.18.1 Infant mouse cholera model

This method was first described by Ujiiye *et al.* (1968) and was used to assess the virulence of *V. cholerae* strains. Infant mice were used at five to six days of age (weight, 2.4-2.7 g) and were removed from their parents about 6 hr before use, to permit the emptying of stomach contents. Mice received 0.1 ml of bacterial suspension that was administered orally by means of a smooth-tipped hypodermic needle. After challenge, the mice were kept on tissue paper in plastic containers at 25°C.

2.18.2 Virulence tests

Serial 10-fold dilutions (in peptone saline–a 0.1% [wt/vol] solution of proteose-peptone [Difco] in 0.85% NaCl) were prepared from the test culture and used to feed groups of 8 to 12 mice. Forty-eight hours after challenge, the number of mice

surviving within each group was noted and these data were used to construct a plot of cumulative percentage mortality vs. the log dose administered (Reed and Muench, 1938). By interpolation, an estimate of the LD_{50} was obtained, that is, the dose of vibrios capable of killing 50% of the mice within 48 hr. Strains with an $LD_{50}>10^9$ were considered nonpathogenic in this model.

2.18.3 Adherence to HEp-2 cells

HEp-2 cells were cultured in RPMI 1640 medium (Gibco Laboratories, N.Y.) containing 10% heat inactivated foetal calf serum (Gibco Laboratories, N.Y.). Approximately 18 hr prior to the experiment, 35 mm tissue culture dishes (Becton Dickinson, Calif.) were innoculated with 5-7 $\times$ 10^5 cells per plate. The plates were incubated at 37^0C, in 5% CO_2. Adherence experiments were performed by incubating HEp-2 monolayers which had been washed in antibiotic free RPMI medium with 10^7/ml of 2 $\times$ washed bacteria (in PBS) at 4^0C for 15 min with gentle agitation. The monolayers were then washed twice with cold PBS, fixed with cold (-20^0C) methanol, dried in air at room temperature and stained with Giemsa.

Table 2.1 *Vibrio cholerae* strains

Strain	Biotype/Serotype	Genotype/Phenotype	Source
569B	Classical Inaba	Sm^R, non-motile	K. Bhaskaran
569B	Classical Inaba	Sm^R, motile	S. Attridge
O17	El Tor Ogawa	Sm^R	K. Bhaskaran
CA401	Classical Inaba		C. Parker
V685	Classical Inaba	Rif^R, 569B	E. Bartowsky

Table 2.2 *Escherichia coli* strains

Strain	Genotype/Phenotype	Source/Reference
LE392	F$^-$, *supF*, *supE*, *hsdR*, *galK*, *trpR*, *metB*, *lacY*, λ^-	L. Enquist
AB1133	F$^-$, *thr*-1, *leu*-6, *proA*2, *lacY*1, *supE*44, *galK*2, *his*-4, *rpsL*31, *xyl*-5, *mtl*-1, *argE*3, *thi*-1, *ara*14, λ^-	P. Reeves
P651	*tolA* mutant of AB1133	P. Reeves
P236	*tolB* mutant of AB1133	P. Reeves
DH1	F$^-$, *gyrA*96, *recA*1, *relA*1, *endA*1, *thi*-1, *hsdR*17, *supE*44, λ^-,	B. Bachmann
DS410	F$^-$, *minA*, *minB*, *rpsL*	D. Sherratt
V271	LE392 (pPM471)	this study
JM101	F′, [*traD*36, *proAB*, *lacI*^{q}, *lacZ*, $\triangle$M15], *supE*, $\triangle$(*lac-proAB*), *supE*, *thi*-1	A. Sivaprasad
C600	F$^-$, *thr*-1, *leu*-6, *tonA*1, *lacY*1, *supE*44, *thi*-1	J. Reeve
537	C600 (pcI857)	J. Pohlner
S17-1	*pro*, *hsdR*$^-$, *hsdM*$^+$, RP4 2-Tc::Mu-Km::Tn7	U. Priefer
MM294	*pro*, *endA*, *hsdR*$^-$, *supF*	B. Bachmann

Table 2.3 Plasmids and cloning vectors

Plasmid	Antibiotic marker	Reference
pBR322	Ap, Tc	Bolivar *et al.* (1977)
pRK290	Tc	Ditta *et al.* (1980)
pHC79	Ap, Tc	Hohn and Collins (1980)
pRU669	Cm, Km, $R_{ts}1$::Tn*1725*	Ubben and Schmitt (1986)
pSUP205	Cm, Tc	Simon *et al* (1983)
pUC8	Ap	Vieira and Messing (1982)
pUC9	Ap	Vieira and Messing (1982)
pcI857	Km	Remaut *et al.* (1983)
M13mp18	Ap	Messing and Vieira (1982)
M13mp19	Ap	Messing and Vieira (1982)
pEv31a	Ap	Pohlner *et al.* (1986b)
pEv31b	Ap	Pohlner *et al.* (1986b)
pEv31c	Ap	Pohlner *et al.* (1986b)
pHIJ1	Gm, Spc, Sm	Ruvkun *et al.* (1982)

Chapter 3

Molecular Cloning of the Mannose-Fucose-Resistant Haemagglutinin of *Vibrio cholerae*

3.1 Introduction

Since 1961 when Bales and Lankford (1961) suggested that the interaction between *V. cholerae* and RBCs may mimic that of this organism with the intestinal epithelium, a number of workers have become interested in the various haemagglutinins of *V. cholerae* and their associated properties. Earlier studies mainly dealt with haemagglutinins as a possible means of establishing an easy differentiation system between organisms of the El Tor and Classical biotypes. However, because of the role haemagglutinins have been shown to play in the adherence process of a number of other organisms, recent studies have taken a new direction.

Hanne and Finkelstein (1982) described four distinct haemagglutinins that were expressed by *V. cholerae*. They found that Classical and El Tor biotypes have in common, a soluble HA as well as a mannose-fucose resistant cell associated haemagglutinin (MFRHA). In addition, each biotype were shown to possess a characteristic cell-associated HA. Thus, El Tor strains were seen to express a mannose-sensitive HA (MSHA) whereas Classical strains expressed a fucose-sensitive HA

(FSHA).

Each haemagglutinin differs with respect to the others in numerous ways: spectrum of RBC activity, sugar sensitivity pattern, Ca^{2+} requirement, growth medium (agar or broth) and phase of expression. Of these haemagglutinins, the soluble HA (SHA) has been studied in greatest detail. The cell-associated haemagglutinins have received little or no attention, with the MFRHA being completely neglected despite being found to be present in both biotypes.

Because of the possible role that the haemagglutinins of *V. cholerae* may play in the adherence and colonization of the intestinal epithelium, further genetic and molecular analysis may assist in unraveling their function in the pathogenic process. This chapter describes the molecular cloning of the gene encoding the MFRHA and subsequent characterization.

3.2 Results

3.2.1 Testing antiserum specificity

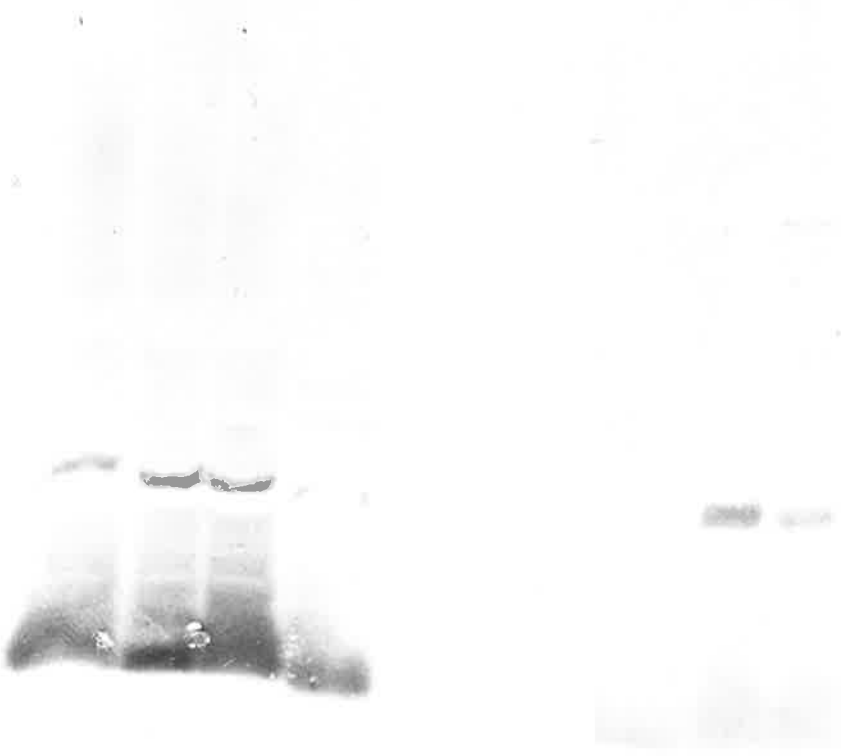
The antiserum to the soluble haemagglutinin (SHA), used in the following experiments was obtained from Professor R. A. Finkelstein. Finkelstein and Hanne (1982) purified the SHA from the Classical Inaba strain CA401 by ammonium sulphate fractionation, gel filtration and preparative isoelectric focussing. Antiserum was then prepared by subcutaneously injecting rabbits with 100 μg of this purified preparation of SHA, together with Freund's complete adjuvant. However, as will be seen, the antiserum not only contained antibodies to the SHA but also to some other protein species, enabling the isolation of the MFRHA.

This antiserum has been used in Western blot analysis with whole cell lysates of *V. cholerae*. In both Classical and El Tor strains, a protein of approximately 30 kDal was detected as the major component capable of reacting with the antiserum (Figure 3.1) although other proteins were observed which reacted/stained less intensely.

Figure 3.1 Western blot analysis following SDS-PAGE of cell envelopes of *V. cholerae* 569B, CA401, 017 and *E. coli* K-12 LE392. The blots were developed with both unabsorbed and absorbed (10^9 *E. coli* organisms/ml) rabbit antiserum to the soluble haemagglutinin, followed by goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase.

UNABSORBED ANTISERUM

ABSORBED ANTISERUM



3.2.2 Detection and isolation of the mannose-fucose resistant haemagglutinin clone

A gene bank was constructed in which whole genomic DNA from *V. cholerae* Classical Inaba strain, 569B, was partially digested with *Bam*HI and cloned into the *Bam*HI site of the vector pBR322 (Bolivar *et al.* 1977). The vector had been treated with alkaline phosphatase to prevent self-ligation. Approximately 2,000 colonies were patched onto ampicillin plates. Of these at least 95% were Ap^R Tc^S implying that inserted *Bam*HI fragments of *V. cholerae* DNA, had inactivated the tetracycline locus of pBR322.

These colonies were transferred to nitrocellulose discs and lysed *in situ* by the method of Henning *et al.* (1979). Colonies that expressed the haemagglutinin were detected using rabbit anti-SHA which had been absorbed with *E. coli* organisms. Visualization of reactive colonies was performed by adding goat anti-rabbit-immunoglobulin G coupled with horse-radish peroxidase as described by Hawkes *et al.* (1982).

One of about 2,000 colonies reacted with the antiserum. This colony together with several surrounding colonies which did not react, were purified and along with the non-haemagglutinating control *E. coli* K-12 strain, LE392 were retested for antiserum reactivity and haemagglutinating activity (Figure 3.2). The positive reactive isolate consistently reacted with the antiserum and exhibited haemagglutination activity, whereas none of the other isolates nor the control (LE392) showed either reaction with the SHA antiserum or an ability to haemagglutinate RBCs.

The plasmid in the positively reacting isolate was designated pPM471 and strain LE392 harbouring pPM471 was designated V271. As shown (Figure 3.2) strain LE392 harbouring either pPM471 or pPM1107 exhibited haemagglutination. pPM1107 is a derivative of the original recombinant plasmid pPM471 but with a reduced coding region. The derivation of pPM1107 is described in Section 3.2.9. Although the haemagglutination titres of V271 and LE392 [pPM1107] were low, these were equivalent titres to that of *V. cholerae* strain 569B from which the

Figure 3.2 Haemagglutination of mouse RBCs by *V. cholerae* 569B, *V. cholerae* 017 and *E. coli* K-12 LE392 harbouring PM471, pPM1107 and pBR322, respectively. The wells contain two fold serial dilutions of bacteria starting with 5 x 10^9 organisms/ml in the left most well. Included are RBCs and diluent (KRT buffer) alone as a control.

BACTERIA TWO-FOLD SERIALLY DILUTED

V271

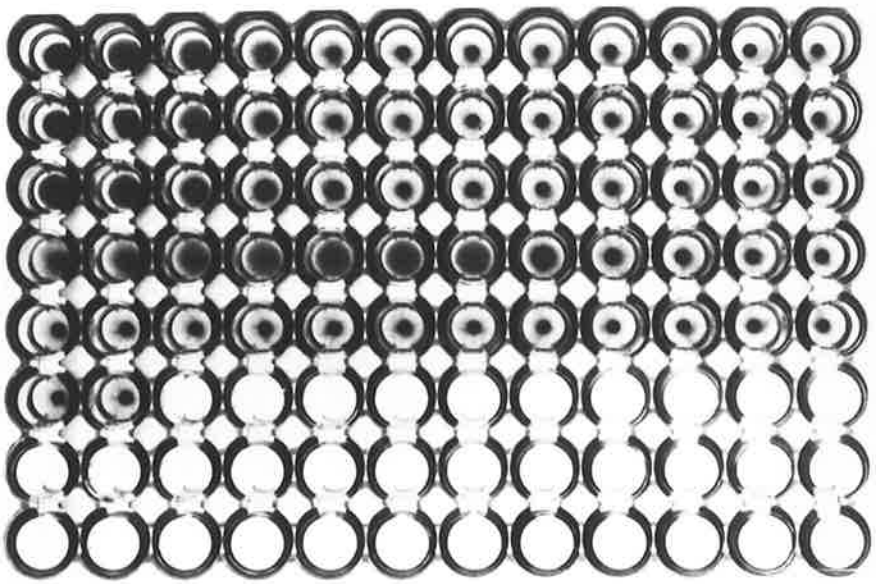
LE392 + pPM1107

V. CHOLERAE 569B

V. CHOLERAE 017

LE392

RBCs + BUFFER ONLY



NO BUTTON = HAEMAGGLUTINATION

BUTTON = NO HAEMAGGLUTINATION

cloned DNA originates. Strain LE392 was haemagglutination negative as expected. Included as a positive control was *V. cholerae* strain O17 (El Tor, Ogawa) which is known to give consistently high titres due to the presence of the cell-associated MSHA and possibly other haemagglutinins (Hanne and Finkelstein, 1982).

Plasmid pPM471 DNA was isolated and used for re-transformation into strain LE392. All transformants were haemagglutination positive, confirming that the haemagglutinating capacity is associated with the presence of plasmid pPM471.

3.2.3 Sugar inhibition and RBC activity

Sugar inhibition tests were performed with strain V271 to further characterize and perhaps identify the haemagglutinin encoded by pPM471.

To assess the capacity of a given sugar to inhibit haemagglutination, a suspension of bacteria containing two haemagglutination units was added to buffer (KRT) containing the appropriate sugars. Haemagglutination mediated by V271 was not inhibited by any of the sugars listed in Table 3.1. This contrasted with a number of *V. cholerae* control strains, such as El Tor strain 017 which was inhibited by both D-mannose and D-fructose.

V271 was also tested for reactivity with a number of RBCs types (Table 3.2). It gave minimal (1 well), if any, haemagglutination activity when tested with chicken and human erythrocytes, whereas maximum titres were obtained with mouse erythrocytes. The haemagglutination titre is defined as the reciprocal of the highest dilution at which haemagglutination is visible.

Haemagglutination by V271 was not dependent on the inclusion of calcium ions in the incubation buffer as compared to haemagglutination by the soluble HA for which maximum activity required the presence of calcium ions.

3.2.4 Western blot analysis

Using antisera raised againgst the SHA, western blot analysis was performed against cell membrane preparations of V271 to see whether the two haemagglutinins (SHA

Table 3.1 Sugars used in haemagglutination inhibition assays (10mg/ml in saline serially diluted).

Sugars tested
N–acetyl galactosamine
D–fructose
L–fucose
D–galactose
D–glucosamine
D–glucose
D–mannitol
D–mannose
D–ribose

Table 3.2 Spectrum of activity of V271 with various red blood cell types.

Source of RBC	Haemagglutination titre
chicken (responder)	0–2
chicken (non-responder)	0–2
guinea pig	0–2
human (type A, B an O)	0–2
mouse (Balb/c)	8
rabbit	0–2
sheep	8

and MFRHA) were immunologically distinct. Hanne and Finkelstein (1982) have suggested that perhaps the MFRHA is a cell-associated form of the SHA, since both exhibit haemagglutination which cannot be inhibited by the addition of sugars.

It was also of interest, to know whether the soluble haemagglutinins purified by both Finkelstein and Hanne (1982) and Svennerholm and co-workers (1983) were identical in both biotypes despite reports of different molecular weights.

Finkelstein and Hanne (1982) have purified the SHA to apparent homogeneity from a Classical Inaba strain, CA401 and shown it to exist as non-covalently associated polymers comprising subunits of size 32 kDal. Svennerholm and co-workers (1983) have also purified the SHA but from an El Tor Ogawa strain, O17. Estimates by SDS electrophoresis in the presence and absence of dithiothreitol revealed a major band of an approximate size 45 kDal.

Western blots have been performed using the two antisera and the purified SHA proteins as well as membrane preparations of V271. Using Finkelstein's anti-soluble HA at a dilution of 1:500, a strong reaction with the 32 kDal protein in both purified protein preparations occurred, with the 34 kDal band reacting to a lesser extent. The same reaction was seen when Svennerholm's antiserum was used (Figure 3.3). Neither of the anti-SHA sera reacted with the MFRHA.

Hence, such results indicate that the SHA purified by Finkelstein and Hanne (1982) and Svennerholm *et al.* (1983), are identical regardless of biotype and despite different reported sizes, both are 32 kDal. Also the SHA and MFRHA are immunologically distinct and therefore it appears unlikely that the MFRHA is a cell-associated form of the SHA.

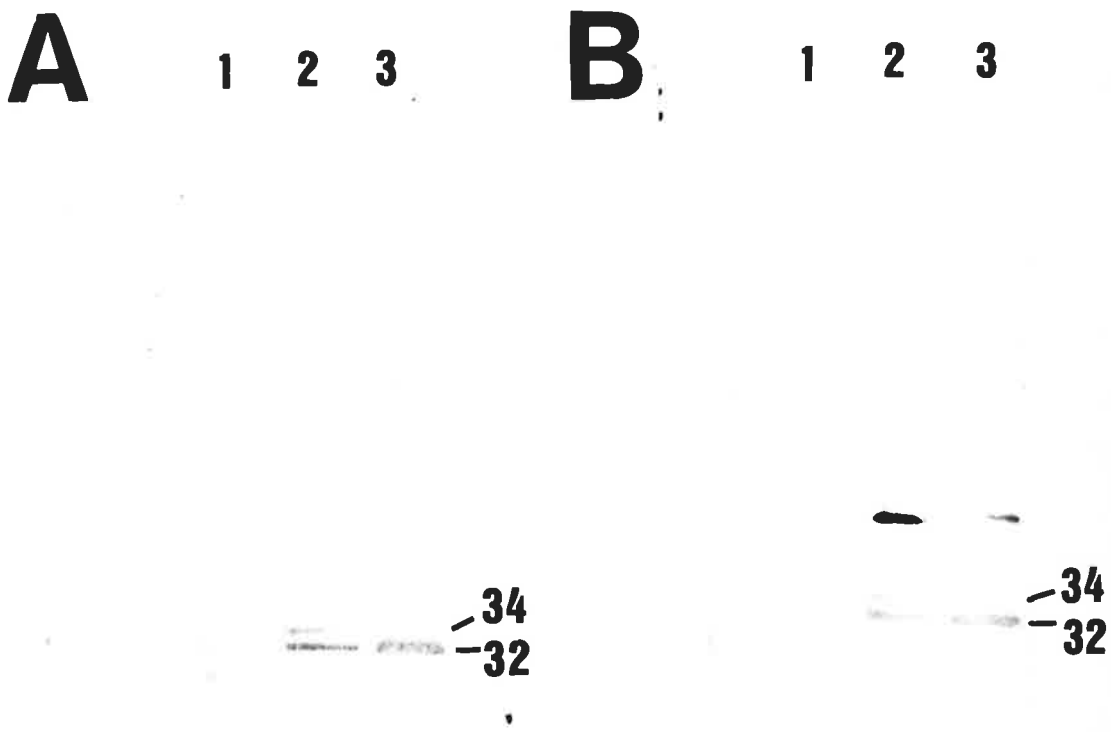
3.2.5 Effect of *tol* mutants on expression and cellular location of the cloned haemagglutinin.

Extracellular proteins of *V. cholerae* such as haemolysin and DNase produce large zones of haemolysis and DNA hydrolysis respectively. However, *E. coli* cells harbouring these cloned genes produce only very small zones of clearing. This is because

Figure 3.3 Western blot analysis of haemagglutinins. **Lane 1:** cell membrane preparation of V271. **Lane 2:** Purified soluble HA supplied by A.-M. Svennerholm. **Lane 3:** Purified soluble HA supplied by R.A. Finkelstein. The blots were developed with rabbit antiserum to the appropriate haemagglutinin followed by goat anti-rabbit IgG coupled with horseradish peroxidase.

A Western blot analysis using antiserum raised against purified SHA, supplied by R.A. Finkelstein.

B Western blot analysis using antiserum raised against purified SHA, supplied by A.-M. Svennerholm.


A
1 2 3
34
32
B
1 2 3
34
32

the majority of molecules become trapped in the periplasm with only a small percentage of molecules actually crossing the outer membrane. Mecurio and Manning (1986) and Focareta and Manning (1987) have shown that when plasmids which encode the haemolysin or DNase are transformed into *tolA* or *tolB* mutants of *E.coli* K-12 then these activities appear in the surrounding medium giving zones approximately equivalent to those detected with the natural host. Strains with *tolA* and *tolB* mutations are described as "leaky" mutants because they allow the release of periplasmic proteins into the external medium (Bernstein *et al.* 1972; Anderson *et al.* 1979).

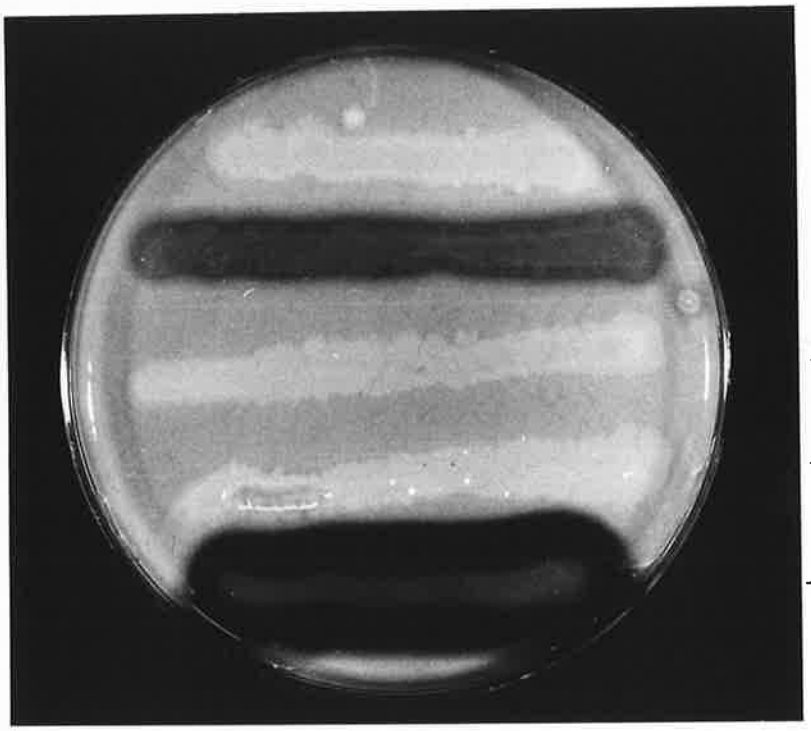
The SHA when expressed by *V. cholerae* is excreted such that haemagglutinating activity is detected in cell-free culture supernatants, therefore reduced or zero proteolysis by V271 could be due to entrapment of molecules in the periplasm. Hence pPM471 was introduced into *tolA* and *tolB E. coli* strains, P651 and P234 respectively and the resulting strains were tested for increased proteolytic activity or increased haemagglutination titres. Both cell cultures and cell-free culture supernatants did not show an increase in either of these activities.

3.2.6 Proteolytic activity

Initially it was thought that the haemagglutinin that had been cloned may have been the soluble HA and consequently V271 was tested for proteolytic activity.

This was performed by streaking cultures, LE392, V271, *V.cholerae* strains O17 and 569B onto casein agar plates and incubating them overnight. No protease activity could be detected as compared to the control strains 017 and 569B (Figure 3.4). It is worth noting that strain 569B had a lower level of proteolytic activity compared to that of 017 which readily degrades the casein contained in the agar. *E. coli* strain P651 (*tolA*) harbouring pPM471 was also tested.

Figure 3.4 Protease production on a skim milk agar plate showing *V. cholerae* 569B, *V. cholerae* 017, *E. coli* K-12 LE392, V271 and *E. coli* K-12 P651 (*tolA*) containing pPM471. The plate was incubated for 24 hours at 37°C.


E.COLI K-12 LE392
V.CHOLERAE 569B
V271
P651 (PPM471)
V.CHOLERAE 017

3.2.7 Zincov inhibition

Zincov (2-(N-hydroxycarboxamido)-4-methyl pentanoyl-L-ala-gly-NH_2) is an inhibitor for zinc-containing metalloproteases (Nishino and Powers, 1979). The addition of Zincov has been shown to inhibit haemagglutination caused by the SHA (Booth *et al.* 1983) and consequently the effect of 220 μM Zincov on the haemagglutinating ability of V271 has been tested. Purified SHA (a gift from Professor R. A. Finkelstein) at a concentration of 20 μg/ml did not agglutinate RBCs when preincubated with Zincov (220 μM), whereas in the absence of Zincov the purified preparation of SHA was able to agglutinate RBCs. This is because haemagglutination by the soluble HA is dependent on a proteolytic event which is inhibited by the presence of Zincov.

The haemagglutinating activity of V271, however, was unaffected by preincubation (30 min) with Zincov. This result taken together with the absence of proteolytic activity, in strain V271, suggests that the cloned haemagglutinin was not the SHA. Furthermore because 569B expresses both a FSHA and MFRHA cell-associated haemagglutinin the lack of sugar inhibition of haemagglutination by V271 implies that this clone was expressing the MFRHA.

3.2.8 Restriction analysis of pPM471

Plasmid pPM471 DNA was digested with a range of restriction endonucleases and the fragments separated by agarose gel electrophoresis. The restriction fragments generated by different enzymes were numbered sequentially from the largest to the smallest according to their relative mobilities on agarose gels (eg. *Eco*RI-1, *Eco*RI-2 etc, Table 3.3). Digestion of pPM471 DNA with *Cla*I, *Eco*RI, *Hin*dIII, *Mlu*I and *Xba*I generated 3, 3, 5, 8 and 2 restriction fragments respectively (Figure 3.5). The sizes of these fragments are summarized in Table 3.3. Both *Bgl*II and *Nru*I cleaved the cloned DNA once. However, digestion of pPM471 with *Nru*I gives two fragments since there is also a site in the vector pBR322.

Plasmid pPM471 was found to carry a 6.1 kb *Bam*HI fragment of *V. cholerae*

Figure 3.5 Restriction cleavage patterns of pPM471 DNA after digestion with the indicated restriction endonucleases followed by electrophoretic separation on a 1% agarose gel. Size standards are SPP1 cut with *Eco*RI. Bands were visualized following staining with ethidium bromide by trans-illumination with UV light.
Restriction endonuclease sites are as follows:
B: *Bam*HI; C: *Cla*I; E: *Eco*RI; M: *Mlu*I; N: *Nru*I; X: *Xba*I.

SPP1/E

B C E M N X

Table 3.3 The sizes of pPM471 fragments produced by digestion with the endonucleases *Cla*I, *Eco*RI, *Hin*dIII, *Mlu*I, *Xba*I.

Restriction	Restriction Enzyme				
Fragment	*Cla*I	*Eco*RI	*Hin*dIII	*Mlu*I	*Xba*I
1	8.23	4.63	5.89	4.96	9.68
2	1.20	4.24	2.45	1.72	0.70
3	0.90	1.50	0.89	1.02	
4			0.65	0.69	
5			0.55	0.65	
6				0.57	
7				0.52	

569B DNA cloned into the *Bam*HI site of pBR322. The cloned DNA in pPM471 did not contain sequences recognized by the restriction endonucleases *Kpn*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sma*I and *Xho*I. By the use of single and double restriction endonuclease digestions, a restriction map of pPM471 was established (Figure 3.6).

3.2.9 Localization of the DNA in pPM471 which encodes the haemagglutination activity

Three approaches were taken to localize the gene encoding for the MFRHA. The first involved transposon mediated mutagenesis. The second involved deleting or subcloning specific restriction fragments. The third involved the use of *Bal*31 nuclease to generate deletions.

Transposon mutagenesis of pPM471

Transposon Tn*1725* (Ubben and Schmitt, 1986) was used to mutagenize pPM471. The advantage of this transposon is that it has recognition sequences for the restriction endonuclease *Eco*RI, 15 bp from each end. This allows simple localization of the point of insertion.

Tn*1725* was transposed into pPM471 from plasmid pRU669 ($R_{ts}1$::Tn*1725*) selecting for chloramphenicol resistant derivatives of pPM471. A total of 20 independent insertions were isolated, mapped by restriction analysis with both *Eco*RI and *Hin*dIII and assayed for their haemagglutinating capacity. Surprisingly, insertions that generated a haemagglutination negative phenotype also had deletions within surrounding DNA. No insertions giving a haemagglutination negative phenotype occurred without such simultaneous deletion. The reason for this is unknown.

However, a number of transposon Tn*1725* insertions which still showed a haemagglutination postive phenotype, were isolated and mapped with restriction endonucleases *Eco*RI and *Hin*dIII (Figure 3.7). The lack of haemagglutinin negative transposon insertions prevented precise delineation of the DNA which encodes for the haemagglutinin.

Figure 3.6 Restriction endonuclease cleavage map of plasmid pPM471. The region of cloned *V. cholerae* DNA is shown by the thick line; the vector pBR322, is shown by the thin line.

Restriction endonuclease sites are as follows:

B: *Bam*HI; **Bg**: *Bgl*II; **C**: *Cla*I; **E**: *Eco*RI; **H**: *Hin*dIII; **M**: *Mlu*I; **N**: *Nru*I; **P**: *Pst*I; **X**: *Xba*I.

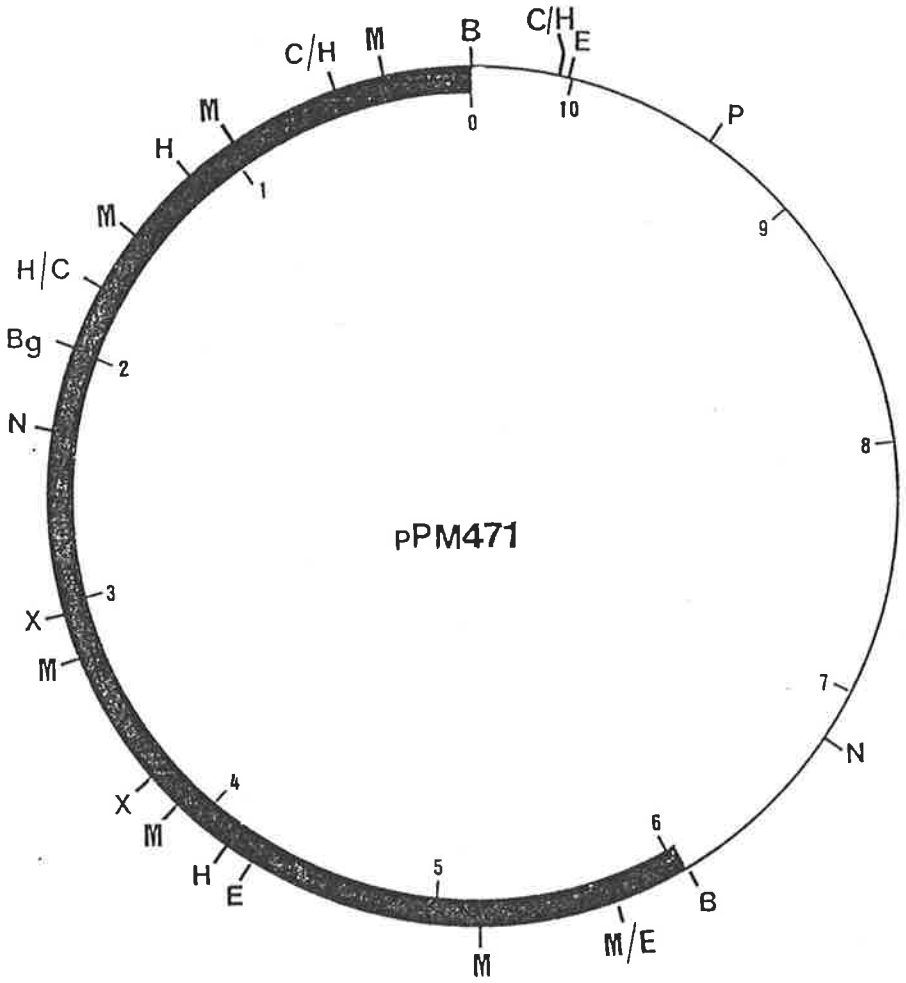
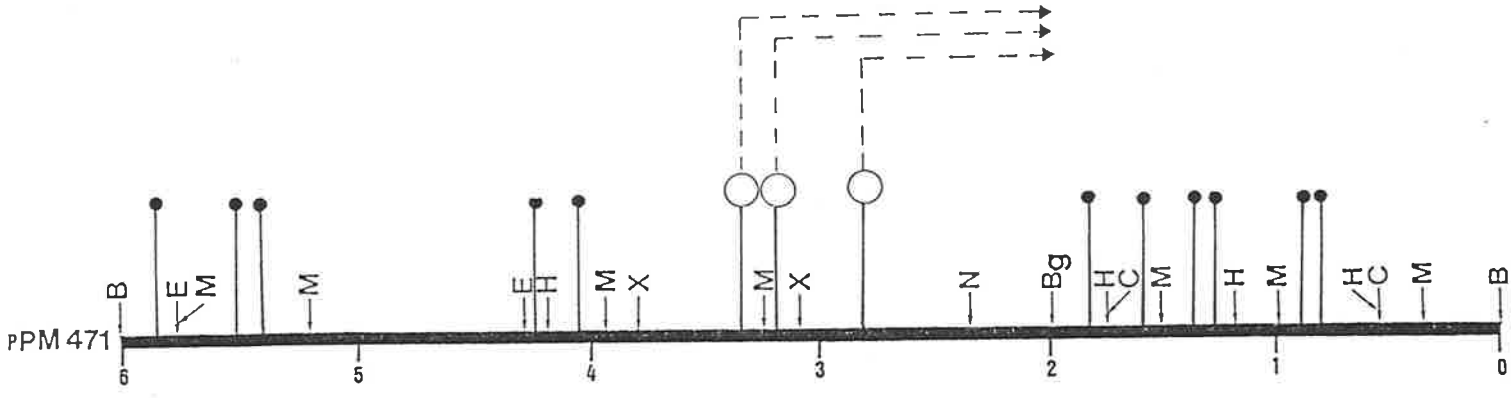

pPM471
B
C/H
E
P
N
B
M/E
M
E
H
X
M
X
M
N
Bg
H/C
M
H
M
C/H
M
0
1
2
3
4
5
6
7
8
9
10

Figure 3.7 A linear map of the *V. cholerae* DNA cloned in pPM471, showing the sites of insertion of a number of the Tn*1725* derivatives of the MFRHA clone. Closed circles represent haemagglutination positive transposon insertions. Open circles represent haemagglutination negative insertions. Dashed lines represent direction of deletions caused by inserted transposon.
Restriction endonuclease sites are as follows:
B: *Bam*HI; Bg: *Bgl*II; C: *Cla*I; E: *Eco*RI; H: *Hin*dIII; M: *Mlu*I; N: *Nru*I; X: *Xba*I.


pPM471
B
E
M
M
E
H
M
X
M
X
N
Bg
H
C
M
H
M
H
C
M
B
6
5
4
3
2
1
0

Restriction endonuclease generated deletions and subclones of pPM471

As can be seen (Figure 3.6) there are a number of conveniently spaced *Hin*dIII sites in pPM471. Thus, by partial digestion of pPM471 DNA with *Hin*dIII, followed by dilution of the resulting DNA and ligation, it was possible to construct plasmids which had lost one or more of the various *Hin*dIII fragments.

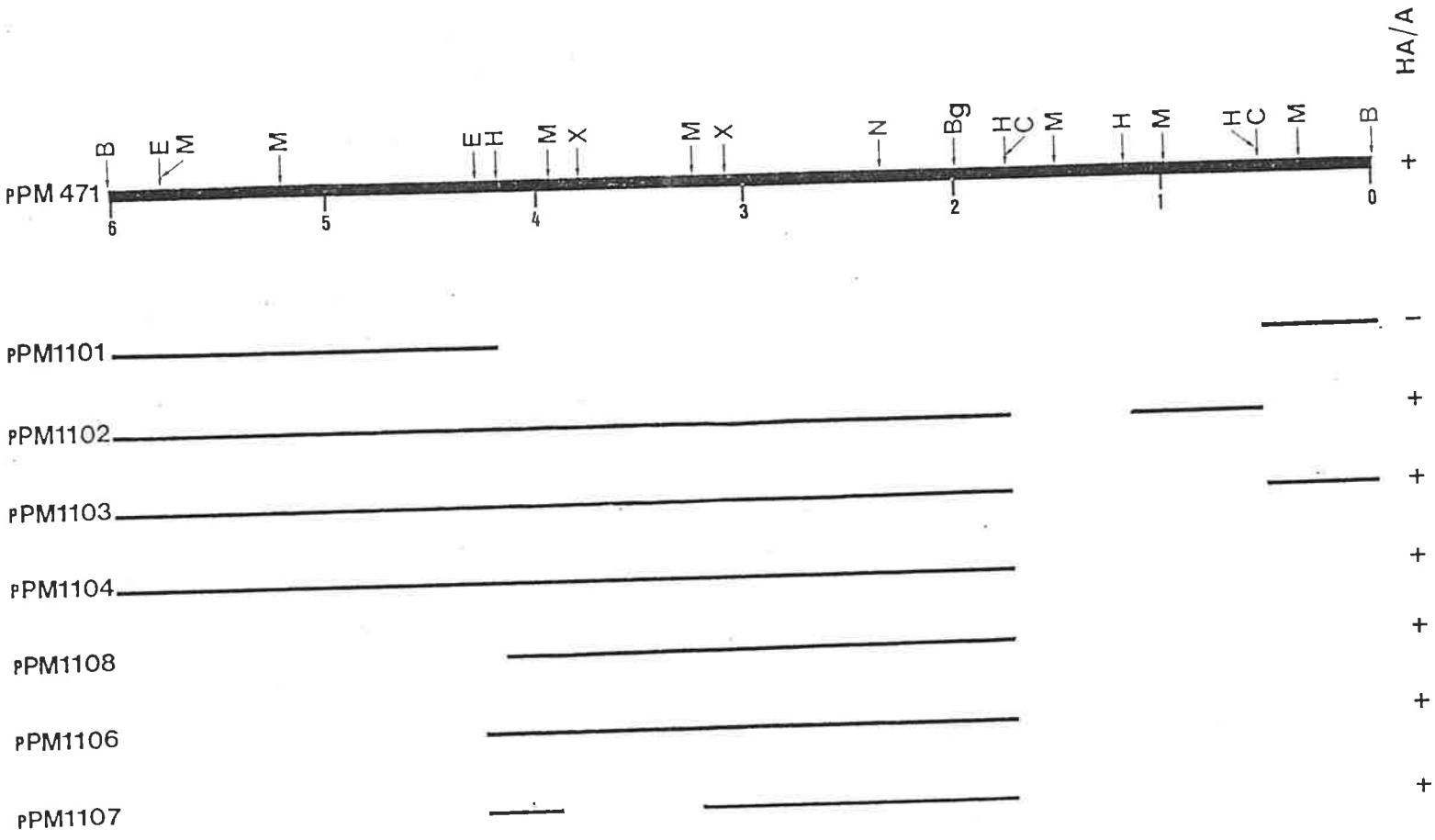
Plasmids pPM1101, pPM1102 and pPM1103 were constructed in this way (Figure 3.8). pPM1101 had *Hin*dIII fragments 2, 4 and 5 deleted; pPM1102 had *Hin*dIII fragments 4 and 3 deleted; pPM1103 had fragments 4 and 5 deleted. Strains harbouring these plasmids were assayed for haemagglutination activity, with pPM1101 being haemagglutination negative and pPM1102 and pPM1103 remaining haemagglutination positive. From such data, it was clear that at least part of the gene expressing haemagglutinating activity must be encoded within the 2.45 kb *Hin*dIII fragment (#2).

A series of subclonings were performed in an attempt to further delineate the limits of the DNA which encodes the haemagglutinin gene. The 2.55 kb *Eco*RI–*Cla*I fragment of plasmid pPM471 was subcloned into *Eco*RI and *Cla*I digested pBR322 to produce plasmid pPM1106. The 2.45 kb *Hin*dIII fragment (#2) of pPM471 was subcloned into the *Hin*dIII site of vector pUC8 (Vieira and Messing, 1982) to generate plasmid pPM1108 (Figure 3.8). Both of these plasmids (pPM1106 and pPM1108) still contained and expressed the MFRHA gene since they exhibited haemagglutination activity identical to that of V271. The gene was further localized by constructing plasmid pPM1107. This plasmid was derived by digesting pPM1106 with *Mlu*I and ligating, thus deleting the 0.69 kb *Mlu*I fragment (#4). Plasmid pPM1107 which still expressed a haemagglutinin positive phenotype (identical to that of pPM471) contained 1.87 kb of contiguous *V. cholerae* 569B DNA (from *Cla*I to *Mlu*I). Hence, the MFRHA gene had been localized to a 1.87 kb region of DNA.

Figure 3.8 Deletion analysis and subclones of pPM471. The lines indicate the region of cloned DNA of plasmid pPM471 retained in the deletion or subclone. Plasmids pPM1106 and pPM1107 were derived by subcloning into pBR322 and pPM1108 was derived by subcloning into pUC8. The various recombinant plasmids were assessed for their haemagglutination capacity. + = haemagglutination, − = no haemagglutination.

Restriction endonuclease sites are as follows:

B: *Bam*HI; Bg: *Bgl*II; C: *Cla*I; E: *Eco*RI; H: *Hin*dIII; M: *Mlu*I; N: *Nru*I; X: *Xba*I.


HA/A
pPM 471
B
E
M
M
E
H
M
X
M
X
N
Bg
H
C
M
H
M
H
C
M
B
+
6
5
4
3
2
1
0
pPM1101
-
pPM1102
+
pPM1103
+
pPM1104
+
pPM1108
+
pPM1106
+
pPM1107
+

Generation of deletions using *Bal*31 nuclease

To define the limits of the DNA encoding the gene for the MFRHA more precisely. *Bal*31 nuclease (Gray *et al.* 1975) was employed.

By varying incubation times, a family of deletions from a given restriction endonuclease cleavage site can be generated (Figure 3.9). A precisely defined minimal coding region can be obtained by isolating deletions entering the region from opposite ends.

Before ligation, synthetic linkers containing an appropriate restriction endonuclease recognition sequence are added. pPM1107 was digested with either *Cla*I or *Eco*RI and then incubated in the presence of *Bal*31 at 30°C. *Cla*I or *Eco*RI linkers were added respectively, to enable the ends of the deletion to be defined precisely. The DNA was then transformed into *E.coli* K-12 LE392.

In this way a number of plasmids with various deletions extending into the cloned DNA from opposite ends have been derived from pPM1107. Some of these are shown in Figure 3.10. Plasmids pPM1120, pPM1121, pPM1122, pPM1123, pPM1124, pPM1125, pPM1126, pPM1127, pPM1132 and pPM1133 all still express a haemagglutination positive phenotype. Plasmids pPM1128, pPM1129, pPM1130 and pPM1131 exhibit a haemagglutination negative phenotype. pPM1127 and pPM1128 differ in size by only 50–100 bp. Since pPM1127 is haemagglutination proficient and pPM1128 is haemagglutination deficient this allowed approximate localization of the start (or end) of the gene encoding haemagglutination.

This has made it possible to localize the MFRHA gene to within a 0.72 kb fragment of DNA (Figure 3.10). Assuming a molecular weight of 110 for the average amino acid, this would give an upper limit of about 27 kDal for the haemagglutinin protein. Interestingly, although a number of deletions could be isolated which extended from the *Cla*I end of the DNA fragment, after digestion from the *Eco*RI site very few transformants could be isolated.

Figure 3.9 *Bal*31 generated deletions of pPM1107. Electrophoresis on a 0.8% agarose gel to illustrate the decrease in size of the DNA molecules following various times of digestion with *Bal*31 exonuclease of linearized (with *Cla*I) pPM1107 DNA.

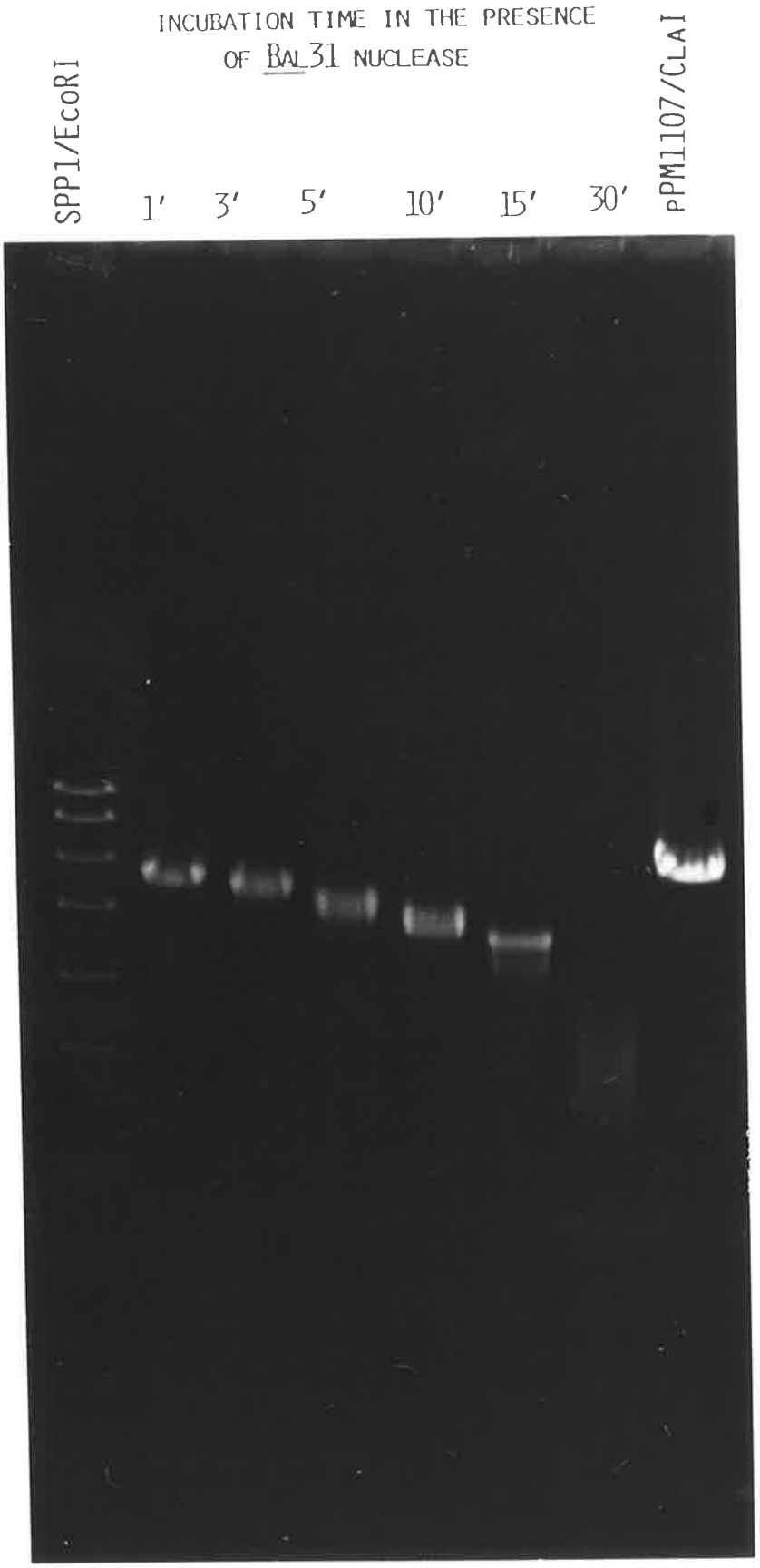
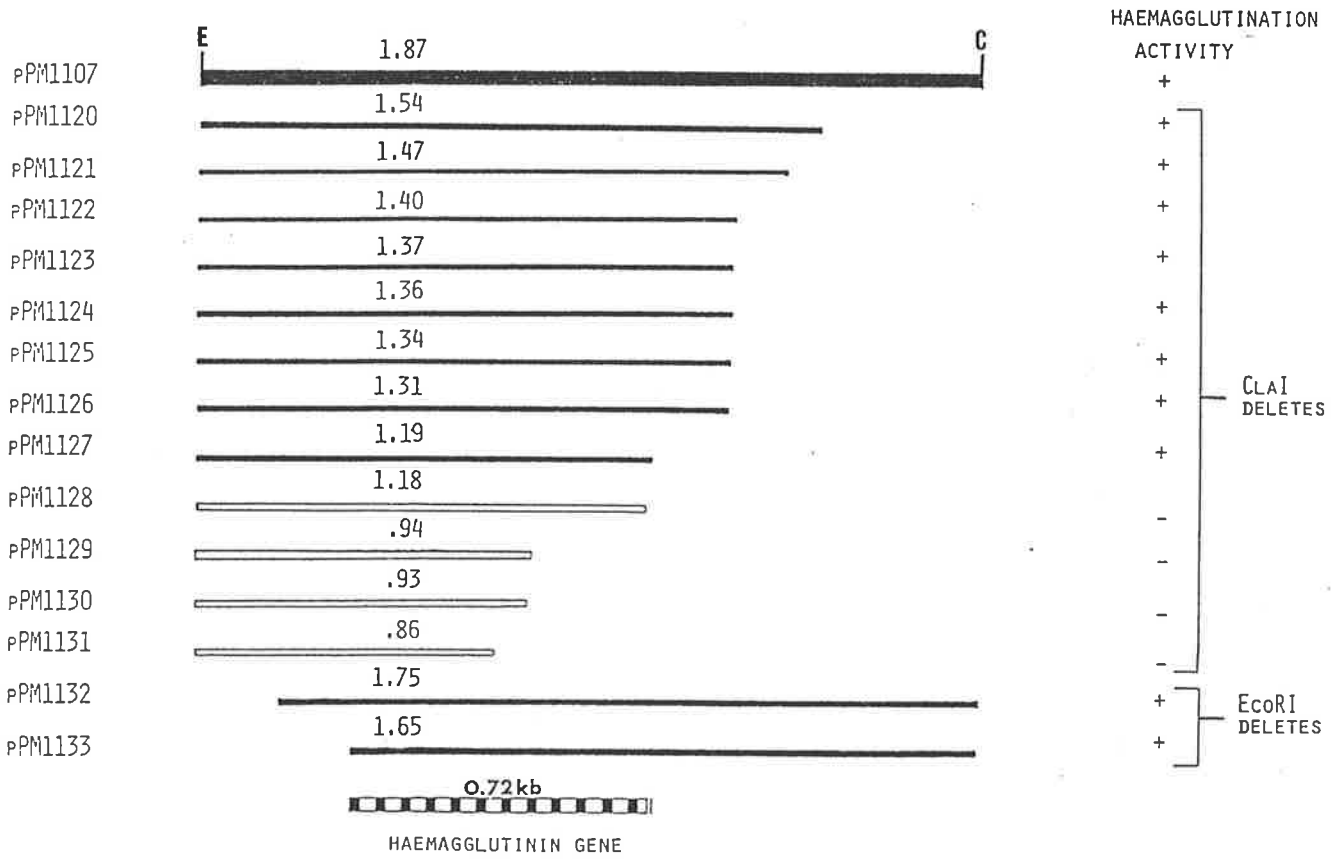

INCUBATION TIME IN THE PRESENCE
OF BAL31 NUCLEASE
SPP1/EcoRI
1'
3'
5'
10'
15'
30'
pPM1107/ClaI

Figure 3.10 Diagram showing the extent of the deletions in the cloned *V. cholerae* DNA in pPM1107 generated by *Bal*31 exonuclease digestion from the *Cla*I and *Eco*RI sites of pPM1107. The solid lines represent the DNA present in plasmids which are capable of mediating haemagglutination. The open lines are haemagglutination negative. The dashed line at the bottom is the maximum region of the DNA available for encoding the gene for the haemagglutinin.

Restriction endonuclease sites are as follows:

C: *Cla*I; E: *Eco*RI.


E
C
1.87
pPM1107
1.54
pPM1120
1.47
pPM1121
1.40
pPM1122
1.37
pPM1123
1.36
pPM1124
1.34
pPM1125
1.31
pPM1126
1.19
pPM1127
1.18
pPM1128
.94
pPM1129
.93
pPM1130
.86
pPM1131
1.75
pPM1132
1.65
pPM1133
0.72kb
HAEMAGGLUTININ GENE
HAEMAGGLUTINATION ACTIVITY
+
+
+
+
+
+
+
+
+
-
-
-
-
+
+
ClaI DELETES
EcoRI DELETES

3.2.10 Identification of the gene products of pPM471

Cell envelope preparations

Cell envelopes were prepared by the small scale method and analyzed by SDS-PAGE. When cell envelope preparations of *E. coli* K-12 strain LE392 and V271 were compared, no additional protein could be detected. This is despite the fact that the haemagglutinin gene being present on a high-copy number plasmid in strain V271. Obviously the protein is being made since haemagglutination activity can be detected, presumably this suggests that haemagglutination assays are more sensitive than protein detection methods. Since it has not been possible to identify the protein product by these means, the various plasmids have been analyzed in *E. coli* K-12 minicells.

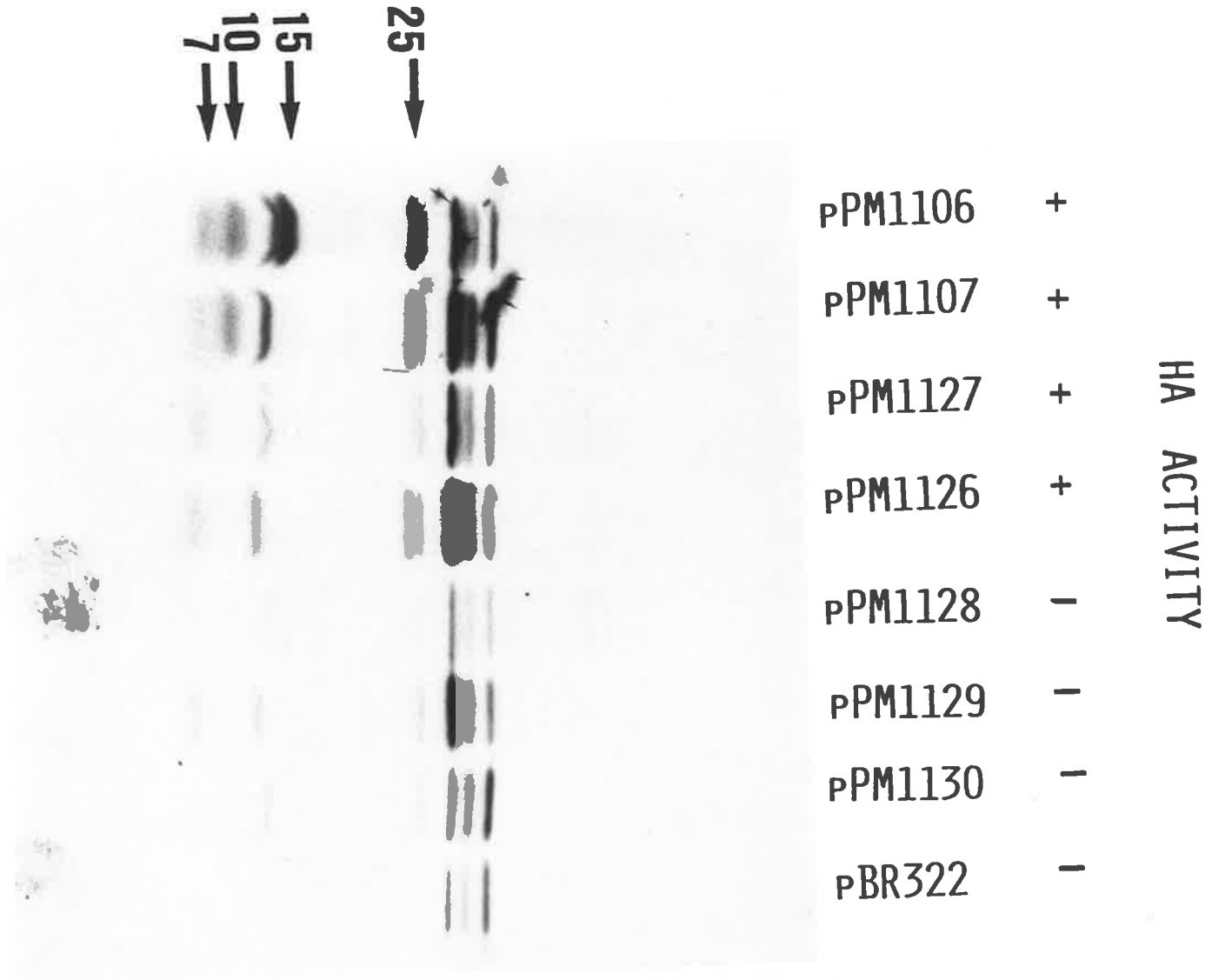
Analysis of plasmid encoded proteins in *E. coli* K-12 minicells

Several of the plasmids were introduced into *E.coli* K-12 minicell-producing strain, DS410 (Dougan and Sherratt, 1977). Minicells were purified from whole cells by centrifugation through sucrose gradients and then labelled with [^{35}S]-methionine (Kennedy *et al.* 1977; Achtman *et al.* 1979). The plasmid encoded proteins were visualized by autoradiography following electrophoresis in SDS through an 11-20% linear polyacrylamide gradient gel.

Minicells containing only pBR322 produced three protein bands (Figure 3.11) (Covarrubias *et al.* 1981; Backman and Boyer, 1983). They have previously been shown to be forms of the β-lactamase, produced by the ampicillin gene. These three polypeptides have sizes of approximately 30 kDal, 28 kDal and 26 kDal. Dougan and Sherrat (1977) have shown the 28 kDal polypeptide to be the active form of the β-lactamase protein, while Achtman *et al.* (1979) showed the 30 kDal polypeptide to be a precursor form of the 28 kDal protein and still possesses the signal sequence. The 26 kDal protein is a breakdown product which cross-reacts with sera directed against the β-lactamase.

Since plasmid pPM471 proved to be unstable in strain DS410 giving rise to

Figure 3.11 Plasmid encoded proteins in the *E. coli* K-12 minicell producing strain DS410. Minicells harbouring the various plasmids were purified on sucrose gradients labelled with [^{35}S]-methionine and solubilized in SDS and the plasmid encoded proteins were visualized by autoradiography after electrophoresis on a 11-20% gradient polyacrylamide gel. Approximate protein sizes (kDal) are indicated in the left-hand margin.


7
10
15
25
pPM1106 +
pPM1107 +
pPM1127 +
pPM1126 +
pPM1128 −
pPM1129 −
pPM1130 −
pBR322 −
HA ACTIVITY

a number of deletion plasmids, it was necessary to introduce plasmids pPM1126, pPM1127, pPM1128, pPM1129, pPM1130 and pPM1131 which had been generated by *Bal*31 nuclease treatment. Several other plasmids (pPM1106 and pPM1107) have also been analyzed. Table 3.4 lists the plasmid encoded gene products identified on SDS-polyacrylamide gels (Figure 3.11). All plasmids which exhibited a haemagglutination positive phenotype expressed a protein with an approximate size of 25 kDal which was absent from minicells harbouring pBR322 or any of the haemagglutination negative plasmids (pPM1128, pPM1129 pPM1130 and pPM1131).

Interestingly plasmid pPM1106 gave the same profile as pPM1107 except the 15 kDal protein was absent. pPM1106 as mentioned before was derived from pPM1107 by the deletion of the *Mlu*I fragment. Therefore the DNA encoding for the 15 kDal protein must be localized to within this region. The *Mlu*I fragment is approximately 0.69 kb which is more than sufficient to encode a 15 kDal protein. Both pPM1106 and pPM1107 still produce the 10 kDal protein, however, all the plasmids derived by *Bal*31 nuclease digestion from the *Cla*I end have lost the ability to produce this polypeptide. This indicates that the gene encoding the 10 kDal protein must be situated somewhere before the gene for the 25 kDal protein. This 10 kDal protein does not seem to be involved in haemagglutination since pPM1124 which is the smallest plasmid capable of mediating haemagglutination, does not produce this 10 kDal protein. The haemagglutination titre of cells harbouring pPM1124 is equivalent to pPM471 which is the largest plasmid.

All the plasmids analyzed in minicells produced a 7 kDal protein. As will become evident in Chapter 4 the gene encoding this polypeptide lies adjacent to the *Mlu*I fragment.

3.2.11 Re-introduction of the cloned HA gene into *V. cholerae*

It was reasoned that perhaps no additional bands were seen when membrane profiles of V271 were compared with *E. coli* LE392, because of the lack of some regulatory

Table 3.4 Protein products of various plasmids when analyzed in [^{35}S] labelled minicells.

Plasmid	Sizes of encoded polypeptides (kDal)
pPM1106	25, 15, 10, 7
pPM1107	25, 10, 7
pPM1126	25, 7
pPM1127	25, 7
pPM1128	7
pPM1129	7
pPM1130	7

mechanism which may be present in *V. cholerae* but not *E.coli*. Therefore if the cloned HA gene was re-introduced into *V.cholerae* 569B, the natural host, expression may be seen to be improved.

Simon and co-workers (1983) have constructed a number of mobilizable vector plasmids. Vectors such as pACYC184, pACYC177 and pBR325 have been modified to include the locus for mobilization, the mobilization (mob) site of plasmid RP4. The mobilization site includes the origin of transfer, which is the recognition site for some RP4 transfer functions. Such vectors can be mobilized at high frequencies into non-*E. coli* hosts because of the broad host range of RP4.

pSUP205 is one such mobilizable vector and is based on the *E. coli* vector pBR325. This vector is Cm^R and Tc^R. pSUP205 was digested with *Bam*HI and phosphatased to prevent re-ligation. pPM471 was also *Bam*HI digested and ligated to *Bam*HI cleaved pSUP205. The ligation mixture was then transformed into S17-1 selecting for Cm^R, Tc^S colonies (Figure 3.12). The appropriate hybrid molecule was designated pPM1150.

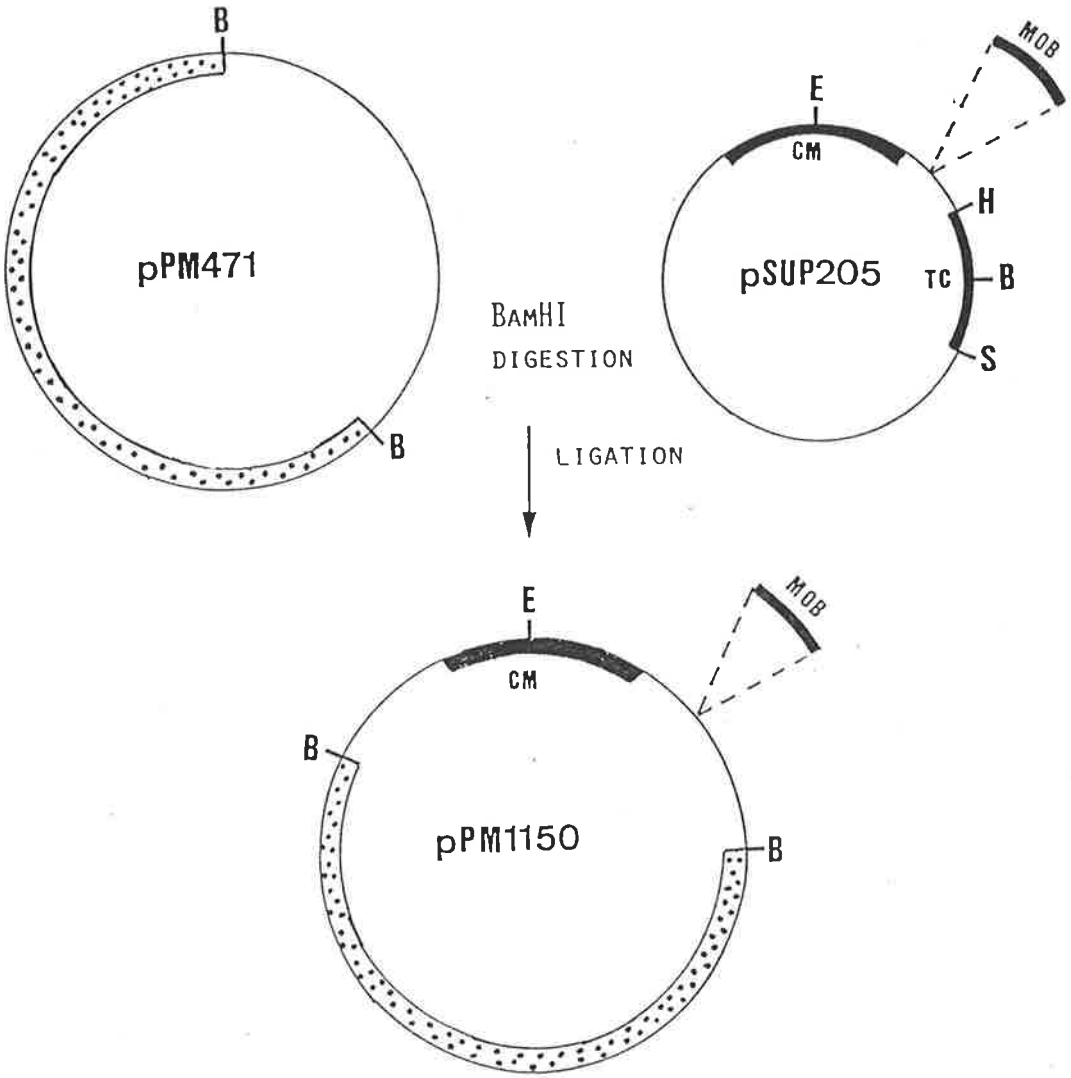
S17-1 is a mobilizing strain which Simon *et al.* (1983) constructed by the integration of RP4-2-Tc::Mu into the *E. coli* chromosome. Hence this strain carries the transfer genes of RP4 and can be used as a donor for conjugative DNA transfer.

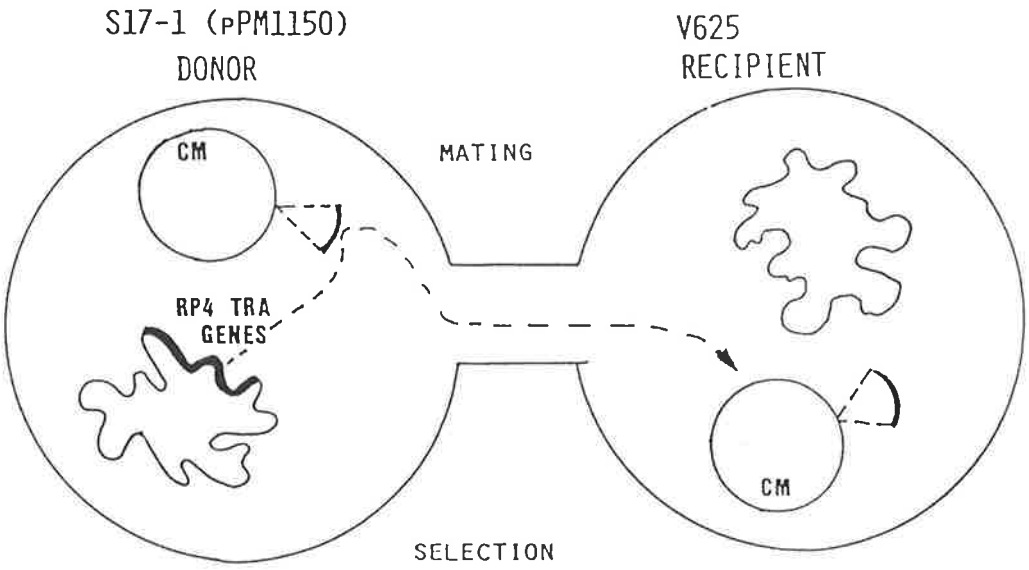
S17-1 (pPM1150) and a spontaneous rifampicin (Rif) resistant derivative of 569B (V625) were mated together for 6 hours and then plated on NA containing Rif and Cm. Rif^R, Cm^R colonies were *V. cholerae* possessing plasmid pPM1150. Membrane preparations of V625 and V625 harbouring pPM1150 were compared by SDS-PAGE electrophoresis. No obvious differences could be distinguished between the membrane profiles on an 11-15% gradient polyacylamide gel.

3.2.12 Cloning of the MFRHA gene from the El Tor biotype

A cosmid gene bank derived from *V. cholerae* 017 (El Tor Ogawa) was constructed using the vector pHC79 (Hohn and Collins, 1980). Chromosomal DNA was partially digested with *Sau*3A to generate fragments of between 30-50 kb. Vector DNA was digested with *Bam*HI inactivating the Tc^R locus and ligated with the partially

Figure 3.12 Construction of plasmid pPM1150. pPM471 was digested with *Bam*HI and ligated overnight to *Bam*HI digested pSUP205. The ligation mixture was transformed into S17-1, a mobilizing strain. The bottom section of the figure shows the mating procedure between S17-1 (pPM1150) the donor mobilizing strain and V685 the recipient. Selection was for Cm^R, Rif^R colonies.
Restriction endonuclease sites are as follows:
B: *Bam*HI; **E**: *Eco*RI; **H**: *Hin*dIII; **S**: *Sal*I.


B
pPM471
B
BamHI
DIGESTION
LIGATION
MOB
E
CM
H
pSUP205
TC
B
S
E
CM
MOB
B
pPM1150
B


S17-1 (pPM1150)
DONOR
V625
RECIPIENT
CM
MATING
RP4 TRA
GENES
CM
SELECTION

digested chromosomal DNA. This was followed by *in vitro* packaging and transduction into the *E.coli* K-12 *recA* strain DH1; selection was for Ap^R colonies. 500 colonies (all Tc^S) were patched onto nutrient agar containing Ap and transferred to nitrocellulose discs. Replicas of these discs were probed with a α–[^{32}P] labelled 6.1 kb *Bam*HI fragment isolated from pPM471 (Figure 3.13).

Several colonies reacted with the probe as judged by subsequent autoradiography. These positive isolates were purified and plasmid DNA was extracted. Several negative clones were included as controls. Digested with various restriction endonucleases (*Bam*HI, *Cla*I, *Eco*RI and *Hin*dIII) demonstrated that their DNA profiles were comparable with that of pPM471 in that all positive clones carried a 2.45 kb *Hin*dIII fragment which co-migrated with the analogous fragment found in pPM471 (Figure 3.14). None of the negative reacting clone contained this *Hin*dIII fragment. Those clones that reacted with the probe and contained the 2.45 kb *Hin*dIII fragment were also able to haemagglutinate red blood cells.

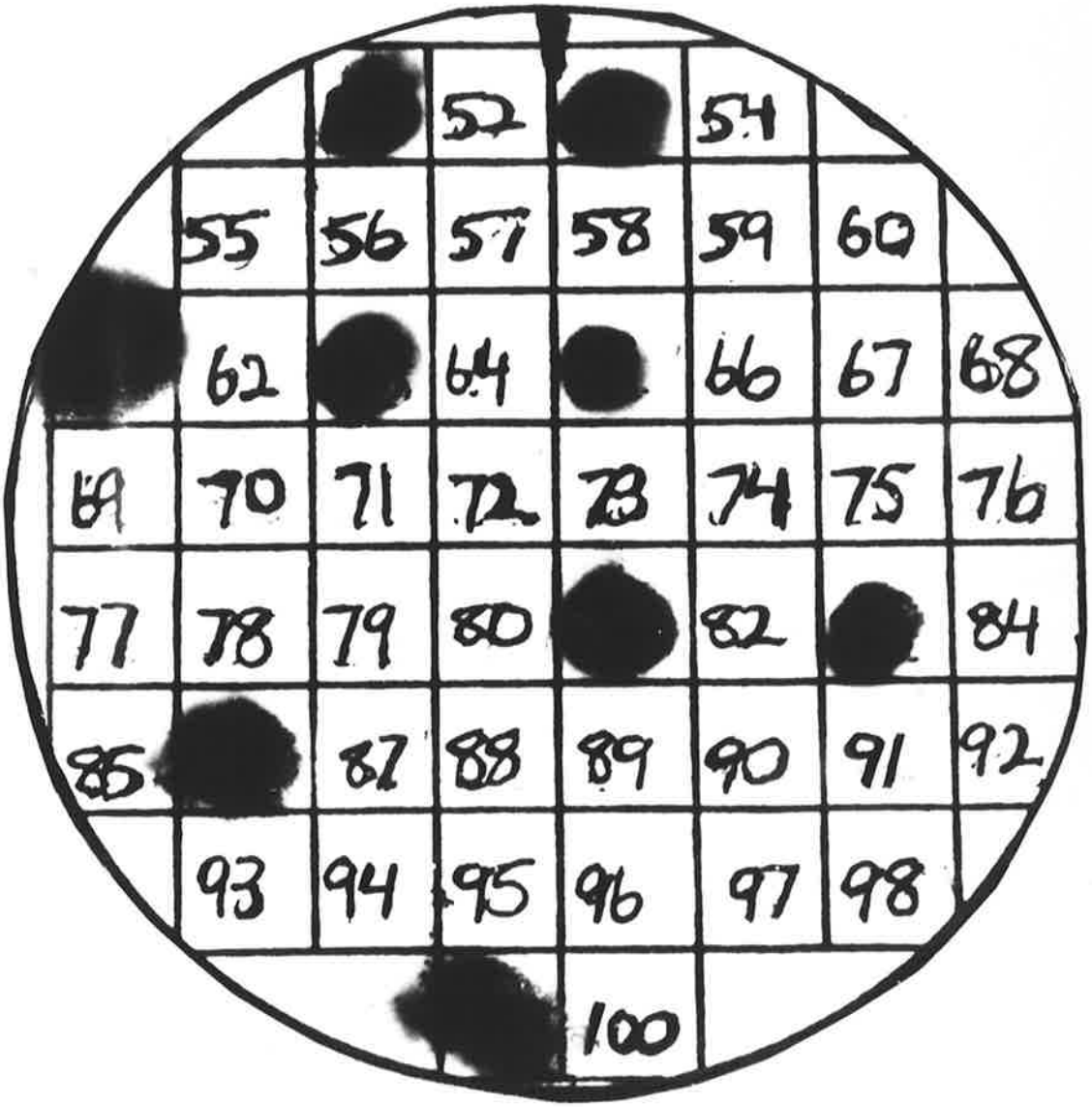
Cell envelopes were prepared from *E. coli* DH1 harbouring various cosmids and analyzed by SDS-PAGE. Interestingly strain DH1 containing plasmid pPM1112 showed the presence of an additional protein of an approximate size of 41 kDal (Figure 3.15).

3.3 Discussion

It has been possible to isolate from a gene bank (derived from *V. cholerae* Classical strain 569B) a clone which has the capacity to haemagglutinate RBCs. The recombinant plasmid present in this clone was designated pPM471.

Initially it was thought that the soluble haemagglutinin had been cloned, however, it has now been demonstrated that the cloned gene encoded the cell-associated mannose-fucose resistant haemagglutinin (MFRHA). A possible reason for this might be that the SHA and MFRHA possess cross reacting antigenic determinants. However this appears not to be the case (see Chapter 5), therefore a more likely explanation is that the preparation of purified SHA contained contaminating

Figure 3.13 Autoradiogram of colony hybridization. 500 colonies of a cosmid bank derived from *V. cholerae* 017 (El Tor biotype, Ogawa serotype) were screened for the presence of a homologous MFRHA gene. Several colonies reacted positively and were patched onto the one grid as shown in the diagram. Colonies were transferred to nitrocellulose paper and probed with the isolated *Bam*HI fragment of pPM471 which had been nick-translated with [^{32}P]-dCTP. No.98 represents the negative control; *E. coli* K-12 LE392 harbouring pBR322. No.99 represents the positive control; V271.



No. 98 = NEGATIVE CONTROL LE392 + pBR322

No. 99 = POSITIVE CONTROL V271

Figure 3.14 Comparison of *Hin*dIII digested DNA profiles of MFRHA$^+$ cosmid isolates, pPM1111, pPM1112, pPM1113, pPM1114 and pPM1115 with pPM471. The common 2.45 kb *Hin*dIII fragment is indicated by an arrow.

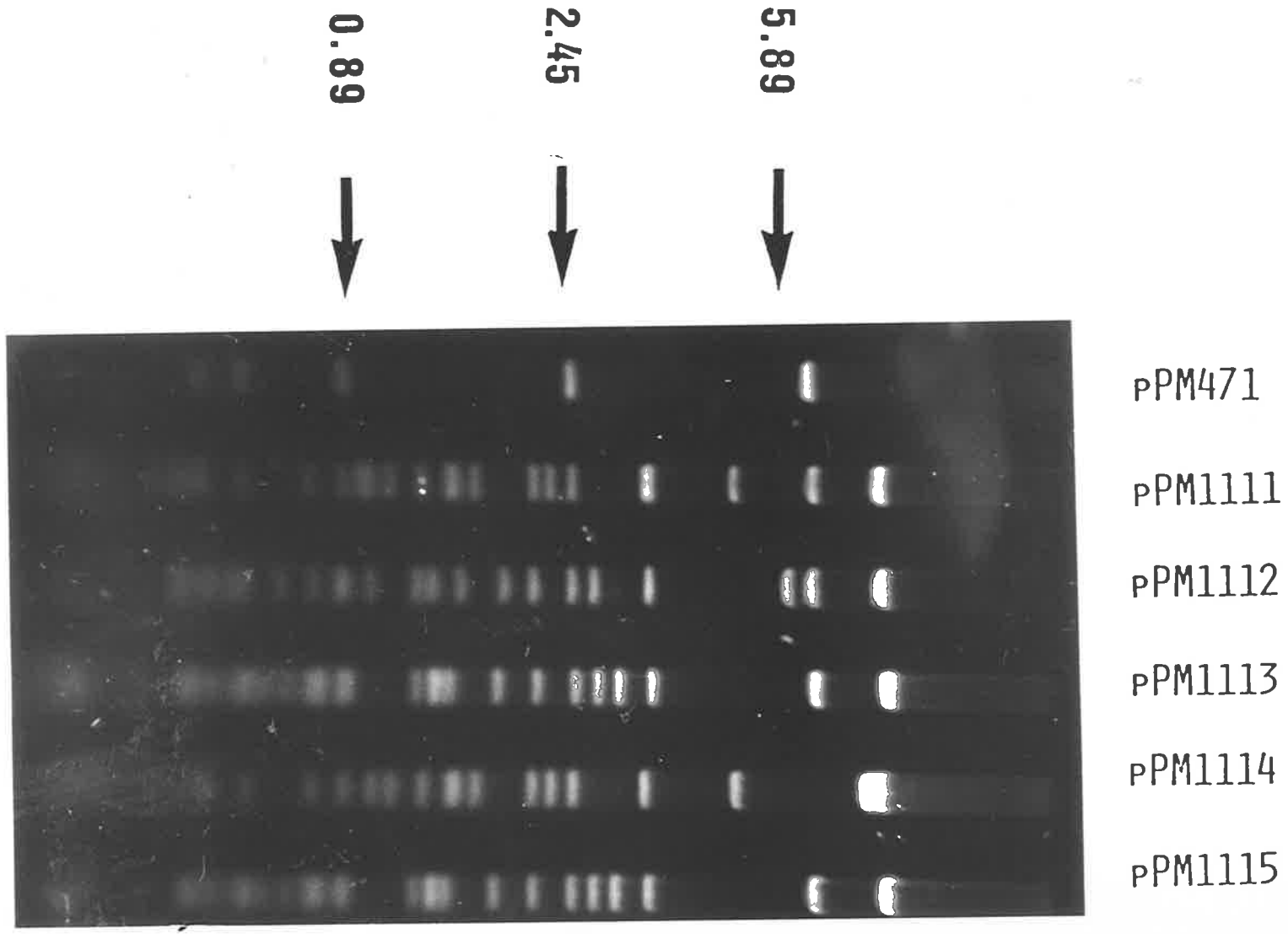
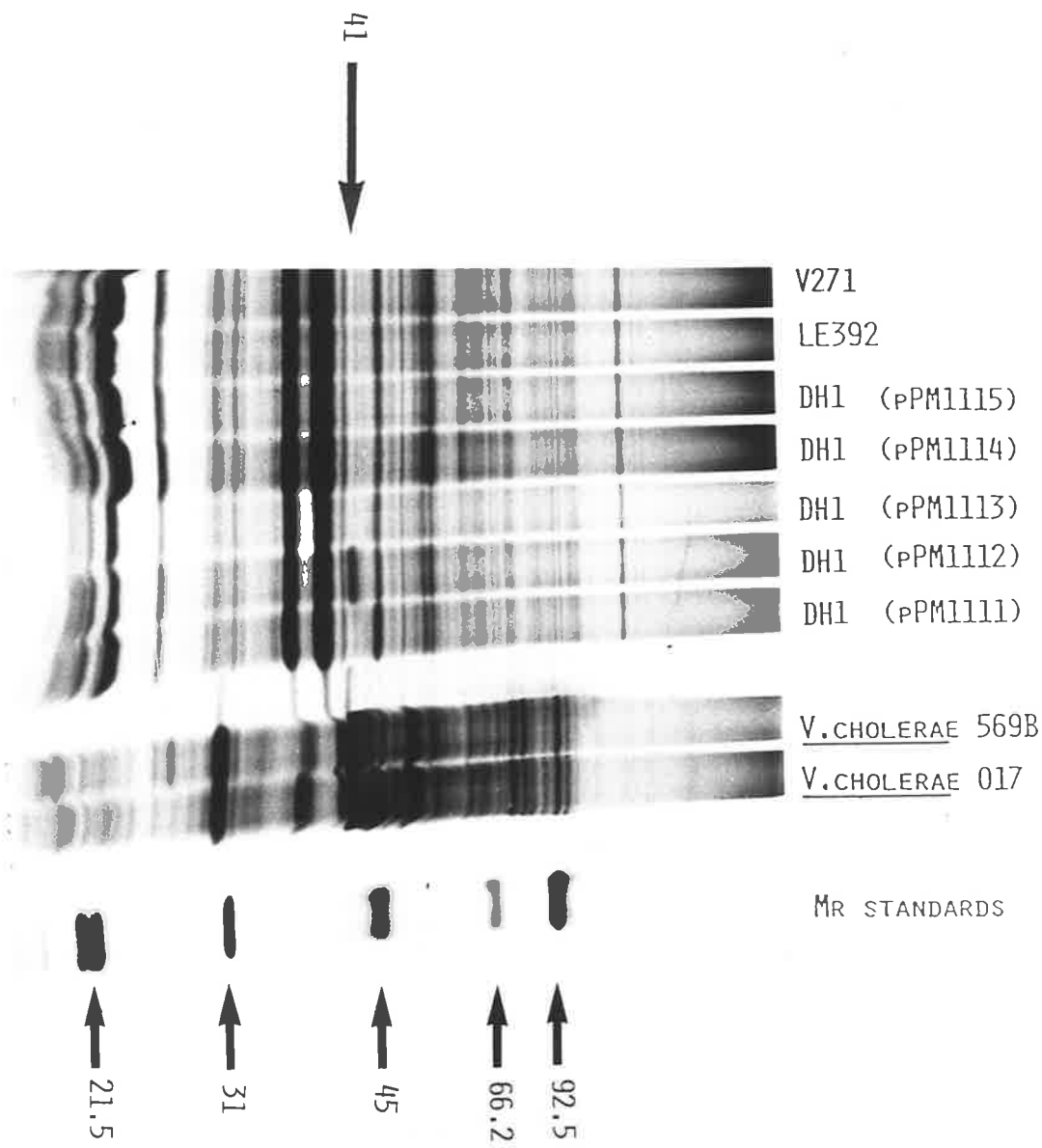

0.89
2.45
5.89
pPM471
pPM1111
pPM1112
pPM1113
pPM1114
pPM1115

Figure 3.15 SDS-polyacrylamide gel electrophoresis of cell envelopes prepared by the small scale method from *E. coli* K-12 strain DH1 harbouring the indicated plasmids or no plasmid (control). The position corresponding to a 41 kDal protein is indicated by an arrow in the left margin. The size markers were phosphorylase B (92.5 kDal), bovine serum albumin (66.2 kDal), ovalbumin (45 kDal), carbonic anhydrase (31 kDal) and soybean trypsin inhibitor (21.5 kDal).


41
V271
LE392
DH1 (pPM1115)
DH1 (pPM1114)
DH1 (pPM1113)
DH1 (pPM1112)
DH1 (pPM1111)
V.CHOLERAE 569B
V.CHOLERAE 017
MR STANDARDS
21.5
31
45
66.2
92.5

proteins which resulted in the presence of corresponding antibodies in the antiserum (R.A. Finkelstein, personal communication).

The haemagglutinin encoded on plasmid pPM471 gave minimal titres with human and chicken erythrocytes and maximum titres with mouse and sheep erythrocytes. It showed no proteolytic activity and haemagglutination was not inhibited by Zincov. Calcium ions were not required for maximum titres. This is in contrast to the SHA which possesses protease activity, gives maximum titres with chicken erythrocytes and whose haemagglutination is enhanced by the presence of calcium ions and inhibited by the addition of Zincov.

Restriction endonuclease mapping of plasmid pPM471 demonstrated the presence of a 6.1 kb *Bam*HI fragment of *V. cholerae* DNA inserted in pBR322. Deletion analysis using both restriction endonucleases and *Bal*31 nuclease, allowed localization of the gene encoding the haemagglutinin to within a region of 0.72 kb. This minimum coding capacity implied that the size of the protein had an upper limit of 27 kDal.

Minicell analysis showed a protein product of 25 kDal correlated with haemagglutinating activity. The MFRHA could be expected to be synthesized with a signal sequence since it must be exported to the OM to enable it to agglutinate RBCs. Hence this would use up nearly all of the available coding capacity. Some of the larger plasmids produced a 10 kDal protein in minicells, in addition to the 25 kDal protein. All plasmids which were haemagglutination positive gave a titre equivalent to the original clone pPM471. That is, pPM1127 which produced only the 25 kDal protein gave exactly the same titre as pPM471.

Using the 2.45 kb *Hin*dIII fragment of pPM1106 as a radioactively labelled probe, allowed the isolation of haemagglutination positive clones from the El Tor Ogawa strain O17, all of which shared a 2.45 kb fragment with pPM471 and exhibited haemagglutinating activity.

Chapter 4

Genetic Organization of the Gene Encoding the MFRHA

4.1 Introduction

The nucleotide sequence of very few *V. cholerae* genes have been determined. These include the genes encoding for the following proteins: cholera enterotoxin (Lockman and Kaper, 1983; Mekalanos *et al.* 1983; Lockman *et al.* 1984), OmpV, a major *V. cholerae* outer membrane protein of 26 kDal (Pohlner *et al.* 1986a), an extracellular DNase (Focareta and Manning, 1987), the ToxR protein, responsible for the coordinate regulation of a number of virulence determinants, (Miller and Mekalanos, 1984; Miller, 1985; Miller *et al.* 1987; Taylor *et al.* 1987a) and the El Tor haemolysin (Alm *et al.* 1988).

Sequence analysis has confirmed the order of the genes *ctx*A and *ctx*B, showing the gene encoding the A subunit (*ctx*A) to precede that for the B subunit (*ctx*B) (Lockman and Kaper, 1983; Mekalanos *et al.* 1983; Lockman *et al.* 1984). It was also shown that the A subunits and the B subunit are synthesized with signal sequences of 18 and 21 amino acids respectively (Lockman and Kaper, 1983; Mekalanos *et al.* 1983).

Miller, Taylor and Mekalanos (1987) have sequenced the *tox*R gene and from this data it has been suggested ToxR is a transmembrane protein since a stretch

of 16 hydrophobic amino acids exist, flanked by charged or uncharged polar amino acids. ToxR also shows substantial amino acid sequence homology to a number of bacterial transcriptional activators.

Thus determination of nucleotide sequence for a given gene or genes can provide substantial information.

In this chapter, the genetic organization of the gene encoding the MFRHA of *V. cholerae* is examined. Using pUC8 and pUC9 vectors, identification of internal promoters can be made. The direction of transcription was determined by using expression vectors pEva, pEvb, pEVc. Finally, the nucleotide sequence of the cloned MFRHA gene of *V. cholerae* was obtained. Hence a detailed analysis of the genetic organization could provide essential information for future work such as the construction of specific mutations.

4.2 Results

4.2.1 Location of promoter

Plasmid pPM471 contains a 6.1 kb *Bam*HI fragment of *V. cholerae* 569B DNA cloned into the *Bam*HI site of pBR322. This cloned DNA encodes for the mannose-fucose resistant haemagglutinin. Five major promoters have been identified in pBR322 (Stüber and Bujard, 1981) (Figure 4.1). The P2 promoter is responsible for tetracycline resistance and is inactivated by *Hin*dIII cleavage, however an insertion into the *Bam*HI site may be under the control of this P2 promoter, if in the appropriate orientation. To establish whether expression of the haemagglutinin was under the control of the vector promoter or whether the natural promoter was cloned intact, the vectors pUC8 and pUC9 were employed to clone fragments in the two possible orientations. pUC8 and pUC9 contain the β-lactamase gene and origin of DNA replication from pBR322 ligated to the *E. coli lacZ* gene. Into the lac region a polylinker with various restriction enzyme sites has been introduced (Vieira and Messing, 1982). pUC8 and pUC9 differ only in the orientation of their

respective polylinkers.

pPM1107 was digested with *Eco*RI and *Cla*I and ligated to both pUC8 and pUC9 which had been digested with *Eco*RI and *Acc*I (Figure 4.2). Transformed *E. coli* K-12 LE392 cells harbouring the resultant plasmids pPM1109 and pPM1110 were tested for their ability to haemagglutinate mouse (Balb/c) RBCs with and without IPTG induction. It was found that haemagglutination was expressed equally in both orientations suggesting the gene(s) is being expressed from its own promoter.

4.2.2 Direction of transcription

To determine the direction of transcription the pEv31 series of plasmids were used. Plasmids pEv31 a, b and c are derivatives of the pEx expression vector system (Strebel *et al.* 1986) which itself is based on pPlc24 (Remaut *et al.* 1981). pEv31 a, b, c, carry a polylinker in three different reading frames relative to the MS2 polymerase frame and contain the P_L promoter of bacteriophage λ which is controlled by a cI_{ts} repressor.

Plasmid pPM1127 was previously generated by *Bal*31 nuclease digestion and cells harbouring this plasmid are haemagglutination positive (Chapter 3, section 3.2.9). Introduction of the cloned DNA from pPM1127 into pEv31 may give a fusion protein in one reading frame or an overproduction of the protein in all frames. Plasmid pPM1127 was digested with *Cla*I and *Hin*dIII and ligated to each of the pEv31 vectors which had been digested with *Acc*I and *Hin*dIII. pPM1127 was also digested with *Eco*RI and *Hin*dIII and ligated to the pEv plasmids cleaved with *Hin*dIII and *Eco*RI. The resulting plasmids were introduced into *E.coli* K-12 537 which contains a temperature sensitive cI repressor on a Km^R plasmid, pcI857. Upon heat induction at 42^0C, no overproduction of proteins was observed. However when the resulting plasmids were tested for haemagglutination capacity only plasmids pPM1138, pPM1139, and pPM1140 were HA positive. These plasmids were pEva, pEvb and pEvc containing the *Cla*I-*Hin*dIII fragment of pPM1127 re-

Figure 4.1 Transcription of pBR322. The upper part of the figure gives a schematic view of pBR322 linearized by *Pvu*II. The transcriptional units, defined by the various promoters (P1 through P5) and corresponding termination signals, are delineated by arrows. The widths of the arrows reflect the relative promoter strengths. It can be seen that the *bla* gene is heavily transcribed because two promoters (P1 and P3) contribute to expression. Under the conditions used, transription from P2 is terminated near the *Sal*I site, leaving most of the tetracycline region untranscribed. The most efficient promoter of the system is P4 which produces the 104 RNA (adapted from Stüber and Bujard, 1981).
Restriction endonuclease sites are as follows:
B: *Bam*HI; **E**: *Eco*RI; **H**: *Hin*dIII; **P**: *Pst*I; **Pv**: *Pvu*II; **S**: *Sal*I.

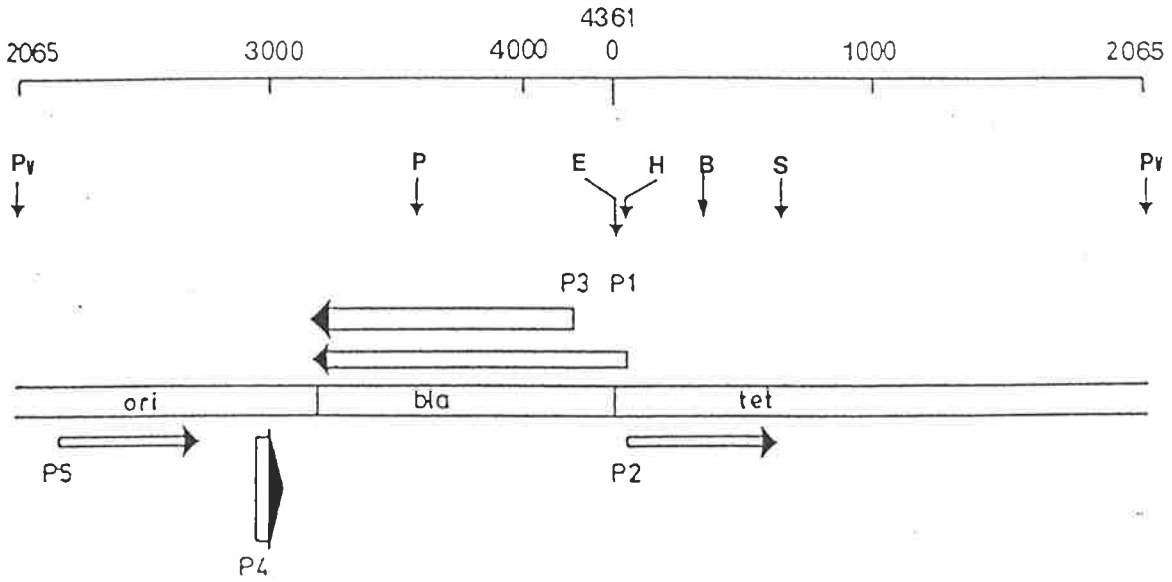
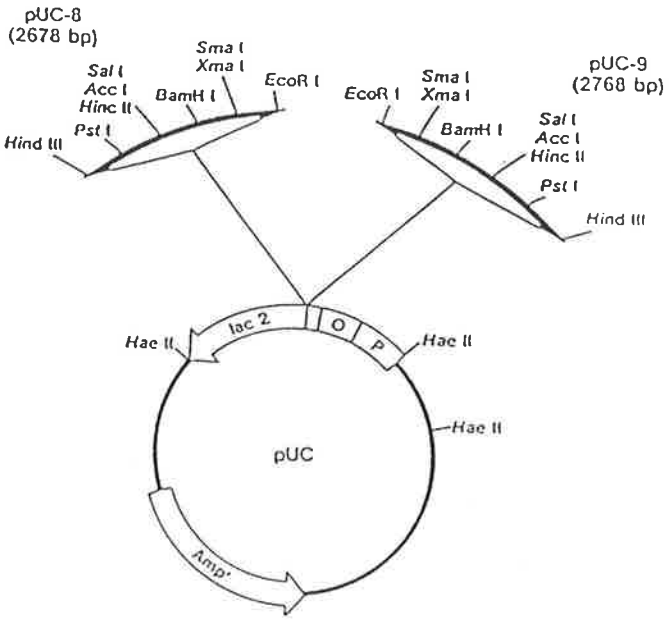
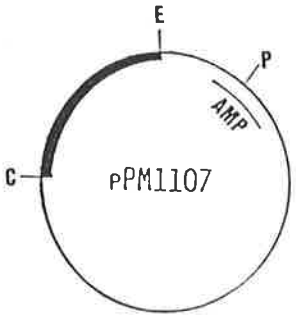

4361
2065
3000
4000
0
1000
2065
Pv
P
E
H
B
S
Pv
P3
P1
ori
bla
tet
PS
P2
P4

Figure 4.2 Construction of plasmids pPM1109 and pPM1110. Vectors pUC8 and pUC9 were digested with *Eco*RI and *Acc*I, plasmid pPM1107 with *Eco*RI and *Cla*I. The cloned insert of pPM1107 is represented by the thick line. Following ligation and transformation into *E. coli* K-12 LE392 two recombinant plasmids were generated. pPM1109 is the *Eco*RI-*Cla*I insert of pPM1107 cloned into the pUC8 polylinker. pPM1110 contains the same insert but cloned into the pUC9 polylinker and therefore the opposite orientation. Both plasmids exhibit haemagglutination.
Restriction endonuclease sites are as follows:
C: *Cla*I; **E**: *Eco*RI; **P**: *Pst*I.

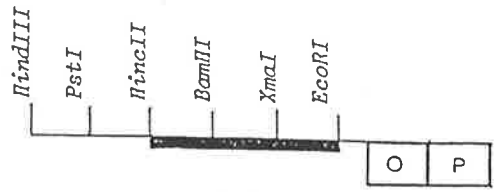


pUC8, pUC9 DIGESTED
WITH EcoRI, AccI

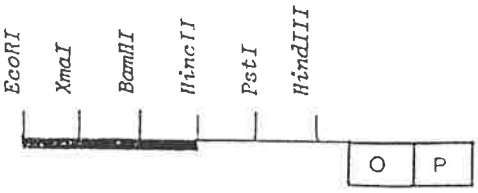


pPM1107 DIGESTED
WITH EcoRI, ClaI

LIGATE



pPM1109
pUC8, EcoRI- ClaI ORIENTATION
= HAEMAGGLUTINATION POSITIVE



pPM1110
pUC9, ClaI- EcoRI ORIENTATION
= HAEMAGGLUTINATION POSITIVE

spectively (Figure 4.3). This therefore suggests that the haemagglutinin gene is transcribed from the *Cla*I end to towards the *Eco*RI end of the insert.

A number of attempts have been made using different expression vectors to obtain overproduction of the MFRHA, but all have failed. The reasons for this could be the poor Shine-Dalgarno sequence present before the start of the MFRHA gene (see Section 4.2.5).

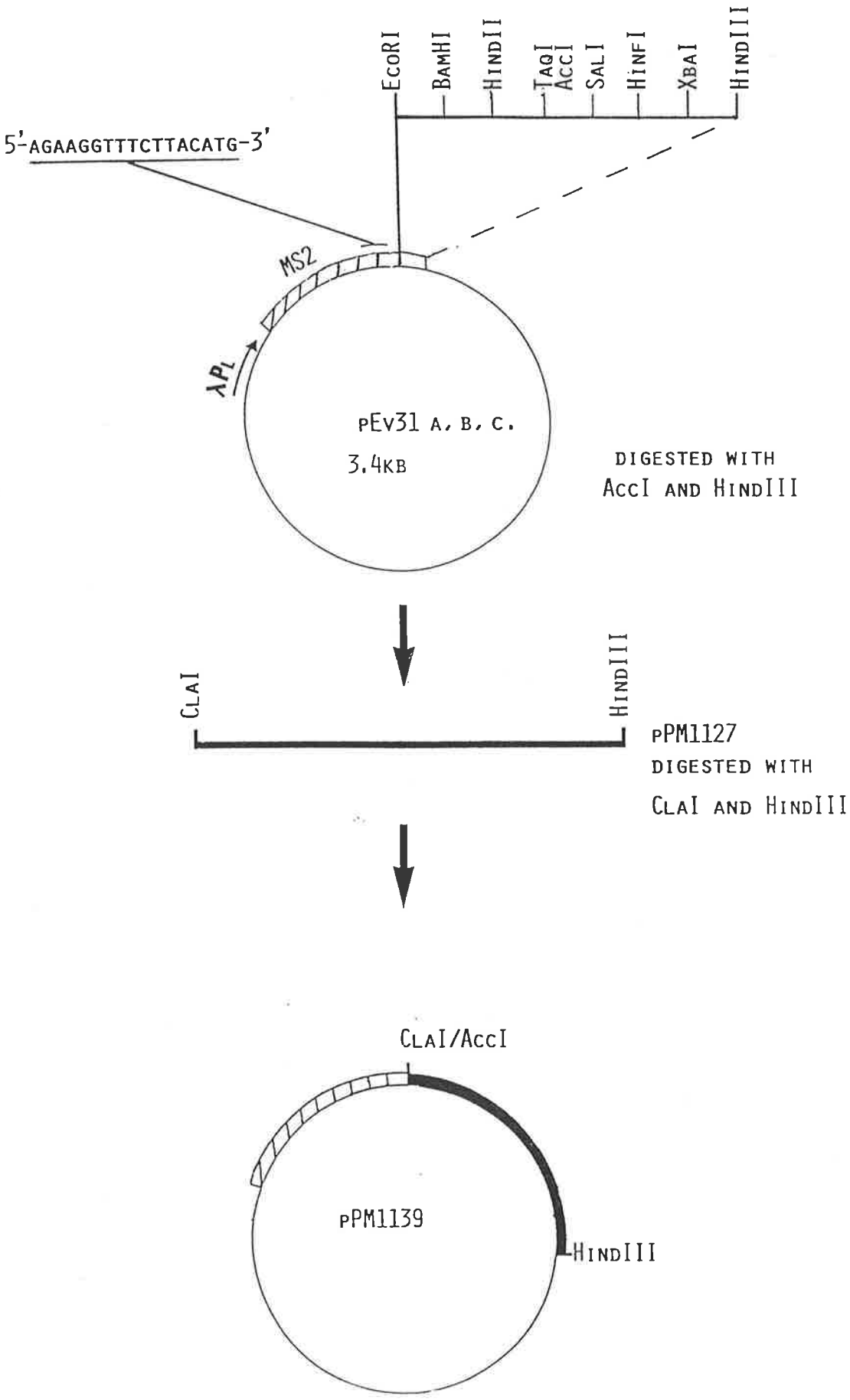
4.2.3 Generation of fragments for nucleotide sequencing

The gene encoding the MFRHA has been localized within plasmid pPM1107 which still produces a haemagglutination positive phenotype (Chapter 3, Section 3.2.9). As can be seen from Figure 3.6, there exists no six base pair restriction endonuclease cleavage sites between the *Nru*I and *Xba*I sites. This necessitated that fragments for cloning into the single stranded DNA phages M13mp18 and M13mp19 (Messing and Vieira, 1982) for use in dideoxy sequencing reactions, be generated in two ways.

Use was made of the deletion plasmids which had been generated by incubation in the presence of *Bal*31 nuclease (Chapter 3, Section 3.11). The smallest plasmid (i.e. largest deletion) still capable of mediating haemagglutination, pPM1127, was cleaved with *Cla*I and *Eco*RI and cloned into the *Acc*I–*Eco*RI sites of the replicative form M13 vector DNA. After transfection of JM101, white plaques were screened for inserts containing *V. cholerae* DNA. Plasmids pPM1128, pPM1129 and pPM1130 were also digested with *Cla*I and *Eco*RI and ligated to M13 vectors digested with *Acc*I and *Eco*RI.

In addition, sequence information was obtained from *Taq*I fragments present in this region. The *Eco*RI–*Cla*I fragment of pPM1126 was extracted from a low melting point agarose gel. This isolated fragment was then incubated with *Taq*I at 65^{0}C and a sample of the digested DNA was end-labelled with α–[^{32}P]–dCTP using Klenow fragment. This was then added to the remaining digested sample and run on a 30% polyacrylamide gel. DNA bands were visualized by autoradiography after 30 min and the appropriate bands excised from the gel and used for cloning into the

Figure 4.3 Construction of plasmid pPM1139. pEv31b a modified version of a vector system used for expression of bacterial fusions in *E. coli* (Remaut *et al.* 1981; Klinkert *et al.* 1985; Strebel *et al.* 1986) was digested with *Acc*I and *Hin*dIII. This was ligated to pPM1127 (haemagglutination positive *Bal*31 deletion) which had been digested with *Cla*I and *Hin*dIII. The resulting plasmid was transformed into *E. coli* K-12 strain 537 which contains a temperature sensitive cI reppressor.


EcoRI
BamHI
HindII
TaqI
AccI
SalI
HinfI
XbaI
HindIII
5'-AGAAGGTTTCTTACATG-3'
MS2
λPL
pEv31 A, B, C.
3.4kb
DIGESTED WITH
AccI AND HindIII
ClaI
HindIII
pPM1127
DIGESTED WITH
ClaI AND HindIII
ClaI/AccI
pPM1139
HindIII

M13mp vectors which had been cleaved with *Acc*I (*Acc*I and *Taq*I have compatible ends). *Taq*I digestion of the *Eco*RI-*Cla*I insert of pPM1126 generated four bands of sizes 62 bp, 168 bp, 241 bp and 652 bp (Figure 4.4).

Included as a control (to ensure digestion was complete) was pPM471 incubated with *Hae*III, since pBR322 has 22 known *Hae*III restriction endonuclease sites present.

4.2.4 Nucleotide sequence determination

Sequencing reactions were carried out according to the dideoxy chain termination procedures of Sanger *et al.* (1977). Randomly terminated chains were separated on ultra thin 6% polyacrylamide gels, in the presence of 8M Urea. Autoradiography was used to visualize DNA fragments. A section of a sequencing gel is illustrated in Figure 4.5.

In order to sequence the complementary strand 5′-3′ synthetic oligonucleotides were synthesized using an Applied Biosystems Model 381A DNA synthesizer. These oligonucleotides span bases 645-627, 500-482, 410-392 and 150-132. The strategy used in sequence analysis is indicated in Figure 4.6.

The complete nucleotide sequence of 1,398 base pairs has been determined in both directions and shows two open reading frames (ORF1 and ORF2). This is not true contiguous DNA but, as can be seen from the construction of pPM1107, represents two regions which are actually joined by a *Mlu*I fragment. Thus, it is reasonable to treat the regions either side of the *Mlu*I site independently. As will be discussed below it seems likely that ORF1 extends from nucleotide position 76 to the termination codon at nucleotides 745-747 (Figure 4.7). This represents the MFRHA gene.

Firstly, the features of the MFRHA nucleotide sequence will be discussed and then briefly some features of the sequence of the following protein.

Figure 4.4 Samples of digested DNA which have been end-labelled using α-[^{32}P]-dCTP were electrophoresed onto a 30% polyacrylamide gel. The DNA fragments were then visualized by autoradiography.

Track 1: the *Eco*RI-*Cla*I insert fragment of pPM1126 was isolated, then digested with *Hae*III.

Track 2: the same isolated fragment digested with *Taq*I. This generated four visible fragments which were used for nucleotide sequence determination.

Track 3: pPM471 digested with *Hae*III.

pPM1126/HaeIII
pPM1126/TaqI
pPM471/HaeIII
FRAGMENT 1
FRAGMENT 2
FRAGMENT 3
FRAGMENT 4

Figure 4.5 Portion of the nucleotide sequence obtained by the dideoxy sequencing method and electrophoresis through a 6% polyacrylamide denaturing gel (8M Urea). The region expanded corresponds to the complementary strand of the sequence from positions 274-246 of ORF2.

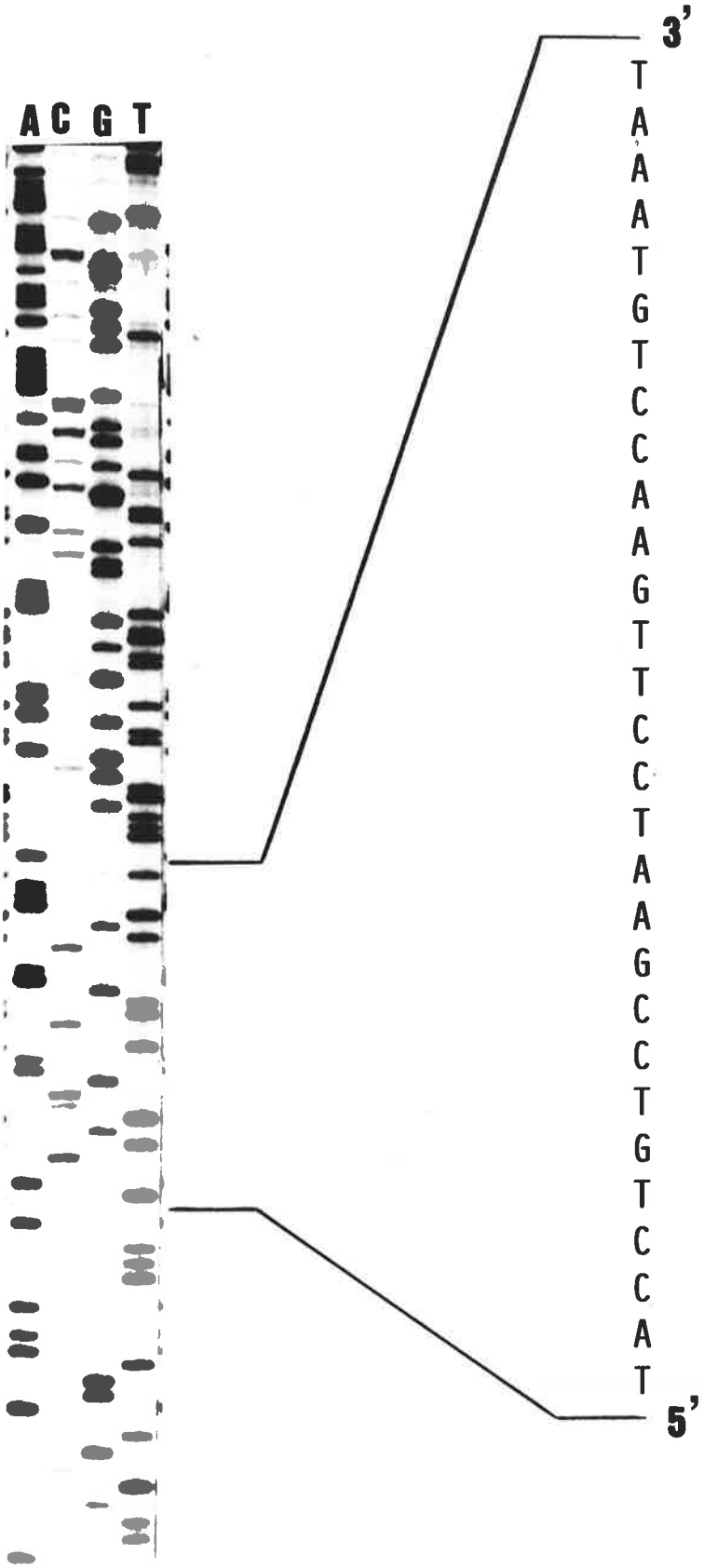

A C G T
3'
T
A
A
A
T
G
T
C
C
A
A
G
T
T
C
C
T
A
A
G
C
C
T
G
T
C
C
A
T
5'

Figure 4.6 M13 cloning strategy employed for dideoxy sequencing of the 1,398 bp region bounded by a *Nru*I and a *Eco*RI site. At the top is a restriction map of the *Bam*HI fragment of *V. cholerae* located in pPM471. Below pPM471, the *V. cholerae* DNA in plasmid pPM1107 is shown. Plasmid pPM1107 was derived by first subcloning the *Eco*RI-*Cla*I fragment of pPM471, followed by a *Mlu*I generated deletion of the resulting plasmid. The insert region in plasmid pPM1107 has then been expanded to show the section that was sequenced. The arrows below the restriction map show the direction sequencing proceeded and the length (in base pairs (bp)) of the nucleotide sequence.

1, **2** and **3** denote the sequence obtained from the *Bal*31 generated deletions.

4 denotes the sequence obtained by *Taq*I isolated fragments.

5, **6** and **7** denote sequence obtained in the opposite direction through using synthetic oligonucleotides as primers.

Restriction endonuclease sites are as follows:

B: *Bam*HI; Bg: *Bgl*II; C: *Cla*I; E: *Eco*RI; H: *Hin*dIII; M: *Mlu*I; N: *Nru*I; X: *Xba*I.

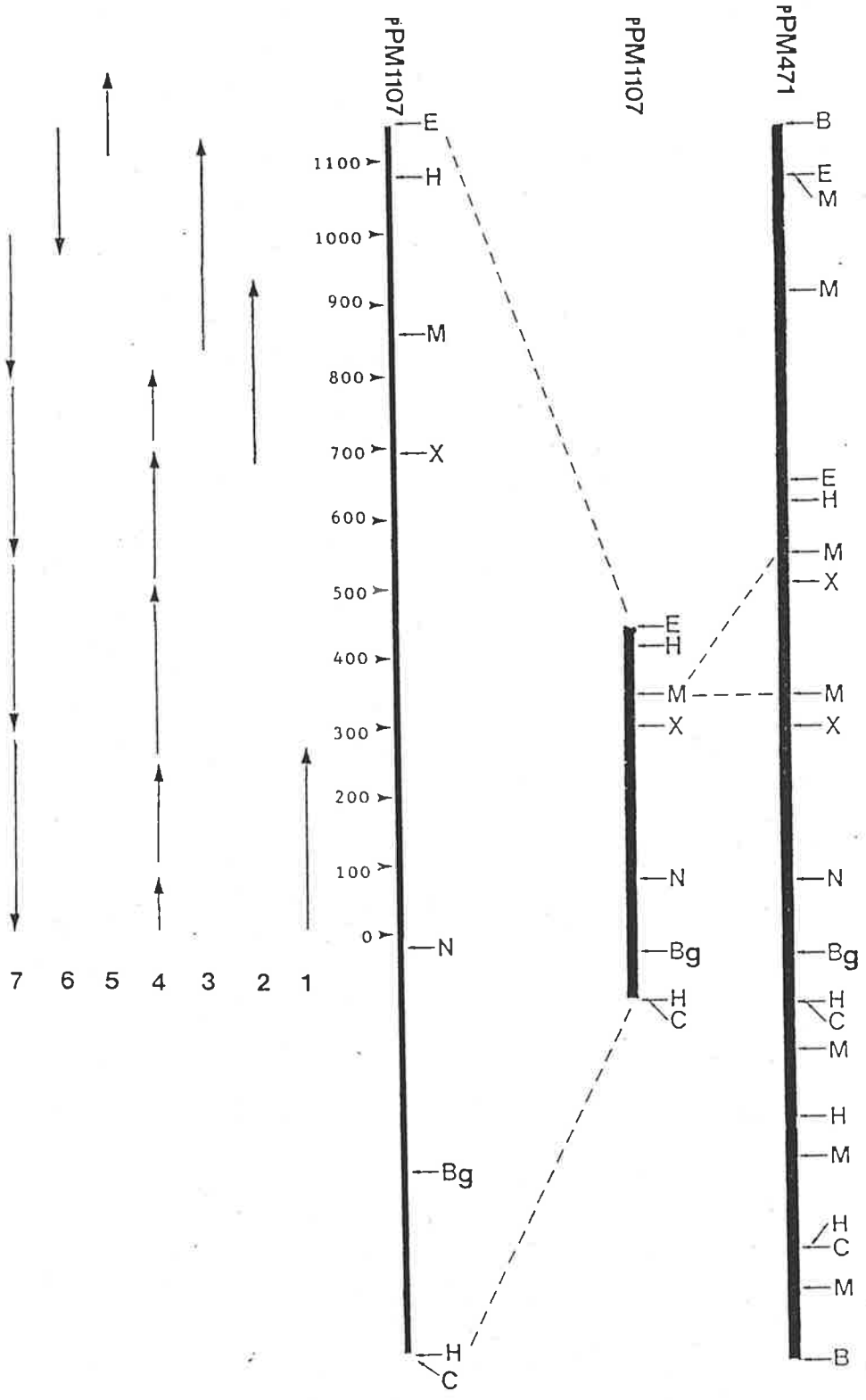

pPM1107
pPM1107
pPM471
E
H
M
X
N
Bg
H
C
B
1100
1000
900
800
700
600
500
400
300
200
100
0
7
6
5
4
3
2
1

Figure 4.7 Nucleotide sequence of a 756 bp region of cloned *V. cholerae* DNA is shown. The region contains an open reading frame (ORF1), which starts at bp 76 and ends at bp 745 with the termination codon TAA. ORF1, when translated, was found to encode a 25 kDal polypeptide which implied that it was the MFRHA. The base pairs are numbered on the right. Amino acids are indicated by three letter designation under each triplet (Ala, alanine; Arg, arginine; Asn, asparagine; Asp, Aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, trptophan; tyr, tyrosine; Val, valine).

The termination codon is denoted by $\star\star\star$.

```
GTAATGACAGATACGATTTTAACGTATCACTGTTTCATGTTAGGTTATGCGTTTCCCTCG      60

ATATTAGGCAATTTA ATG TCA AAA ATT TAT CAA ATG GAT GAG GTT AGT      108
                Met Ser Lys Ile Tyr Gln Met Asp Ala Val Ser
                                                        10
TGG CTT AAA ACA CTT GAA AAT TGT AGT GTT GAT CTG TTC ATC ACT      153
Trp Leu Lys Thr Leu Glu Asn Cys Ser Val Asp Leu Phe Ile Thr
                                20
GAT CCA CCA TAT GAA TCG CTA GAA AAA TAT AGA CAA ATA GGT ACG      198
Asp Pro Pro Tyr Glu Ser Leu Glu Lys Tyr Arg Gln Ile Gly Thr
            30                                          40
ACT ACA CGG TTA AAA GAG AGT AAA TCA TCG AGC AAT CAA TGG TTT      243
Thr Thr Arg Leu Lys Glu Ser Lys Ser Ser Ser Asn Gln Trp Phe
                                50
AGT GTT TTT CCT AAC ACT AGG TTT GAA GAG TTG TTT CGT GAA GTT      288
Ser Val Phe Pro Asn Thr Arg Phe Glu Glu Leu Phe Arg Glu Val
            60                                          70
TAT AGA GTG CTA AAA AAA GGT TCT CAT TTC TAT TTA TTT TGC GAC      333
Tyr Arg Val Leu Lys Lys Gly Ser His Phe Tyr Leu Phe Cys Asp
                                80
CAG GAA ACT ATG TTT TTG GCG AAA CCA ATA GCG GAA AGT GTA GGC      378
Gln Glu Thr Met Phe Leu Ala Lys Pro Ile Ala Glu Ser Val Gly
            90                                          100
TTT AAA TTT TGG AAG CCT ATA GTT TGG GAT AAG TGT GCT ATA GGT      423
Phe Lys Phe Trp Lys Pro Ile Val Trp Asp Lys Cys Ala Ile Gly
                               110
ATG GGA TAT CAT TAT CGT GCT AGA TAT GAA TTT ATT CTA TTT TTC      468
Met Gly Tyr His Tyr Arg Ala Arg Tyr Glu Phe Ile Leu Phe Phe
            120                                         130
GAG AAA GGA AAG AGA AAG TTA AAT GAT TTA AGT GTT CCT GAT GTG      513
Glu Lys Gly Lys Arg Lys Leu Asn Asp Leu Ser Val Pro Asp Val
                               140
TTG GAA TAT AAG AGA GTT TGG AAA GGC TAC CCA ACA GAA AAG CCA      558
Leu Glu Tyr Lys Arg Val Trp Lys Gly Tyr Pro Thr Glu Lys Pro
            150                                         160
GTT GAA CTT CTG GAG GTT TTG ATT AGG CAA AGC TCT TCA GAA AAT      603
Val Glu Leu Leu Glu Val Leu Ile Arg Gln Ser Ser Ser Glu Asn
                               170
GAA ATT GTA GCG GAT TCA TTT TTT GGT TCA GGC TCA ACT TTA ATT      648
Glu Ile Val Ala Asp Ser Phe Phe Gly Ser Gly Ser The Leu Ile
            180                                         190
GCA GCT AAT AAT CTC TCT AGA AAA TAC ATT GGT TGT GAT ATA TCA      693
Ala Ala Asn Asn Leu Ser Arg Lys Tyr Ile Gly Cys Asp Ile Ser
                               200
AGT TCT GCA CAT GAG TAT TTT AAG AAT AGA GCT TGC AAC ACT ATT      738
Ser Ser Ala His Glu Tyr Phe Lys Asn Arg Ala Cys Asn Thr Ile
            210                                         220
TAT GCC TAA CAAACGCGT                                           756
Tyr Ala ***
```

4.2.5 Regulatory sequences affecting expression of the MFRHA

Analysis of various promoter sequences have shown two regions of homology. One exists 10 base pairs upstream from the initiation site and is termed the "Pribnow box". The other is located 35 bp upstream and is referred to as the "−35 region" or "recognition sequence" (Maniatis *et al.* 1975; Pribnow, 1979; Hawley and McClure, 1983; Studnicka, 1987). The consensus sequence for each are:

T_{80}, A_{95}, t_{45}, A_{60}, a_{50}, T_{96} and

T_{82}, T_{84}, G_{78}, A_{65}, C_{54}, a_{45}

respectively, where the subscript represents the percent occurrence of the base which is most frequently found at that position. Capital letters represent bases which are >54% conserved. Lower case letters represent bases which are less conserved (Siebenlist *et al.* 1980). Using these consensus sequences as a guide, potential promoter regions can be detected prior to the start point. These suggested −35 and −10 regions are TATCAC and ATGACA at bp 25-30 and 4-9, respectively. The −35 region shows excellent homology with the consensus with, only the first base differing. The −10 region shows reasonable homology with two bases varying. The location of this suggestive promoter is consistent with observations using pUC and pEv31 vectors (Sections 4.2.1 and 4.2.2). Information gained from use of the pEv31 vectors indicates that transcription proceeds from the *Cla*I end to towards the *Eco*RI end.

Experiments by Dr. Sue Williams (personal communication) have confirmed that this region may possess a putative promoter. Schneider and Beck (1986) have constructed a vector pCB192, which contains promoterless indicator genes, *lacZ* and *galK*. pPM1127 was digested with *Dde*I and *Xmn*I, end-filled and ligated to *Sma*I digested pCB192. There is a *Dde*I site just prior to the sequence shown in Figure 4.7. The ligation mixture was then transformed into a $galK^-$ *E. coli* strain. The presence of promoters are tested by plating colonies onto indicator plates ie. McConkey-galactose plates. Expression of *galK* activitiy was produced

with the inserted *Dde*I-*Xmn*I fragment, indicating promoter presence. However, the strength of these promoter sequences was not determined.

Theoretically the allowed spacing between the two regions is 15 to 21 base pairs with the promoter strength being maximized with a 17 ± 1 bp spacing (Hawley and McClure, 1983). The spacing seen between the -35 and -10 regions of the MFRHA gene is 15 bp.

Upon examination of the sequence upstream from the initiation codon, one does not see the presence of a stem-loop structure capable of blocking transcription of the DNA encoding the 10 kDal protein, which precedes the haemagglutinin protein. Perhaps there exists a dyad symmetry before the sequence shown in Figure 4.7. Another possibility could be that transcription is not terminated after the 10 kDal protein but reads through to include the MFRHA on the same transcript. This latter explaination seems more likely as will become evident.

As mentioned briefly above, further analysis of the nucleotide sequence upstream of the initiation codon AUG also reveals the presence of a possible Shine-Dalgarno sequence (Shine and Dalgarno, 1974). This is represented by the sequence AGG as shown in Figure 4.7 and shows homology to the consensus Shine-Dalgarno sequence AGGAGG (Gold *et al.* 1981). This sequence is present 7 bp before the start codon AUG. Translation of the first open reading frame may be initiated at four positions 4, 37, 76, and 94. Since NH_2-terminal amino acid analysis was unattainable one cannot be totally positive about which methionine residue translation actually commences. However, if the regions preceeding each of these residues are examined, only position 76 has a sequence which resembles the consensus of a ribosome-binding site. The other potential initiation codons are not preceeded by a region of significant homology to the consensus. This suspected initiation site correlates well with the predicted size observed in minicell analysis.

The complementary strand of the sequence shown in Figure 4.7 contains numerous termination codons and therefore cannot be considered as a potential gene. The MFRHA gene terminates with the codon UAA at position 745 of the sequence, whether a termination stem-loop follows cannot be deduced, since 0.69

kb DNA has been deleted following the *Mlu*I site at position 751.

4.2.6 ORF1 signal sequence

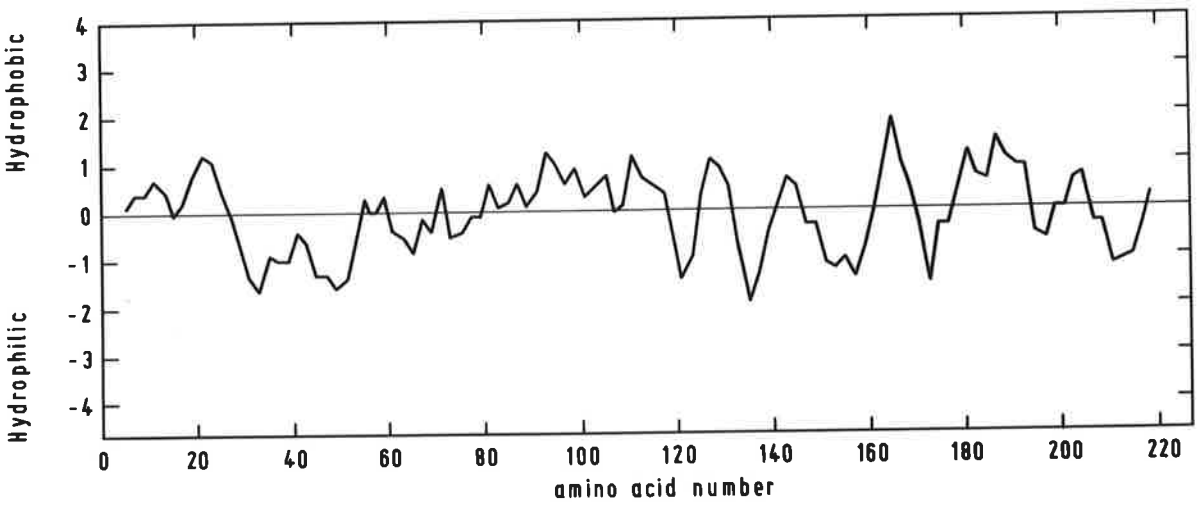
The nucleotide sequence encoding for the MFRHA is a single ORF of 669 bp. corresponding to a protein of 26.065 kDal. A plot of the hydropathic nature of the hypothetical protein precursor was made according to Kyte and Doolittle (1982) and is shown in Figure 4.8. Similar results were obtained when the hydrophobicity was analyzed according to Hopp and Woods (1981) and Eisenberg *et al.* (1984).

Since the MFRHA is clearly found on the bacterial cell surface as demonstrated by its ability to mediate haemagglutination, one would predict the presence of a NH_2-terminal signal sequence

The general pattern of a signal sequence seems to include within the first five amino acids a positively charged residue, which is normally followed by a core of at least nine hydrophobic residues, which would enable spanning of the membrane. Generally, the amino acid at position -1 from the cleavage site is an Ala or Gly or at lower frequencies a Ser, Cys or Thr. Usually an Ala, Gly or Ser is also found at position -3 according to the -3,-1, rule defined by von Heinje (1984; 1985). Comparison of the MFRHA with this general format shows it to be atypical. If cleavage occurs it must be before Asp at amino acid 8 since negatively charged amino acids are never found in signal sequences. Thus cleavage most likely occurs between the Gln and Met amino acids (a.a. 6 and 7). Therefore the signal sequence of the MFRHA is only 6 amino acids in length. Cleavage of this signal sequence would give a mature protein of 25.296 kDal consistent with the observed size of the protein detected in *E. coli* K-12 minicells (Chapter3, Section 3.2.10).

The leader sequences for a number of pilin genes have been compared (Marrs *et al.* 1985) and shows the presence of signal sequences six amino acids in length, prior to the begining of the mature protein. In addition Taylor and co-workers (1987a) have reported that the first amino acid after the signal sequence in the TcpA major pilin protein, which is one of the pili types found in *V. cholerae*, is a

Figure 4.8 Hydropathic nature of pro-MFRHA. The amino acid sequence of the entire precursor form of MFRHA was analyzed according to Kyte and Doolittle (1982) using a window of nine amino acids.


Hydrophobic
Hydrophilic
4
3
2
1
0
-1
-2
-3
-4
0
20
40
60
80
100
120
140
160
180
200
220
amino acid number

methionine.

It is apperent from the hydropathicity plot that there are a number of markedly hydrophobic domains within the MFRHA, consistent with its observed outer membrane location in *E. coli* K-12.

A predicted secondary structure according to Chou and Fasman (1974a, 1974b, 1978) enables further speculation as to the organization of the protein. Such a structure is shown in Figure 4.9.

The predicted structure in combination with the hydropathic plot suggests that the MFRHA protein is highly ordered. In particular, the arrangement of the Cys residues is interesting. Cys_{19} and Cys_{85} are both located within regions of extended β sheet whereas, Cys_{113}, Cys_{203} and Cys_{218}, are closely linked to turn regions. It seems likely that the former and possibly Cys_{203} and Cys_{218} are cross-linked. Thus Cys_{113}, would be unlinked. Possibly it is involved in subunit/subunit interactions.

4.2.7 Codon usage

A summary of codon usage in MFRHA mRNA is shown in Table 4.1. Table 4.2 shows the MFRHA gene codon usage as compared to the predominant usage seen in other sequenced *V. cholerae* genes. These genes include: *toxR* (Miller *et al.* 1987), *ctxAB* (Mekalanos *et al.* 1983), *ompV* (Polhner *et al.* 1986a), *xds* (Focareta and Manning, 1987), *hlyA*, (Alm *et al.* 1988). As can be seen the codon uasge of MFRHA conforms to the predominant usage within *V. cholerae* genes. However, the codon usage presented is slightly biased since they represent a select group of proteins, namely OM and secreted proteins.

4.2.8 Restriction endonuclease cleavage sites

The position of cleavage sites of various restriction endonuclease sites in the MFRHA nucleotide sequence is shown in Figure 4.10. This confirms the data obtained from restriction endonuclease mapping.

Figure 4.9 Predicted secondary structure of the MFRHA protein according to Chou and Fasman (1974a, 1974b, 1978).

The various regions are represented as follows:

α helix, 

β sheet, 

turn, 

random coil

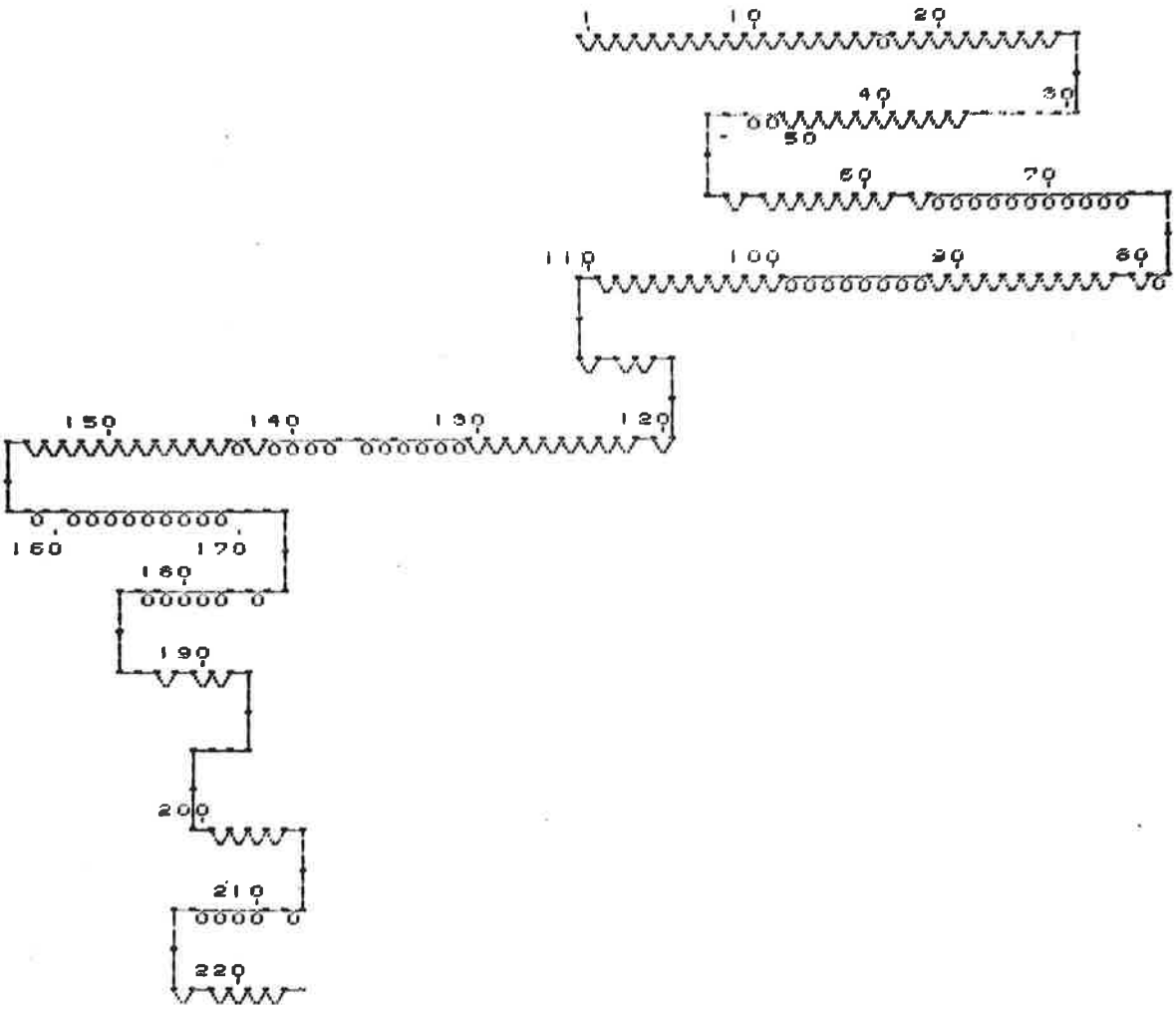

1
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
210
220

Table 4.1 Codon usage within MFRHA.

	U^2	C^2	A^2	G^2	
U^1	13 Phe	4 Ser	11 Tyr	3 Cys	U^3
	3 Phe	0 Ser	2 Tyr	2 Cys	C^3
	5 Leu	7 Ser	1 ochre	0 opal	A^3
	4 Leu	2 Ser	0 amber	5 Trp	G^3
C^1	3 Leu	3 Pro	3 His	2 Arg	U^3
	1 Leu	0 Pro	0 His	0 Arg	C^3
	3 Leu	5 Pro	4 Gln	0 Arg	A^3
	2 Leu	0 Pro	1 Gln	1 Arg	G^3
A^1	7 Ile	6 Thr	7 Asn	7 Ser	U^3
	1 Ile	0 Thr	2 Asn	2 Ser	C^3
	5 Ile	3 Thr	12 Lys	7 Arg	A^3
	4 Met	1 Thr	7 Lys	2 Arg	G^3
G^1	9 Val	4 Ala	8 Asp	5 Gly	U^3
	0 Val	1 Ala	1 Asp	3 Gly	C^3
	2 Val	2 Ala	13 Glu	2 Gly	A^3
	2 Val	4 Ala	5 Glu	0 Gly	G^3

1: Denotes the first base of the three base codon.

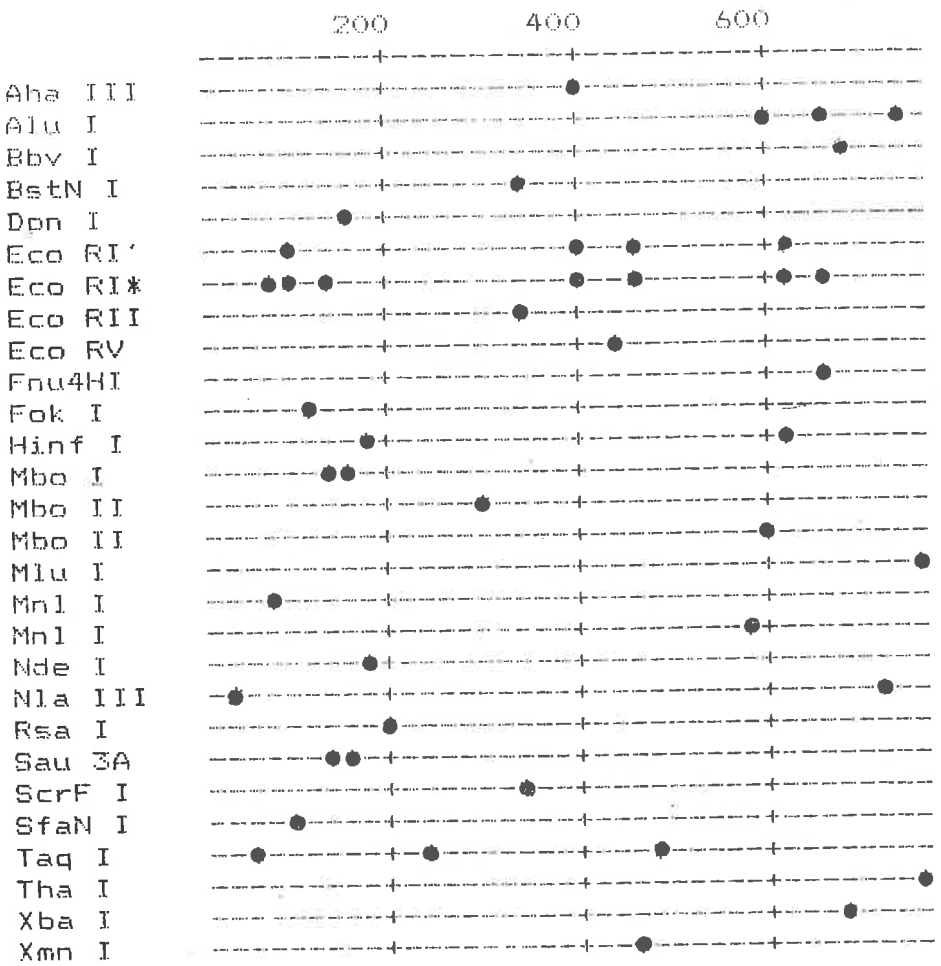
2: Denotes the second base of the three base codon.

3: Denotes the third base of the three base codon.

Table 4.2 Comparison of the MFRHA gene codon usage with the predominant codon usage amongst the sequenced *V. cholerae* genes.

Codon	Amino Acid	Percent MFRHA	Percent *V. cholerae*	Codon	Amino Acid	Percent MFRHA	Percent *V. cholerae*
TTT	Phe	81.2	64.5	GCT	Ala	36.4	31
TTC	Phe	18.8	35.3	GCC	Ala	9.1	22.8
				GCA	Ala	18.2	23.9
TTA	Leu	27.8	27.4	GCG	Ala	36.4	22.3
TTG	Leu	22.2	20.5				
CTT	Leu	16.7	13.7	TAT	Tyr	84.6	54.9
CTC	Leu	5.5	8.4	TAC	Tyr	15.4	45.1
CTA	Leu	16.7	11.1				
CTG	Leu	11.1	18.9	CAT	His	100	72.4
				CAC	His	0	27.6
ATT	Ile	53.8	45.9				
ATC	Ile	7.7	33.8	CAA	Gln	80	69.6
ATA	Ile	38.5	20.3	CAG	Gln	20	30.4
ATG	Met	100	100	AAT	Asn	77.8	50.7
				AAC	Asn	22.2	49.3
GTT	Val	69.2	31.5				
GTC	Val	0	21.2	AAA	Lys	63.2	71.7
GTA	Val	15.4	16.4	AAG	Lys	36.8	28.3
GTG	Val	15.4	30.8				
				GAT	Asp	88.9	73
TCT	Ser	18.2	13.0	GAC	Asp	11.1	27
TCC	Ser	0	8.3				
TCA	Ser	31.8	20.8	GAA	Glu	72.2	67.2
TCG	Ser	9.1	12.5	GAG	Glu	27.8	32.8
AGT	Ser	31.2	26.6				
AGC	Ser	9.1	18.8	TGT	Cys	60	58.1
				TGC	Cys	40	41.9
CCT	Pro	37.5	24.7				
CCC	Pro	0	8.6	TGG	Trp	100	100
CCA	Pro	62.5	30.9				
CCG	Pro	0	35.8	CGT	Arg	16.7	36.2
				CGC	Arg	0	16.4
ACT	Thr	60	32.0	CGA	Arg	0	15.5
ACC	Thr	0	25.0	CGG	Arg	8.3	6.0
ACA	Thr	30	20.3	AGA	Arg	58.3	19.8
ACG	Thr	10	22.7	AGG	Arg	16.7	6.0
GGT	Gly	50	43.5				
GGC	Gly	30	30.6				
GGA	Gly	20	15.6				
GGG	Gly	0	10.2				

Figure 4.10 Restriction endonuclease cleavage sites of the nucleotide sequence, from base number 1 to base number 756. Site of cleavage is represented by a circle.


200
400
600
Aha III
Alu I
Bbv I
BstN I
Dpn I
Eco RI'
Eco RI*
Eco RII
Eco RV
Fnu4HI
Fok I
Hinf I
Mbo I
Mbo II
Mbo II
Mlu I
Mnl I
Mnl I
Nde I
Nla III
Rsa I
Sau 3A
ScrF I
SfaN I
Taq I
Tha I
Xba I
Xmn I

Restriction endonucleases for which there are no cleavage recognition sites in the nucleotide sequence are: *Aat*II, *Acc*I, *Aha*II, *Apa*I, *Asu*II, *Ava*I, *Ava*II, *Avr*II, *Bal*I, *Bam*HI, *Ban*I *Ban*II, *Bbv*I, *Bcl*I, *Bgl*I, *Bgl*II, *Bsp*1286, *BssH*II, *Bst*XI, *BstE*II, *BstX*I, *Cla*I, *Dde*I, *Eco*B, *Eco*K, *EcoP*15, *EcoP*I, *Eco*RI, *Hae*I, *Hae*II, *Hae*III, *Hga*I, *Hgi*AI, *Hgi*DI, *Hha*I, *Hind*dIII, *HinP*I, *Hinc*II, *Hpa*I, *Hpa*II, *Hph*I, *Kpn*I, *Mst*I, *Mst*II, *Nae*I, *Nar*I, *Nci*I, *Nco*I, *Nla*IV, *Not*I, *Nru*I, *Nsi*I, *Pst*I, *Pvu*I, *Pvu*II, *Rru*I, *Rsh*I, *Sal*I, *Sau*96I, *Sca*I, *Sfa*NI, *Sfi*I, *Sma*I, *Sna*BI, *Sst*I, *Sst*II, *Stu*I, *Tok*I, *Tth*111I, *Tth*111II, *Xho*I, *Xho*II, *Xma*I, *Xma*III, and *Xor*II.

4.2.9 ORF 2

Following the terminaton of the region coding for the MRFHA protein, there is another ORF encoding for a possible outer membrane protein. However one must remember that sequencing was performed on a DNA segment which has the *Mlu*I piece of pPM471 removed. The sequence shown in Figure 4.11 is 642 bp. The ORF commences at bp 140 and ends at bp 388. Therefore the ORF is 248 representing 83 amino acids which corresponds to a protein size of 9.370 kDal. 7 bp, before the initiation codon UTG, is a Shine-Dalgarno sequence AGG.

A plot of the hydropathic nature of the hypothetical protein precursor was made according to Kyte and Doolittle (1982) and is shown in Figure 4.12. Similar results were obtained when the hydrophobicity was analyzed according to Hopp and Woods (1981) and Eisenberg *et al.* (1984). The signal sequence for this protein is in excellent agreement to the general format. The positively charged amino acid, Lys, occurs straight after the Met which is then followed by 16 hydrophobic amino acids. Cleavage occurs between the Gly and Leu amino acids (a.a. 19 and 20). Removal of this signal sequence would give a mature protein of 7.242 kDal.

The sequence shown in Figure 4.11 starts at the restriction endonuclease cleavage site for *Mlu*I which is ACGCGT. The *Eco*RI restriction cleavage site of pPM1107 is situated at bp 377. An oligonucleotide which spanned bases 336 to 353 was used to obtain the remaining 265 bases. ORF 2 terminates at a UGA codon.

4.3 Discussion

The nucleotide sequence of 1,398 bp of *V.cholerae* DNA has been determined. Two open reading frames for two different polypeptides have been identified.

Sequence was obtained from the artificial *Cla*I site of pPM1127 to 250 bp past the *Eco*RI site. The gene encoding the MFRHA of *V. cholerae* is suggested to start at position 76 and to terminate at the UAA codon at position 745 of the sequence. This 669 bp region consists of 223 amino acids residues giving a protein product of 26.065 kDal. This gene shows the presence of an unusual signal sequence which when cleaved gives a mature protein product of 25.296 kDal. Although the initiation codon has been placed at position 76, there are four other possible start codons. However, if we examine the sequence preceeding each of the Met codons for putative promoter regions as well as ribosome binding sites, the initiation codon begining at nucleotide 76 seems the most logical choice. The polpeptide size deduced from this initiation also correlates best with minicell data obtained in Chapter 3.

A region of dyad symmetry is not seen following the MFRHA gene, because the sequence in which it is likely to be positioned has been deleted. Just before the termination codon of this ORF is a possible initiation codon for another ORF. In fact there is an overlap of 8 nucleotides before the *Mlu*I site is reached. Prior to this is an excellent Shine-Dalgarno site AAGAA. Therefore this could be the start of another ORF which would encode the 15 kDal protein detected from minicell analysis. This is also consistent with RNA analysis which indicates that the MFRHA and 15 kDal are transcribed on the same RNA transcript and therefore form an operon or possibly are part of a larger operon. An overlap such as this has also been seen in the case of *fanG* and *fanH* which are genes encoding fimbrial-like proteins involved in the biosynthesis of K99 fimbriae (Roosendaal, 1987). This situation could involve translational coupling (Schümperli *et al.* 1982) where a ribosome terminating translation of the MFRHA gene, could reinitiate translation of the gene encoding the 15 kDal protein without being released from the mRNA.

Following the *Mlu*I segment is an ORF for a mature protein of 7.242 kDal.

This ORF is 248 bp consisting of 83 amino acid residues. There is the presence of a 20 amino acid signal sequence with the cleavage site occurring between the alanine and aspargine residues.

Figure 4.11 Nucleotide sequence of a 642 bp region of cloned *V. cholerae* DNA is shown. The region contains an open reading frame (ORF), which starts at bp 140 and ends at bp 389 with the termination codon TAA.

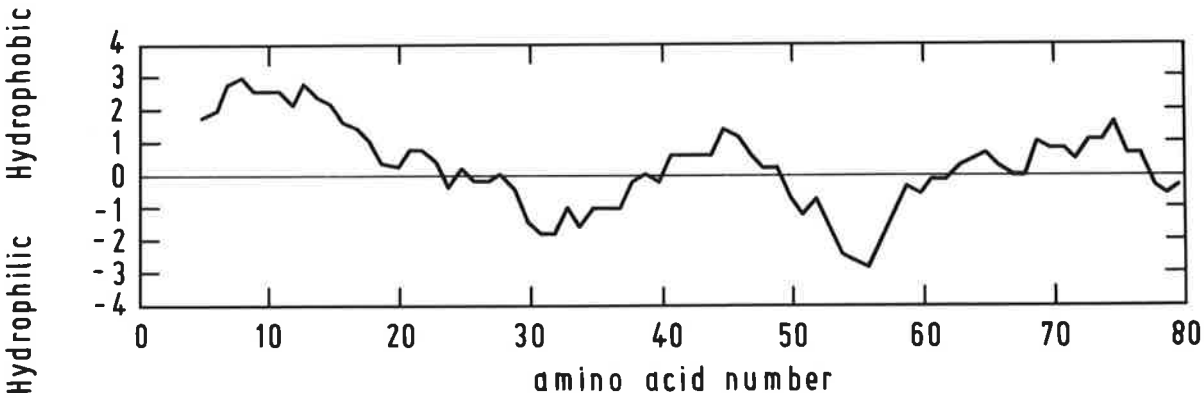
The base pairs are numbered on the right.

Amino acids are indicated by three letter designation under each triplet (Ala, alanine; Arg, arginine; Asn, asparagine; Asp, Aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, trptophan; tyr, tyrosine; Val, valine).

The termination codon is denoted by $\star\star\star$.

```
ACGCGTCAATACGATGCACAGTGGCGTTTCCAGTCCCATTGAGCCGCGGTGGTTTCGGTT      60

GTTGTGTTTGAGTTTAGTGTGTGCGTTGTCATCCCTTAGCGGCGTTAGGTGAATGTAAGT     120

GATTGAACAAGGACATGTT ATG AAA TGT TTT TTA GCT TTT TGG CTC GTT      169
                    Met Lys Cys Phe Leu Ala Phe Trp Leu Val
                                                          10
TTC GTC AGC TTT TTT TCG GTC GGC TTG CAT GCC AAT GAC GCC GTT      214
Phe Val Ser Phe Phe Ser Val Gly Leu His Ala Asn Asp Ala Val
                                              20
TTA CAA CAA GCT TAT CAA TCG CAA CAA AGT GAT TTA CAG GTT CAA      259
Leu Gln Gln Ala Tyr Gln Ser Gln Gln Ser Asp Leu Gln Val Gln
                    30                                    40
GGA TTC GGA CAG GTA GTG AAA GTG TTA CCT GAC GAC AAT GAT GGT      304
Gly Phe Gly Gln Val Val Lys Val Leu Pro Asp Asp Asn Asp Gly
                                              50
TCA AAG CAT CAA AAA TTC ATC TTA AAG CTC AAT AGC GGA CAA ACA      349
Ser Lys His Gly Lys Phe Ile Leu Lys Leu Asn Ser Gly Gln Thr
                    60                                    70
TTG CTG GTT GCT CAT AAC ATG GAC TTA GAA TTC CGA ACT TGA AAGT    395
Leu Leu Val Ala His Asn Met Asp Leu Glu Phe Arg Thr ***
                                              80
TGGCGATAGTGTTGAGTTTTATGGTGAATATGAATGGAACAAAAAAGGTGGGGTTCTTCA    455

CTGGACTCATAAAGATCCTCAAAATCGTCATGCTCATGGTTGGTTGAAACACAATGGGCA    515

GGTGTACGAGTAAATTCACCTAAGGGGCGCCTCAAGCGGGACTGTCAACGCGGCGTTTCC    575

AGTCCCATTGAGCCGCGGTGGTTTCGGTTGTTGTGTTTGAGTTTAGTGGTAATGCGTTGC    635

CAGCCCC                                                          642
```

Figure 4.12 Hydropathic nature of the pro–7 kDal protein. The amino acid sequence of the entire precursor form of the 7 kDal protein was analyzed according to Kyte and Doolittle (1982) using a window of nine amino acids.


Hydrophilic
Hydrophobic
4
3
2
1
0
-1
-2
-3
-4
0
10
20
30
40
50
60
70
80
amino acid number

Chapter 5

Construction of Defined Mutations in the *Vibrio cholerae* chromosome

5.1 Introduction

To evaluate the contribution that a particular component of *V. cholerae* makes in the process of eliciting disease, it is necessary to construct specific mutants in the respective molecules. However, very few defined mutations have been introduced into the *V. cholerae* chromosome.

By repeated rounds of NTG mutagenesis Honda and Finkelstein (1979) isolated a mutant with a defect in the gene encoding the A subunit (*ctxA*) of the cholera enterotoxin, whilst leaving the B subunit gene (*ctxB*) intact. This *V. cholerae* strains was named Texas Star SR. Schneider and Parker *et al.* (1978) also used NTG mutagenesis to construct protease deficient strains and compared them with wild-type parent strains. However, mutations obtained in this fashion have a number of disadvantages in that the precise alteration is unknown and therefore one cannot rule out the possibility of reversion, or the introduction of mutations into genes for other components which are unrecognized but which may affect virulence. Therefore, using such a method for generating mutations will not allow one to

specifically associate any loss of virulence with a specific gene product.

More recently, the use of recombinant DNA techniques have allowed the incorporation *in vivo* of precise deletion mutations into otherwise wild-type strains. This avoids the possibility of reversion and allows specific genes to be eliminated whilst leaving those for other components untouched. This method has now been used by a number of workers (Mekalanos *et al.* 1983; Kaper *et al.* 1984).

After sequencing the cholera toxin operon, Mekalanos *et al.* (1983) constructed an internal deletion *in vitro* in the *ctxA* gene. This was then recombined *in vivo* into the chromosome of a *V. cholerae* strain as the basis of producing a potential live vaccine strain. Kaper and colleagues have also introduced specifically constructed cholera toxin gene deletions into the *V. cholerae* chromosome.

This chapter deals with the construction of a mutation in the MFRHA gene which is then introduced by allelic exchange into the *V. cholerae* chromosome. resulting in a strain which is isogenic, except for the locus encoding the MFRHA gene. This allows analysis of strains possessing and lacking this haemagglutinin and the effect of the mutation on colonization and adherence which are necessary for the onset of disease. Hence an indication of what role the MFRHA may play as a putative adhesin can be obtained.

5.2 Results

5.2.1 Construction of a MFRHA deletion: type1

Plasmid pPM471 contains very few cleavage sites for enzymes which cleave only once or twice. It has a unique site for the restriction endonuclease *Bgl*II and two sites for *Xba*I. Both of these restriction endonucleases do not cleave the vector, pBR322, from which pPM471 is derived. If pPM471 is digested with both *Bgl*II and *Xba*I, 1.8 kb of DNA, part of which encodes the MFRHA, will be deleted.

Hence pPM471 was digested with *Bgl*II and *Xba*I and the ends were then filled using Klenow fragment and deoxynucleotides. Phosphorylated *Bgl*II linkers

were then added to the blunt ends which had just been created.

In parallel, the 3.3 kb *Hin*dIII fragment encoding the chloramphenicol transacetylase of Tn*1725*, was purified. *Hin*dIII ends of this fragment were also end-filled and phosphorylated *Bgl*II linkers were added to the blunt ends.

The *Bgl*II linkers were digested with the *Bgl*II restriction endonuclease and the two preparations were ligated together at 4°C overnight. The ligation mix was transformed into *E. coli* K-12 strain DH1 selection being made for Ap^R, Cm^R colonies.

However, such a construct proved to be unstable, with transformants having further deletions, for example the adjacent DNA containing the *Eco*RI site was readily deleted. Numerous attempts failed to produce the desired construct or one which still had sufficient flanking DNA for recombination. The reasons for this failure are unknown.

5.2.2 Construction of a MFRHA deletion: type 2

Because of the instability of the first construct, a second type was decided upon. As mentioned previously, plasmid pPM471 contains two sites for the restriction endonuclease *Xba*I. One site is situated at nucleotide 664 of the MFRHA sequence (Figure 4.7). A second *Xba*I site is approximately 0.7 kb away from the first. pPM471 was digested with *Xba*I thus removing 92 bp of the MFRHA carboxy-terminal coding sequence plus the gene for the following protein. The DNA was then end-filled using Klenow fragment and deoxynucleotides. Phosphorylated *Pst*I linkers were then added by ligation followed by restriction with *Pst*I and further ligation. The resulting plasmid pPM1145 has two *Pst*I sites, one artificially introduced by means of a linker, the second is in the β-lactamase gene from the original pBR322 cloning vector.

5.2.3 Insertion of a kanamycin resistance cartridge

At first the antibiotic cartridge being used was one which encoded Cm^R. This choice proved to be unfortunate since pPHIJ1 exhibited Cm^R. To check whether pPHIJ1 was Cm^R or the strain (MM294) which harboured the plasmid was, pPHIJ1 was transfered to a Cm^S strain. Upon recieving pPHIJ1 the strain became Cm^R. This was an unusual finding since nowhere in the literature is pPHIJ1 reported as being Cm^R. This proved to be a crucial finding since it meant recombinant events could not be selected by Cm^R. Hence new constructs using a kanamycin cartridge were made.

pPM1143 is pUC8 which has the 1,500 bp Km^R cartridge (Pharmacia) derived from Tn*903* cloned into the *Eco*RI site of the polylinker. *Pst*I sites flank the Km cartridge. pPM1143 was digested with *Pst*I and ligated to pPM1145 which had been partially digested with *Pst*I so that the *Pst*I site in the Ap^R gene remained intact. The ligation mix was transformed into *E. coli* K-12 strain DH1, selecting for Ap^R, Km^R colonies and the resultant plasmid in which the Km^R fragment has replaced the *Xba*I fragment of pPM471 was designated pPM1146 (Figure 5.1).

5.2.4 Subcloning into plasmid pRK290

No generalized system exists for transformation of plasmid DNA into *V. cholerae* strains, necessitating the introduction of plasmids by conjugal mobilization. Ditta and co-workers (1980) developed a broad host range cloning vehicle, RK290. This plasmid can be mobilized at high-frequency into various Gram-negative organisms using a helper plasmid. RK290 confers Tc^R and contains the RK2 replicon, it also has single cloning sites for restriction endonucleases *Eco*RI and *Bgl*II.

pPM1146 was cleaved with *Bam*HI and ligated to *Bgl*II digested RK290 and the resultant ligation was transformed into *E. coli* K-12 strain S17-1 (Sm^R) selecting for Km^R, Tc^R and Sm^R colonies. This produced pPM1147 in which the *Bam*HI fragment of pPM1146 has been cloned into the *Bgl*II site of RK290 (Figure 5.1).

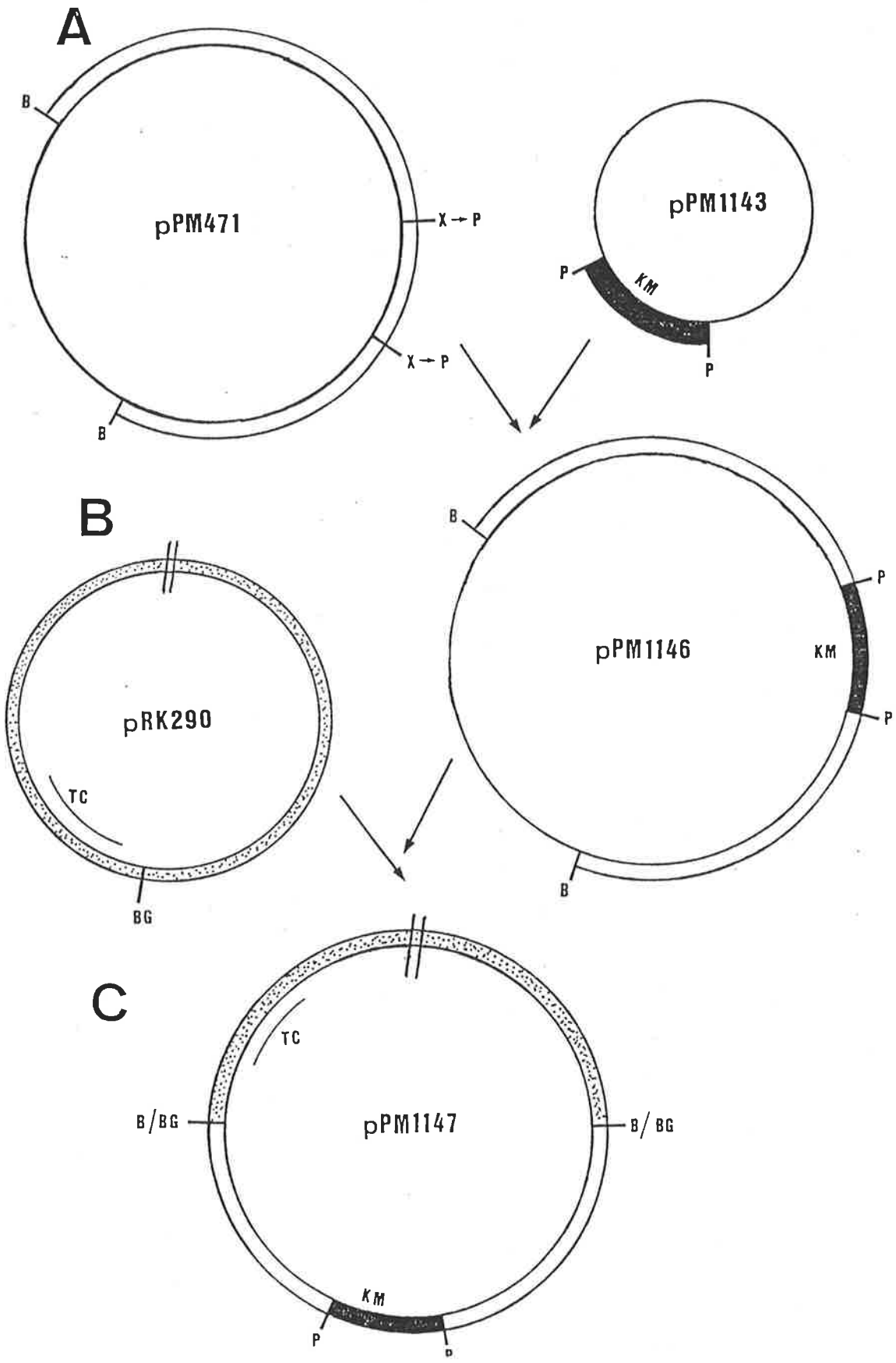
Figure 5.1 Construction of plasmids pPM1146 and pPM1147.

(A) pPM471 was digested with *Xba*I, the ends were then filled using Klenow fragment and deoxynucleotides. Phosphorylated *Pst*I linkers were ligated onto the blunt ends. This was followed by partial digestion and ligation to pPM1143 which had been digested to completion with *Pst*I. The mixture was transformed selecting for Ap^R, Km^R colonies. The resulting plasmid was designated pPM1146 in which the 0.7 kb *Xba*I fragment of pPM471 had been substituted by a 1.5 kb *Pst*I fragment encoding Km resistance.

(B) pPM1146 was digested with *Bam*HI and ligated to *Bgl*II digested pRK290. The mixture was transformed selecting for Km^R, Tc^R colonies.

(C) The resulting plasmid was designated pPM1147.

The restriction endonuclease sites are as follows:
B: *Bam*HI; **Bg**: *Bgl*II; **P**: *Pst*I; **X**: *Xba*I


A
B
pPM471
X → P
X → P
B
pPM1143
P
KM
P
B
pRK290
TC
BG
B
pPM1146
KM
P
P
B
C
TC
pPM1147
B/BG
B/BG
KM
P

5.2.5 Mobilization of pPM1147 from *E. coli* into *V. cholerae*

Plasmid pPM1147 was transferred to *V. cholerae* strain V685 (Classical, Inaba) by selection for Rif^R, Km^R and Tc^R conjugants. V685 is a spontaneous Rif^R mutant of the *V. cholerae* strain 569B. *V. cholerae* strains which had obtained plasmid pPM1147 were purified twice and tested for an oxidase positive reaction, sensitivity to *V. cholerae* specific bacteriophages and agglutination by *V. cholerae* specific antiserum.

5.2.6 Construction of a *V. cholerae* MFRHA$^-$ strain

The Gm^R plasmid pHIJ1, (Ruvkun and Ausubel, 1981) was transferred to strain V685 [pPM1147] by conjugation. Like pPM1147 which is derived from RK290, pHIJ1 is also an Inc P group plasmid and therefore the two plasmids are incompatible, preventing both from being maintained in the same cell. Seventy-five transconjugants were streaked out onto plates containing Rif, Gm and Km and patched onto Tc plates. Seventy of these colonies were Tc^S. These conjugants have potentially had the Km^R cartridge in pPM1147 introduced into the chromosome of V685, by recombination between the flanking homologous DNA present in the cloned DNA (Figure 5.2).

5.2.7 Colony hybridization

Fifty Gm^R, Km^R, Rif^R, Tc^S colonies were repatched onto Rif, Km plates and transferred onto nitrocellulose. Colony hybridization was performed using the 0.7 kb *Xba*I fragment of pPM471 (Figure 5.3). Suprisingly all the Tc^S colonies still hybridized with the probe, but if the Km^R cartridge had been incorporated and a deletion mutant isolated, then such colonies should have lost the *Xba*I fragment. One Tc^S colony did seem to be negative, however this may have been due to poor transfer to the nitrocellulose. The negative control S17-1 did not react. This observation necessitated a more detailed analysis in order to explain the result.

Figure 5.2 Introduction of a defined mutation into the chromosome of *V. cholerae* strain, V685.

(A) Plasmid pPM1147 was mobilized from *E. coli* strain S17-1 into *V. cholerae* strain, V685. Resulting transconjugants were Rif^R, Km^R and Tc^R.

(B) Plasmid pHIJ1 (Gm^R) belongs to the IncP group and was mated into *V. cholerae* V685 [pPM1147]. Gm^R, Rif^R and Km^R colonies were selected and screened for Tc sensitivity.

(C) *V. cholerae* cells in which the $MFRHA^-$ deletion mutation had recombined into the chromosome were isolated.

The restriction endonuclease sites are as follows:
B: *Bam*HI; Bg: *Bgl*II; N: *Nru*I; X: *Xba*I.

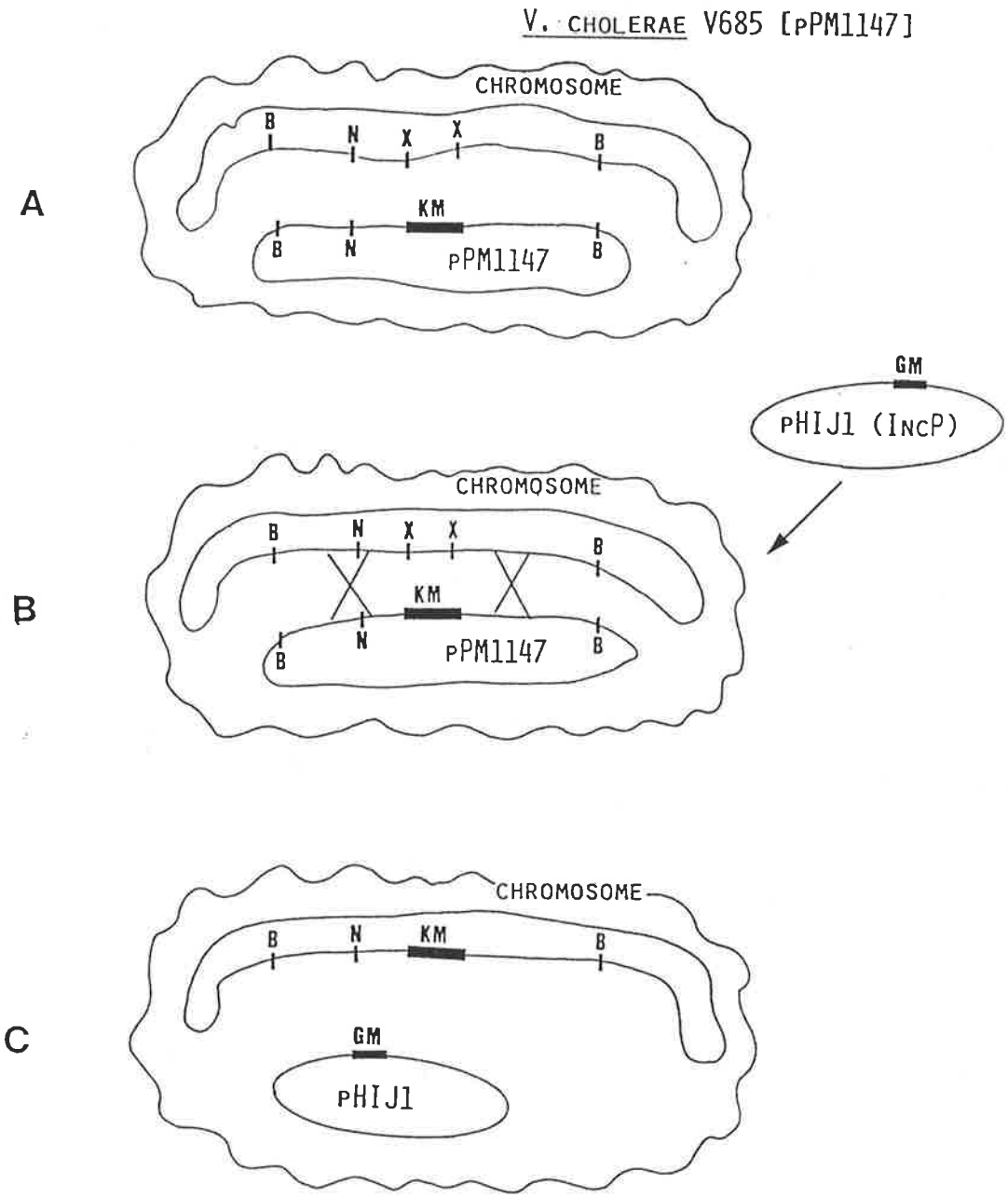
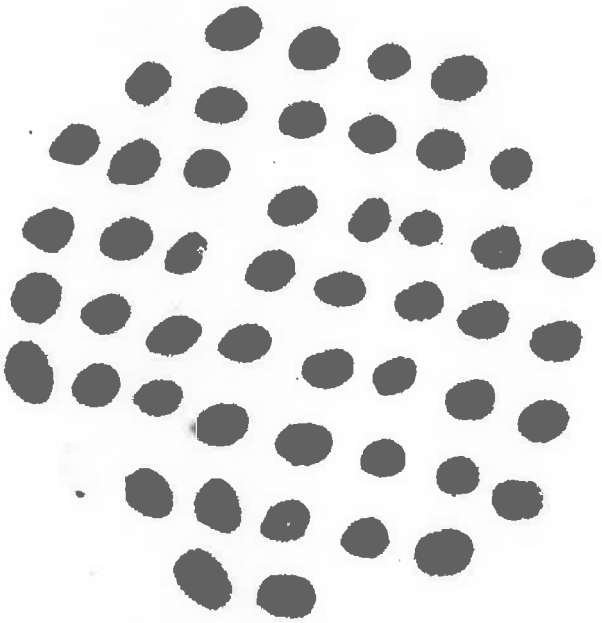
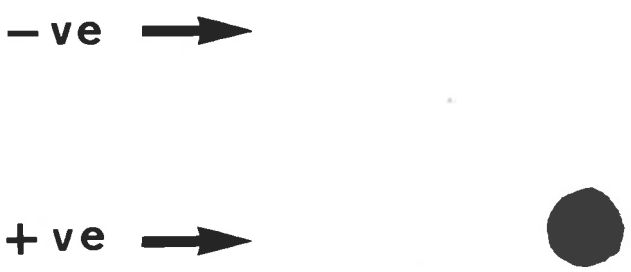

V. CHOLERAE V685 [pPM1147]
A
CHROMOSOME
B
N
X
X
B
KM
B
N
pPM1147
B
GM
pHIJ1 (IncP)
B
CHROMOSOME
B
N
X
X
B
KM
N
B
pPM1147
B
C
CHROMOSOME
B
N
KM
B
GM
pHIJ1

Figure 5.3 Autoradiogram of colony hybridization. Fifty Gm^R, Km^R, Rif^R, Tc^S colonies were patched (Filter A) and then transferred to nitrocellulose paper and probed with the 0.7 kb *Xba*I fragment of pPM471, which had been nick-translated with α-[^{32}P]-dCTP. Filter B shows the positive control, V685 and the negative control, *E. coli* strain S17-1.

A



B



5.2.8 Southern hybridization

Whole genomic DNA was isolated from five Rif^R, Km^R, Gm^R, Tc^S colonies and the one colony which reacted poorly in the colony hybridization. DNA from strain V685 and plasmids pPM471 and pPM1146 were used as controls. These DNAs were digested with *Xba*I and electrophoresed in agarose and transferred to nitrocellulose. The filters were then probed with the isolated 0.7 kb *Xba*I fragment which had been radioactively labelled. The *Xba*I fragment was not detected in DNA from the Rif^R, Km^R, Gm^R, Tc^S colonies when the filters were probed (Figure 5.4), indicating that the Km^R cartridge had inserted into the chromosome to replace this fragment. V685, the parental strain however, still shows the presence of this *Xba*I piece.

The question thus arises as to why the Tc^S colonies reacted in the colony hybridization even though the *Xba*I fragment was not detected in the Southern hybridization. This can be answered by further analysis of the Southern hybridizations shown in Figure 5.4. pPM471 cut with *Xba*I gives two bands, however, when the smaller fragment is used as a probe, it hybridizes to itself and also to the larger *Xba*I fragment. pPM1146 does not possess the 0.7 kb *Xba*I fragment but still reacts with the probe. In those tracks containing the digested chromosomal preparations, the small *Xba*I fragment was not seen but, other multiple bands are detected. This unusual result seems to indicate that within the 0.7 kb *Xba*I fragment must be sequences which have homologous regions located elsewhere in the chromosome.

pPM471 was digested with a range of restriction endonucleases in single as well as double combinations, electrophoresed in agarose, transferred to nitrocellulose and probed with the *Xba*I fragment. Figure 5.5 shows the fragments which can hybridize with the *Xba*I fragment. No hybridization to the vector DNA was observed. Since the hybridizations were done at high stringency, this result clearly indicates that there are other sequences found within the *Bam*HI region cloned in pPM471 which are related to the *Xba*I region.

Figure 5.4 Southern hybridization analysis of whole genomic DNA of *V. cholerae* strain V685 and various Rif^R, Km^R, Gm^R, Tc^S isolates (numbered 1-6). Chromosomal DNA was digested with *Xba*I and electrophoresed on an agarose gel (1%) and the bands visualized by UV irradiation after staining with ethidium bromide. After transfer to nitrocellulose filter the blot was probed with α–[^{32}P]-dCTP labelled 0.7 kb *Xba*I of pPM471.

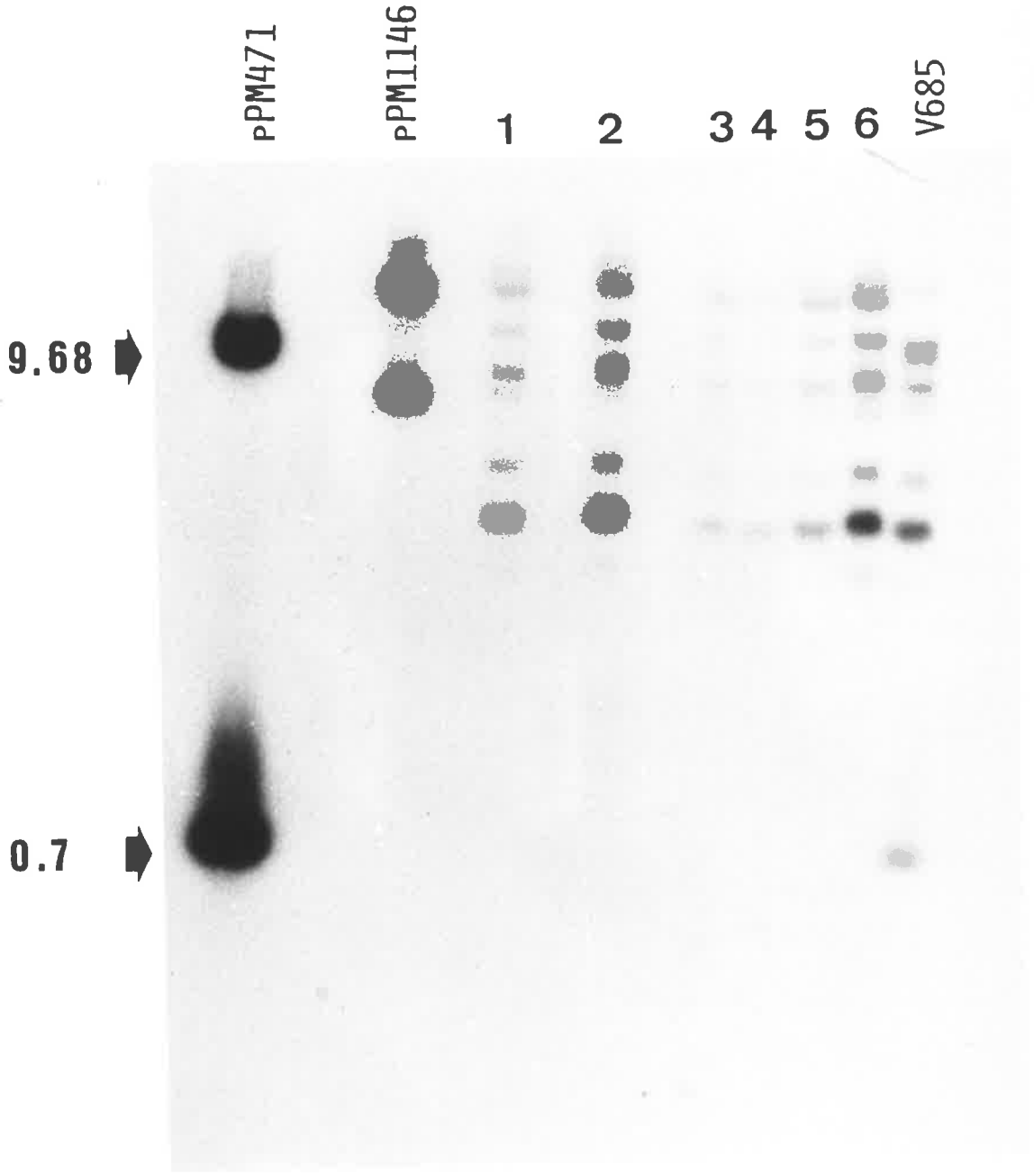

pPM471
pPM1146
1
2
3
4
5
6
V685
9.68
0.7

Figure 5.5 Southern hybridization analysis of pPM471 DNA digested with a range of restriction endonucleases in single as well as double combinations for the presence of DNA homologous to sequences found in the 0.7 kb *Xba*I fragment of pPM471. DNA was digested and electrophoresed on an agarose gel (0.8%) and the bands visualized by UV irradiation after staining with ethidium bromide. After transfer to nitrocellulose filter the blot was probed with α–[^{32}P]-dCTP labelled 0.7 kb *Xba*I fragment of pPM471. The left panel shows the digested genomic DNA stained with ethidium bromide and the right panel shows an autoradiogram of the Southern blot. Restriction endonuclease sites are as follows:
B: *Bam*HI; C: *Cla*I; E: *Eco*RI; H: *Hin*dIII; M: *Mlu*I; X: *Xba*I.


B C H M X B B B B C C C H H X
C H M X C H M X H M X M X M


B C H M X B B B B C C C H H X
C H M X H M X H M X M

5.2.9 Distribution of MFRHA gene in *V. cholerae*

MFRHA activity has been previously found in both biotypes of *V. cholerae* (Booth and Finkelstein, 1986), however, there appear to be a multiplicity of haemagglutinins with this sugar sensitivity and consequently it is not possible to say whether the activities are due to the same HA. This can be analyzed at the DNA level.

Probing of chromosomal DNA from El Tor and Classical biotypes

To assay for the presence of homologous DNA in the chromosomes of both biotypes, the 2.45 kb *Hin*dIII fragment of pPM1106 was purified from a low-melting point agarose gel, radiolabelled and used to probe *Hin*dIII digested whole genomic DNA extracted from various *V. cholerae* strains of both biotypes and serotypes, as well as non-01 *V. cholerae* strains (Table 5.1).

As has been suggested above, this probe which includes the 0.7 kb *Xba*I fragment also detects related sequences throughout the chromosome.

The 2.45 kb *Hin*dIII fragment was detected in each of the *V. cholerae* 01 strains (Figure 5.6) indicating that DNA encoding the MFRHA is conserved between the Classical and El Tor biotypes as well as the Inaba, Ogawa and Hikojima serotypes. Interestingly, related sequences were also detected in the non-01 vibrios, however the 2.45 kb *Hin*dIII fragment was not observed except. No homologous DNA was detected in strains of either *Escherichia coli*, *Salmonella typhimurium* or *Vibrio parahaemolyticus*.

Probing MFRHA$^+$ cosmid isolates

DNAs from MFRHA$^+$ cosmid isolates (Chapter 3) were digested with *Hin*dIII and electrophoresed in agarose, transferred to nitrocellulose and probed with the radiolabelled 2.45 kb *Hin*dIII fragment used above. All of the cosmids contained a 2.45 kb *Hin*dIII which reacted with the probe, however, several other *Hin*dIII fragments also reacted (Figure 5.7).

Table 5.1 Strains which were probed with the radiolabelled 2.45 kb *Hin*dIII fragment of pPM471.

Track Number	Strain	Strain Type	Biotype/Serotype
1	O3 K4	*Vibrio parahaemolyticus*	
2	T51	*Vibrio cholerae*	El Tor Inaba
3	KB152	non-cholera vibrio	
4	KB153	non-cholera vibrio	
5	KB154	non-cholera vibrio	
6	KB155	non-cholera vibrio	
7	1621	*Vibrio cholerae*	El Tor Ogawa
8	CA411	*Vibrio cholerae*	Classical Ogawa
9	CA401	*Vibrio cholerae*	Classical Inaba
10	O17	*Vibrio cholerae*	El Tor Ogawa
11	11689	*Vibrio cholerae*	El Tor Hikojima
12	569B	*Vibrio cholerae*	Classical Inaba
13	SL5519	*Salmonella typhimurium*	
14	LE392	*Escherichia coli*	

Figure 5.6 Southern hybridization analysis of whole genomic DNA of various *V. cholerae* and non-O1 strains (Table 5.1) for the presence of DNA homologous to that encoding the MFRHA. Chromosomal DNA was digested with *Hin*dIII and electrophoresed on an agarose gel (0.8%) and the bands visualized by UV irradiation after staining with ethidium bromide. After transfer to nitrocellulose filter, the blot was probed with α–[^{32}P]-dCTP labelled 2.45 kb *Hin*dIII fragment (#2) of pPM471.

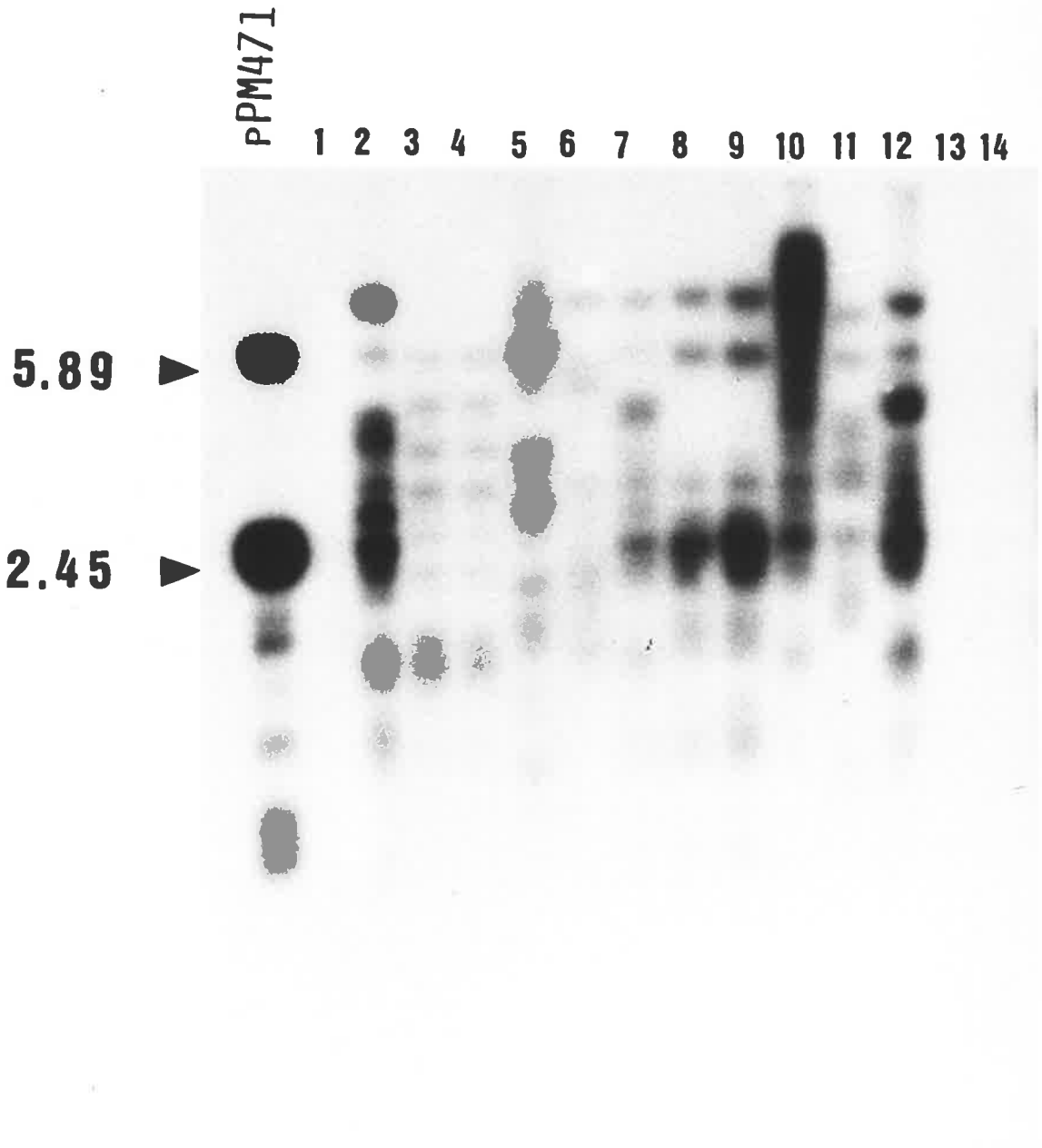
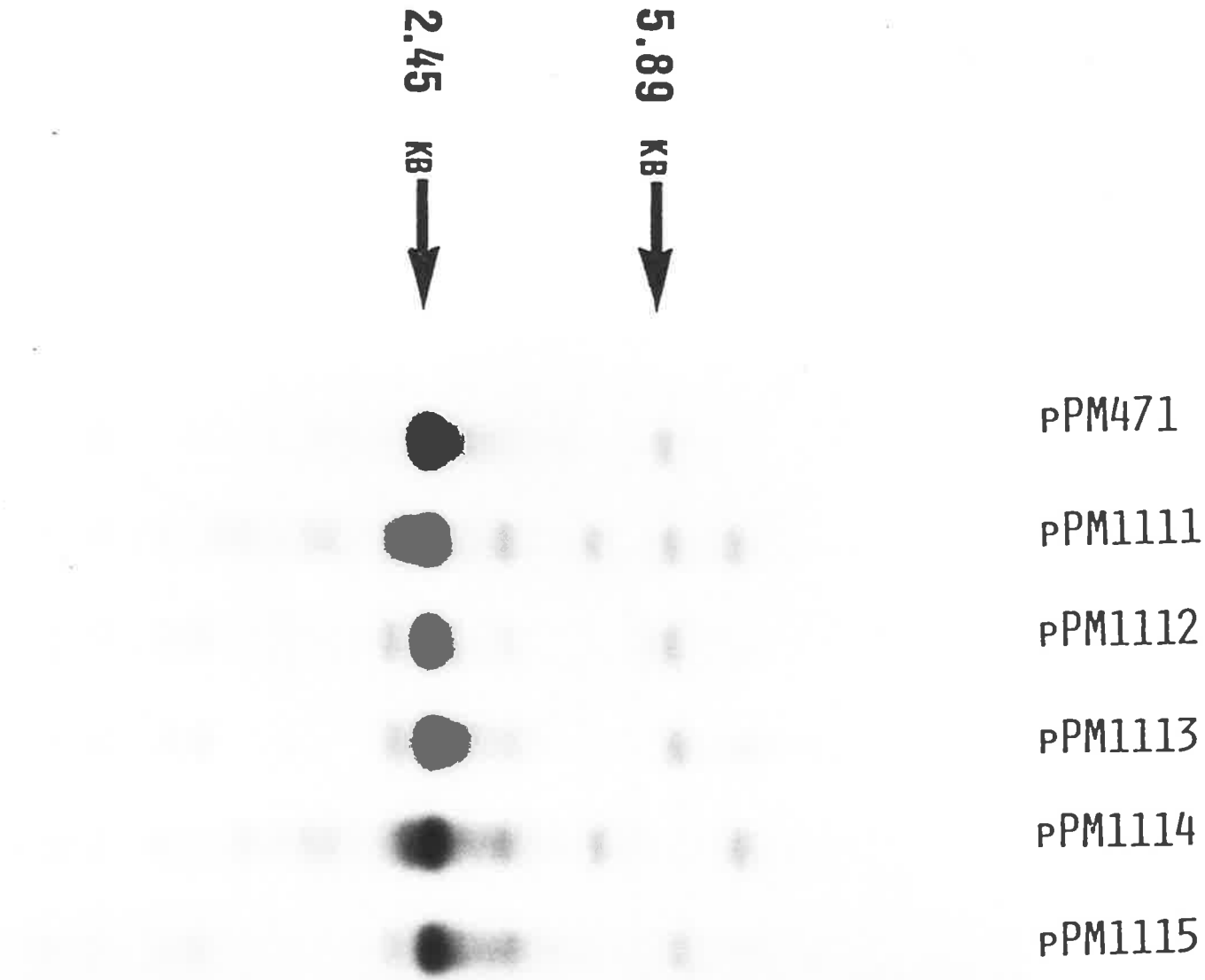

pPM471
1 2 3 4 5 6 7 8 9 10 11 12 13 14
5.89
2.45

Figure 5.7 Southern hybridization analysis of MFRHA$^+$ cosmid isolates for the presence of DNA homologous to that encoding the MFRHA. DNA was digested with *Hin*dIII and electrophoresed on an agarose gel (0.8%) and the bands visualized by UV irradiation after staining with ethidium bromide. After transfer to nitrocellulose filter, the blot was probed with α–[^{32}P]-dCTP labelled 2.45 kb *Hin*dIII fragment (#2) of pPM471.


2.45 KB
5.89 KB
pPM471
pPM1111
pPM1112
pPM1113
pPM1114
pPM1115

5.2.10 Adherence to HEp-2 cells

Haemagglutination is often used as an indicator of adhesive capacity of a strain. Thus since the presence of pPM471 renders a haemagglutinating phenotype on *E. coli* K-12 LE392, it was of interest to see whether V271 (*E. coli* K-12 LE392 [pPM471]) specifically could adhere to cultured HEp-2 epithelial cells. Incubation of HEp-2 monolayers with *E. coli* K-12 LE392 and V271 at 4^0C at a final concentration of 10^7 bacteria per ml, showed a marked difference in the ability of the organisms to be retained on the HEp-2 cells. The presence of pPM471 enables *E. coli*K-12 LE392 to adhere.

5.2.11 Virulence in the infant mouse cholera model

V685 and its MFRHA$^-$ variant of this strain were fed to infant mice at a range of concentrations up to 5×10^8 organisms/mouse (Attridge and Rowley, 1983a) and the mice were incubated at 25°C for 48 hours. No difference was detected in the suvival of the mice. In fact, no mice died in any of the groups, suggesting that V685 was totally avirulent.

5.2.12 Affect of motility

It was subsequently discovered that V685 and the parent strain 569B were non-motile. Previous studies (Attridge and Rowley, 1983a) have shown that such non-motile variants are less virulent than their motile counterparts. This is probably the reason why no infant mice died when giving the parent strain V685.

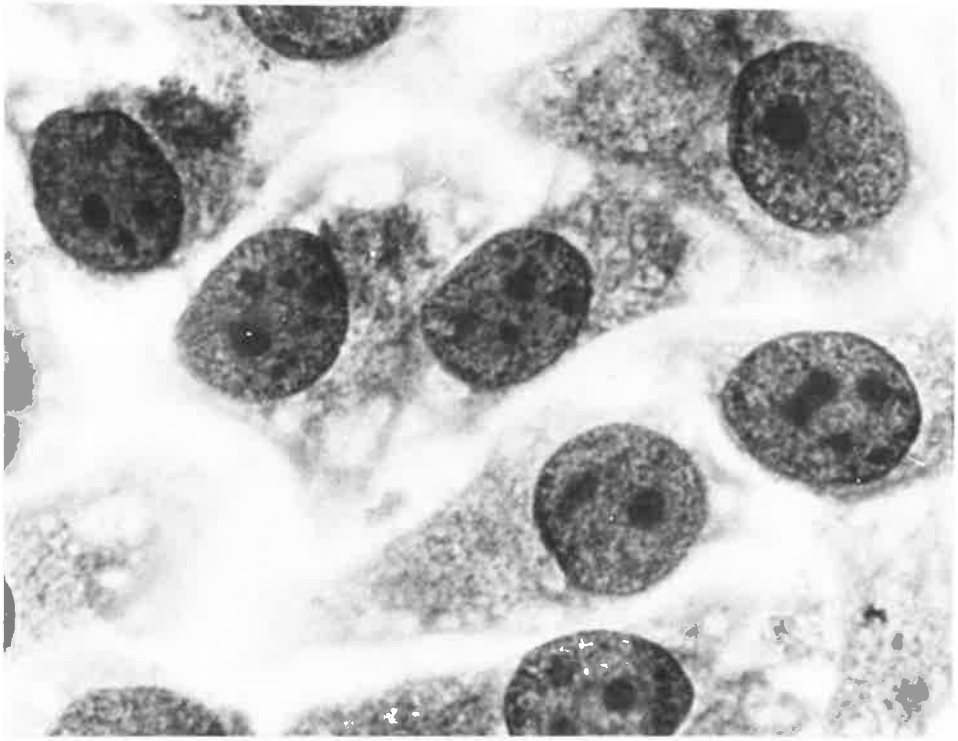
Therefore a motile 569B was obtained (S. Attridge) and the whole process of introducing a defined mutation in the chromosome was repeated. Motility was confirmed by swarming in soft agar as well as microscopic observation.

5.2.13 Chemotaxis

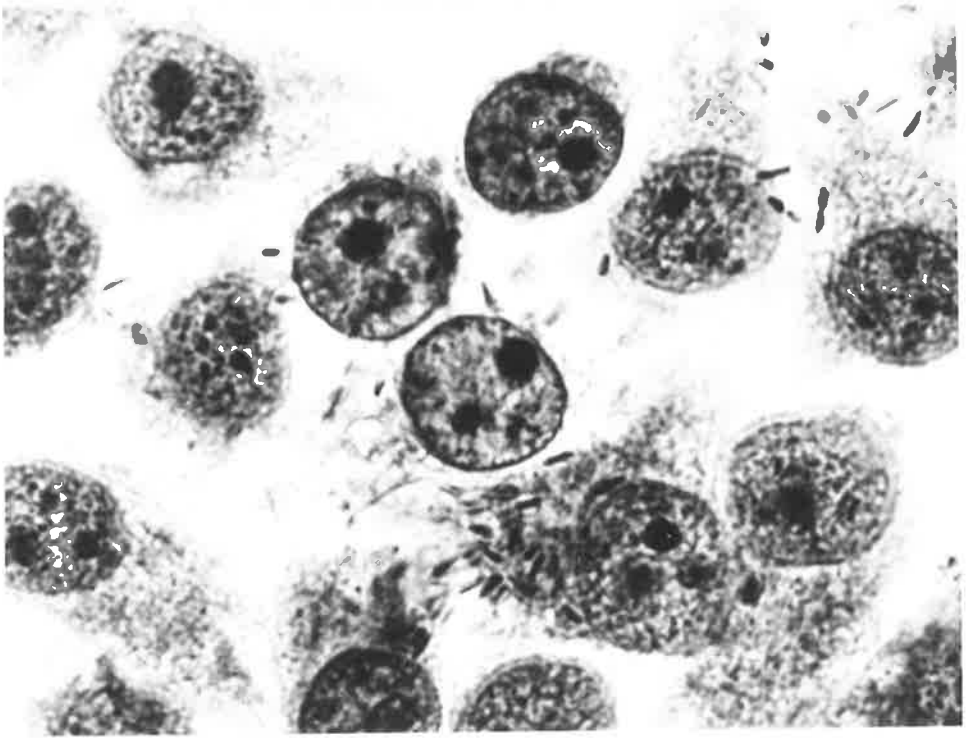
The chemotactic reaction of both the motile 569B and the MFRHA$^-$ derivative were tested. The capillary test for chemotaxis as descibed by Freter and Jones (1981a)

Figure 5.8 Adherence to HEp-2 cells. Light microscopic analysis under oil immersion of HEp-2 monolayers after incubation with, A: *E. coli* K-12 LE392 [pBR322] and B: V271 (*E. coli* K-12 LE392 [pPM471]).

A



B



was used. The MFRHA$^-$ derivative exhibited unaltered chemotactic reactivity to the two stimuli tested (glucose and L-methionine).

5.2.14 Virulence of motile strains in the infant mouse cholera model

Motile 569B and its MFRHA$^-$ variant were grown in TSB at 37°C overnight. Groups of mice were orally administered with bacterial suspensions at varying concentrations (Table 5.2) and after 48 hours, the number of mice surviving within each group was noted. Concentrations were checked by both cell counts as well as viable counts. The LD_{50} value for 569B has previously been calculated to be 5.5 $\times$ 10^5 (Sharma *et al.* 1987). The LD_{50} value of its MFRHA$^-$ derivative is $>10^9$. Therefore the introduction of a mutation as described above into the *V. cholerae* chromosome resulted in a marked decrease in virulence.

5.3 Discussion

The previous chapters have described the isolation of a recombinant plasmid encoding the MFRHA and the characterization of the gene. This chapter has dealt with the construction of firstly an *in vitro* mutation in the MFRHA gene, followed by the introduction of this mutation into the *V. cholerae* chromosome.

Initially several attempts were made to construct a mutant, by first making a deletion in pPM471 which extended from the *Bgl*II site to the second *Xba*I site and then inserting a DNA fragment which encodes an antibiotic resistance. However, all efforts to isolate such a construct failed. The reason for this still remains unclear. Perhaps the extent of the deletion results in some deleterious effect on the cell.

An alternative method was to construct a deletion just between the *Xba*I sites of pPM471. Selection of the region to delete, is limited firstly by the location of convenient restriction endonuclease sites and secondly the DNA sequences flanking the MFRHA gene must be long enough to permit the required double crossover event to occur at a detectable frequency. The deleted DNA was then replaced at

Table 5.2 Comparison of virulence between *V. cholerae* 569B and its MFRHA$^-$ derivative in the infant mice cholera model

Bacterial concentration (organisms/mouse)	569B survival[a]	569B MFRHA$^-$ survival[a]
5×10^8	0	8
1×10^8	0	7[b]
5×10^7	0	8
1×10^7	0	8
5×10^6	0	8
1×10^6	0	8

[a] Baby mice (groups of 8) surviving 48 hours after oral administration of bacteria.

[b] Assays were repeated three times with each concentration. Shown is the group in which 7 survived, however, when repeated twice more 8 survived in each group.

first with the *cat* gene encoding Cm^R and then a second construct was made using a Km^R cartridge.

The marker exchange procedure of Ruvkun and Ausubel (1981) was then used to recombine this construct back onto the *V. cholerae* chromosome. It was decided to use an antibiotic insertion, instead of constructing a base pair insertion which would cause a shift in reading frame, since one can easily screen recombination events of a normally Km^S *V. cholerae* strain.

The *Bam*HI fragment of pPM471 which has the *Xba*I fragment deleted and the antibiotic resistance inserted, was cloned into pRK290 and mobilized into *V. cholerae* strain V685. By using plasmid pPHIJ1 which is incompatible with pRK290, strains in which the Km^R cartridge had recombined into the *V. cholerae* genome can be selected.

The Km^R, Gm^R, Tc^S conjugants were screened by Southern hybridization analysis which confirmed that the constructed mutation had been introduced, but also revealed that sequences around the chromosome or at least a region flanking the MFRHA gene are related. This shall be discussed further in Chapter 6.

Using the infant mouse cholera model as a gauge of virulence it was clearly shown that a mutation in the MFRHA gene effectively renders the strain non-pathogenic. However, it should be pointed out that although we refer to the constructed mutation as a $MFRHA^-$ derivative it is actually a strain with a mutation in the MFRHA gene and the following gene which encodes the 15 kDal protein. Therefore there is the possibility that either one is responsible for the decrease in virulence, or perhaps both, since other data show that both genes probably belong to an operon.

Chapter 6

Discussion

6.1 Introduction

Despite the efforts of a number of different workers, the development of a safe and successful cholera vaccine has failed. Although parentally administered killed cholera vaccines have been used for many years, it has now been established that such vaccines are basically ineffective (Joo, 1974; Feeley and Gangarosa, 1980).

Since cholera is a toxin-mediated disease it was reasoned that perhaps the infection could be prevented by inducing protective antibodies by vaccinating with the enterotoxin (Curlin *et al.* 1975; Noriki, 1976; Svennerholm *et al.* 1982). However this approach has proved disappointing. The residual diarrhoea produced by strains in which the genes for one or both subunits of cholera toxin have been deleted may be due to a Shiga-like toxin or from the colonization process of the organism itself (O'Brien and Holmes, 1987; Taylor *et al.* 1988b).

For the manifestation of the disease, *V. cholerae* must firstly overcome the gastric acid barrier, followed by colonization and adherence of the intestinal epithelium and lastly, release of the cholera enterotoxin in close proximity to its receptor. Pierce *et al.* (1988) have shown that the colonizing capacity of *V. cholerae* is the major determinant of the immunogenicity of the organism. Since colonization is an essential step in the infectious process, attention has turned recently to what factors may be responsible for adhesion. Evidence so far has implicated a number of

different molecules which could serve the purpose of adhesins. Fimbriae have been undisputably shown to play a role in the adherence of a number of bacteria including enterotoxigenic *E. coli*: K88, K99, CFA/I, CFA/II (Evans *et al.* 1975; Rutter *et al.* 1975; Evans and Evans, 1978; Gaastra and de Graaf, 1982; Lindahl *et al.* 1982; Smit *et al.* 1984; Roosendaal, 1987); *Pseudomonas aeruginosa* pili (Woods *et al.* 1980); uropathogenic *E. coli*, Pap pili (van Die, 1986; Lindberg. 1987); *Bacteroides nodosus* pili (Every and Skermam, 1982; Stewart *et al.* 1982; Stewart *et al.* 1983): most of these pili act as haemagglutinins and therefore by analogy, the haemagglutinins of *V. cholerae* have been suggested as likely candidates for colonization factors.

A number of haemagglutinins have been identified in *V. cholerae*. The definitive study by Finkelstein and Hanne (1982) showed that all strains produced a soluble HA/protease. Apart from this soluble factor there are a number of cell-associated molecules. They found El Tor strains produced a major cell-associated HA which was mannose sensitive. This is a potent HA and masks two other HA/s which were only identified when spontaneous MSHA$^-$ mutants were obtained. In MSHA$^-$ mutants a fucose-sensitive HA was detected in early exponential phase and also a cell-associated HA in late exponential phase which was not inhibited by any sugars tested. The latter HA has since been referred to as the MFRHA. Classical biotype strains exhibit fucose-sensitive HA which is expressed transiently in early exponential phase. Later in the growth cycle a MFRHA can also be detected. Whether such haemagglutination activities are mediated by fimbrial structures remains unclear.

Recently four different fimbrial types were identified on *V. cholerae* (Hall *et al.* 1988). This is an interesting finding since a number of workers have had difficulty in identifying pilus structures which may be present on the surface of *V. cholerae*. Hale and co-workers have grown Classical and El Tor *V. cholerae* strains under different growth conditions and examined them by electron and immuno-electron microscopy. It was found that Classical strains expressed three fimbrial types. One type was the previously identified Tcp pilus (Taylor *et al.* 1987a). The

other two were named Type B and Type C. Tcp production is under the control of ToxR and was expressed when cells were grown on CFA agar at 25°C. Growth at 37°C seemed to inhibit its production. In contrast, Type B and C were expressed at both temperatures, 25°C and 37°C.

El Tor strains did not manifest Tcp fimbriae, however, they expressed a fourth fimbrial type designated D, as well as producing Type B and C pili. Type D pili were expressed under the same conditions as Tcp but are immunologically distinct. Tcp fimbriae have been shown to mediate fucose-resistant haemagglutination (Taylor *et al*, 1987a). Whether Type B, C and D fimbriae have haemagglutination activities has yet to be examined. This cannot be effectively evaluated until specific mutants become available.

6.2 Cloning and characterization of the gene encoding the MFRHA

In this study an *E. coli* K-12 LE392 clone expressing the MFRHA of *V.cholerae* was obtained from a pBR322 gene bank constructed using *V.cholerae* 569B (Classical, Inaba) DNA. One reactive clone was identified after screening with a rabbit antiserum raised against the *V. cholerae* soluble HA and the plasmid it contained was designated pPM471. The *E. coli*K-12 strain LE392 carrying pPM471 was identified as V271. pPM471 consisted of a 6.1 kb *Bam*HI insert of *V. cholerae* DNA cloned into the *Bam*HI site of pBR322.

Although the antiserum used for screening was raised against the soluble HA/protease, the cloned haemagglutinin was different. This was first indicated by the lack of proteolytic activity which is normally associated with the SHA. This observation initiated a series of experiments to positively confirm that the MFRHA and not the SHA had been cloned.

V. cholerae 569B has previously been reported to express three and possibly four haemagglutinins (Booth and Finkelstein, 1986). The SHA and two cell-associated HAs; a MFRHA and the FSHA. Also 569B express Tcp pili which also

mediate MFRHA activity. The relationship between Tcp pili and the MFRHA will be discussed below. Since haemagglutination encoded by pPM471 could not be inhibited by either mannose or fucose this eliminated the possibility of pPM471 encoding the FSHA.

The soluble HA activity is also not inhibited by sugars (Hanne and Finkelstein, 1982), however, the RBC spectrum of haemagglutination differs between the SHA and the MFRHA. In addition, the SHA/protease is a zinc containing Ca^{++} ion activated enzyme requiring calcium ions for maximum HA titres and this haemagglutination is inhibited by Zincov (Booth *et al.* 1983). The MFRHA does not require Ca^{++} ions to be included in the assay buffer and its haemagglutination activity is not inhibited by the addition of Zincov.

6.3 Localization of the coding region

The minimum coding region for the gene encoding the MFRHA was deduced to be 0.72 kb. This was achieved through subcloning regions of pPM471 in addition to using *Bal*31 nuclease to generate a family of deletion derivatives. Unfortunately transposon mutagenesis proved unsuccessful since no stable transposon insertion-haemagglutination negative isolates could be obtained. This information together with the difficulty in isolating transformants when constructing deletions from the *Eco*RI site with *Bal* 31 nuclease seems to suggest certain regions of pPM471 cannot be mutated due to reasons which remain unclear. Perhaps the Tn insertions lead to polar mutations affecting functions which may prove lethal to the cell. Alternatively, the MFRHA gene could form part of an operon so that a mutation in the MFRHA gene causes more extensive deletions of the operon.

6.4 The MFRHA is distinct from the Tcp pilus

As previously mentioned Tcp pili are found on *V. cholerae* strains of the Classical biotype. A tentative organization of the Tcp region has been proposed by Tay-

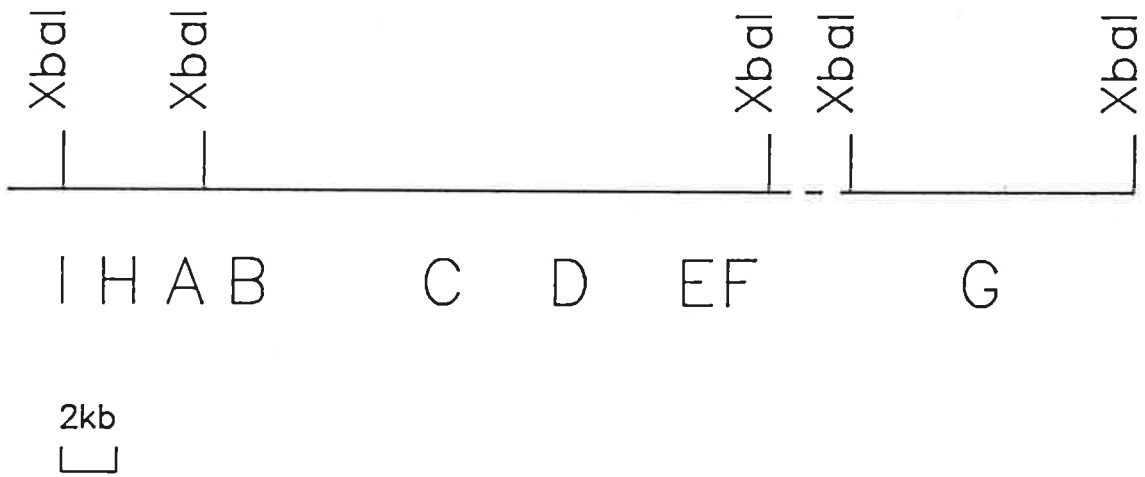
lor and colleagues (1988b) (Figure 6.1). The *tcpA* gene produces the major pilin subunit, with *tcpG* representing a possible tip adhesin. The organization of the Tcp pilus correlates extremely well with that the *E. coli* Pap pilus (Lindberg, 1987; Section 6.8). The Tcp pilus is also known to mediate mannose-fucose resistant haemagglutination and consequently it was suspected that perhaps the MFRHA was also synomonous with the TcpG protein. However, fine restriction endonuclease mapping of pPM471 and cosmids encoding the MFRHA show that the MFRHA and Tcp are distinct. Also Western blot analysis using antiserum against the Tcp pilus supplied by D. Sharma showed that pPM471 did not encode any of the Tcp related proteins.

6.5 Identification of protein products

Various recombinant plasmids were introduced into minicell strain DS410. Subsequent visualization of the plasmid encoded products showed that at least four proteins were encoded in the approximate 1.95 kb between the *Nru*I site and the *Eco*RI sites of pPM471. Firstly there exists left of the *Nru*I site a gene encoding a 10 kDal protein. This is followed by the MFRHA gene whose protein product is approximately 25 kDal. Next maps the genes for 15 kDal and 7 kDal polypeptides respectively.

The size of the MFRHA differs from the 32 kDal reported for the SHA by nkelstein and Hanne (1982). On the other hand Svennerholm and co-workers 983) have calculated the SHA size to be 43 kDal. Using antisera and purified SHA preparations supplied by both groups, PAGE analysis and Western transfer experiments showed that both preparations were immunologically identical. Under our conditions the reported size of 32 kDal seems to be the correct estimate.

Figure 6.1 Proposed organization of the genes associated with the production of the Tcp pilus (Taylor *et al.* 1988b). It is not known whether this constitutes a single operon, however, transcription proceeds from left to right.


XbaI
XbaI
XbaI
XbaI
XbaI
I H A B C D EF G
2kb

6.6 Nucleotide sequence determination

In Chapter 4 the sequence of the MFRHA gene is presented. The nucleotide sequence of the *Cla*I-*Eco*RI fragment of the last *Bal*31 nuclease deletion to give a haemagglutination positive phenotype, was determined using sub fragments cloned into M13 in conjunction with specifically synthesized oligonucleotide primers.

A total of 1,398 base pairs of pPM471 DNA was sequenced and two open reading frames could be identified. The first ORF comprises 669 base pairs representing 223 amino acids and corresponding to a protein size of 26.065 kDal. When the sequence is analyzed for the presence of an amino-terminal extension there seems to be a possible 6 amino acid extension which when cleaved would give a mature protein of 25.296 kDal.

This signal peptide does not fit the general format of consensus signal sequences. Marrs *et al.* (1985) compared the signal sequence of the pilin gene of *Moraxella bovis* with a number of other pili, such as *Neisseria gonorrhoeae*, *Bacteroides nodusus* and *Pseudomonas aeruginosa* and observed that they belonged to a family group which have a 6-7 amino acid leader sequence and a methylated Phe on the NH_2-terminus of the mature pilin. The signal sequence of the MFRHA also consists of 6 amino acids, but does not end in a MePhe residue. However, the signal sequence of *V. cholerae* Tcp pilus ends in a methionine (Taylor *et al.* 1987a) which is what is predicted from the MFRHA signal sequence.

The second ORF consists of 245 base pairs encoding a protein of 9.370 kDal. This ORF shows a typical amino terminal signal sequence which conforms to those observed for other exported proteins. The cleavage site is between glycine and leucine and when cleaved gives a mature form of 7.242 kDal, the 7 kDal protein.

RNA analysis using primer extensions and Northern hybridizations indicate that the genes for the 10 kDal, 25 kDal and 15 kDal proteins are translated from the same mRNA and hence represent a single operon. This shall be discussed below.

6.7 Primer extensions

This method involves using a γ-[^{32}P]-dATP labelled synthetic oligonucleotide which is complementary to the sequence of the MFRHA gene. This primer is hybridized to RNA and using reverse transcriptase and deoxynucleotides, forms a copy of the template RNA. Reverse transcriptase is able to transcribe RNA into DNA. The primer is extended until the end of the RNA. From such a technique the position of the 5′ end of the RNA can be determined (McKnight and Kingsbury, 1982; Jones *et al.* 1985).

Dr. Sue Williams has performed primer extensions using an oligonucleotide complementary to the sequence from nucleotides 109 to 89 shown in Figure 4.9. RNA was prepared from strain V271 which harbours pPM471 and *V. cholerae* 569B the strain from which the MFRHA gene was isolated. After electrophoresis on a sequencing gel and autoradiography a primer extension of approximately 400 bps was seen. This would indicate that regulation of the 25 kDal protein is under the control of sequences just prior to the gene encoding the 10 kDal protein which lies immediately prior to the MFRHA gene.

6.8 Northern hybridization

For further analysis of the regulatory properties of the MFRHA, gene Northern hybridizations were performed by Dr. Sue Williams. This protocol involved isolation of RNA, separation on a denaturing agarose gel, followed by transfer to nitrocellulose (Lehrach *et al.* 1977; Thomas, 1980; Hassouna *et al.* 1984; Raynal *et al.* 1984;). This allows determination of the amount and size of intact RNA.

Hybridizations involved RNA from strain V271 and *V. cholerae* strain 569B. Filters were probed with the [^{32}P]-dCTP labelled *Dde*I–*Xmn*I fragment of the MFRHA gene sequence. A band of approximately 1,500 bp was visualized after the film had been exposed for 24 hours. This indicates that the genes encoding the 10 kDal, 25 kDal and 15 kDal may lie on the same RNA transcript and therefore

belong to an operon. Figure 6.2 summarizes the information gained through RNA analysis, nucleotide sequencing and minicell analysis.

6.9 Construction of specific mutations

To analyze the various properties which can be attributed to the MFRHA, specific mutations were introduced into the *V. cholerae* chromosome to examine the effects on the pathogenesis of cholera by such strains. Because of the lack of convienient restriction endonuclease sites, a specific MFRHA gene deletion could not be made.

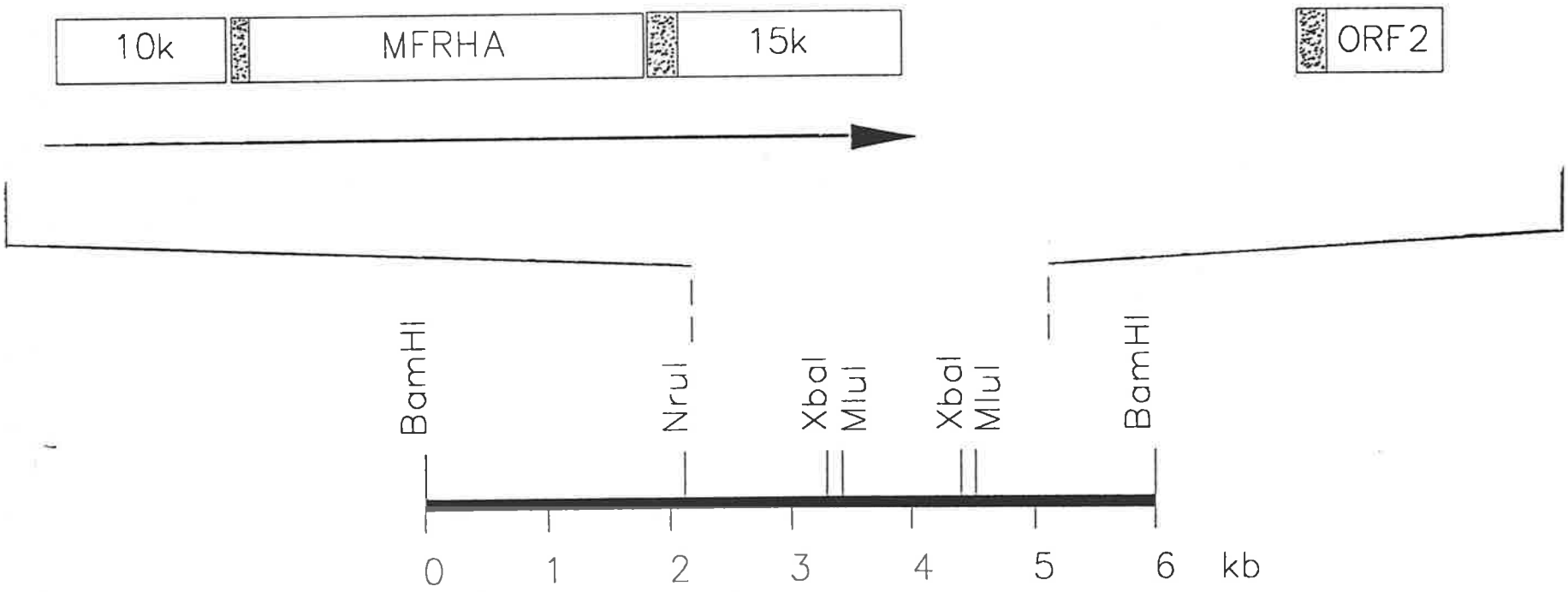
Two constructs were attempted, one a *Bgl*II to *Xba*I deletion which removes both the 10 kDal, the MFRHA and 15 kDal proteins. Secondly a *Xba*I deletion which removes a portion of the MFRHA plus the 15 kDal protein. As mentioned before, numerous attempts were made to delete the *Bgl*II–*Xba*I region and replace it with an antibiotic resistance cartridge. But this construct proved to be unstable and the reasons for this still remain unclear. However, a deletion was obtained in pPM471 in which the deleted *Xba*I fragment had been substituted by a Km^R cartridge. Using this construct and the marker exchange procedure of Ruvkun and Ausubel (1981), an *in vivo* replacement of the *V. cholerae* chromosomal *Xba*I piece with an antibiotic marker was achieved. This strain was then compared to the parent strain for change in virulence patterns. Hence the two strains are isogenic except for the absence of one *Xba*I fragment in the mutant strain.

6.10 Comparison with the Pap pilus

It is worth considering that perhaps the MFRHA could be part of a system such as that associated with the production of Tcp and Pap pili.

The *pap* gene cluster is shown in Figure 6.3. The major subunit is encoded by the *papA* gene. The *papE* and *papF* genes encode what are termed minor pilins which are similar in sequence to the major pilin. PapG is the adhesin which is located at the tip of the pilus and mediates attatchment. Only one or a few copies

Figure 6.2 Genetic organization of the DNA surrounding the locus encoding the MFRHA. The RNA transcripts and direction of transcription are represented by arrows. The boxes represent the size and location of the genes whose products have been identified. Potential signal sequences are shown as hatched regions.


10k
MFRHA
15k
ORF2
BamHI
NruI
XbaI
MluI
XbaI
MluI
BamHI
0
1
2
3
4
5
6
kb

of PapE, PapF and PapG are produced. PapD is required to stabilize the pilus subunits during export and assembly. PapH is involved in regulation of the pilus length. PapI and PapB regulate pilus expression. The product of the *papC* gene forms the assembly platform for pilus growth (Lindberg *et al.* 1987).

Lindberg *et al.* (1987) has postulated a model for the structure of the Pap pilus (Figure 6.4). Lindberg suggests that PapC acts as the base upon which pilus polymerization occurs. PapA, PapE, PapF and PapH are delivered to this base as seperate complexes with PapB, the periplasmic transport protein.

If one considers the operon containing the MFRHA, perhaps the 15 kDal protein may be a minor pilin such as PapE and PapF. This could be the reason it hybridizes with other sections of pPM471, since either the major or other minor pilin subunits could be located elsewhere. The MFRHA could be analagous to the tip adhesin, PapG.

Another hypothesis worth considering is the possible presence of silent copy genes which are seen in the case of the *N. gonorrhoeae* pilus (Meyer *et al.* 1984; Swanson *et al.* 1985; Nicolson *et al.* 1986; Saunders, 1986). The chromosome of *N. gonorrhoeae* contains a number of pilin-related sequences which in Southern transfer experiments hybridize with radiolabelled pilin DNA (Saunders, 1986).

6.11 Virulence

It was of interest to determine whether the MFRHA contributes to the infectious process of *V. cholerae*. Using a MFRHA$^-$ derivative of a motile 569B strain, it was assessed that the introduction of such a mutation, markedly reduces the ability of 569B to kill infant mice. As mentioned previously a specific mutation in the MFRHA alone could not be obtained, therefore whether the decrease in virulence is due to the lack of the MFRHA protein or the 15 kDal protein, either alone or together, remains uncertain.

V. cholerae strain 569B was chosen as the strain in which to construct a MFRHA$^-$ derivative for a number of reasons. Firstly, the gene encoding the

Figure 6.3 The *pap* gene cluster situated on a 9.6 kb *Eco*RI-*Bam*HI fragment of plasmid pPAP5 (Lindberg *et al.* 1987). This represents a single operon with transcription proceeding from left to right.

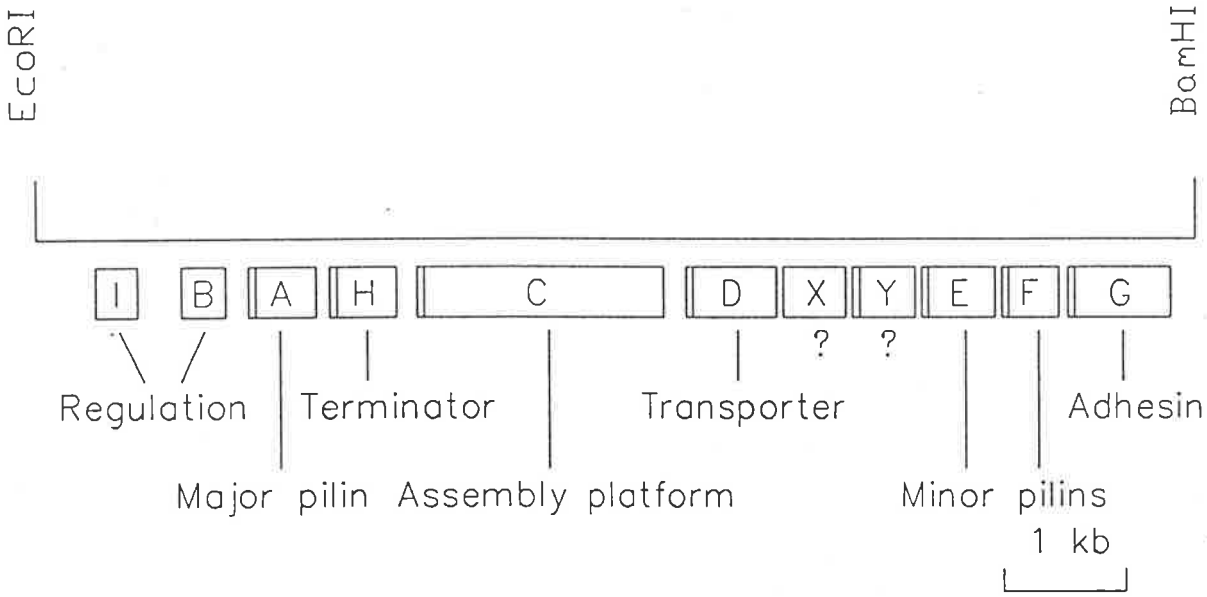
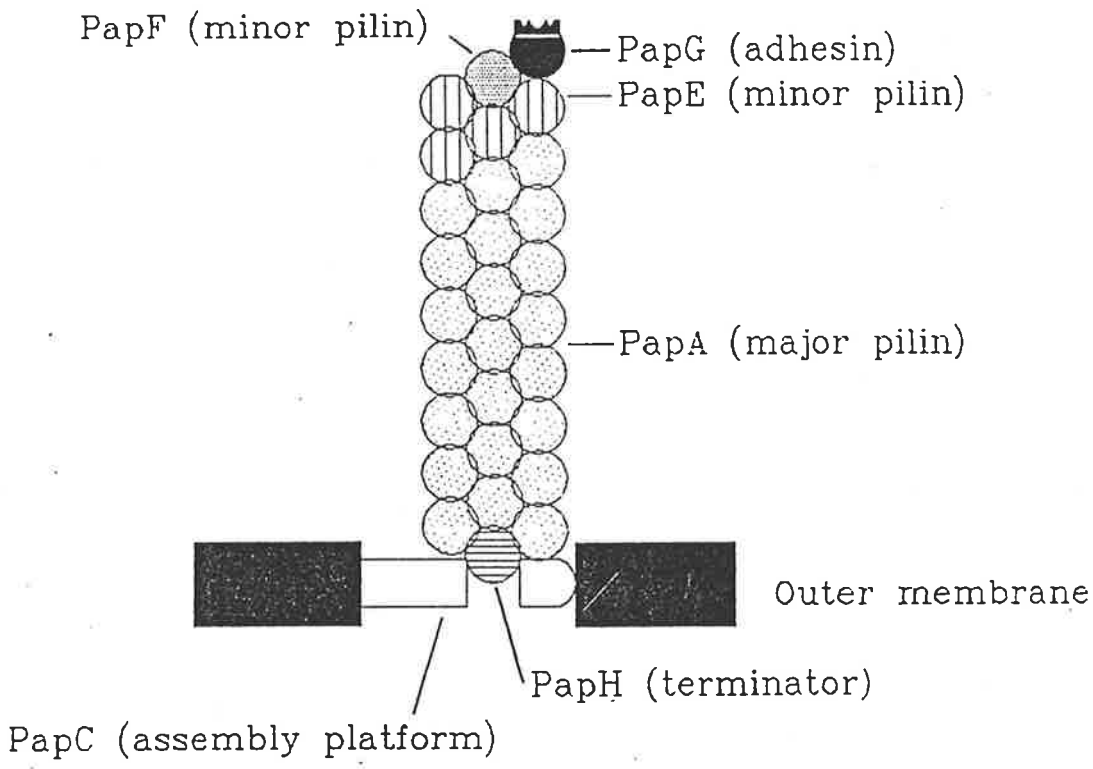

EcoRI
BamHI
I
B
A
H
C
D
X
Y
E
F
G
? ?
Regulation
Terminator
Transporter
Adhesin
Major pilin
Assembly platform
Minor pilins
1 kb

Figure 6.4 Model for the structure of Pap pilus produced by pyelonephritis causing *Escherichia coli*, which is thought to be related to the pilus type produced by *V. cholerae*. The various Pap gene products which form part of the pilus itself are indicated. The pilus is assembled via the assembly platform (PapC) which acts as a channel in the outer membrane through which the various subunits pass. The adhesin (PapG) enters first followed by PapF and then PapE, which then allows the major pilin subunit to be assembled until the terminator protein, PapH, enters the pilus. This then fixes the pilus length. Based on the model of Lindberg *et al.* 1987.


PapF (minor pilin)
PapG (adhesin)
PapE (minor pilin)
PapA (major pilin)
Outer membrane
PapH (terminator)
PapC (assembly platform)

MFRHA was isolated from 569B. Secondly, 569B is well characterized and used by many workers in their research of *V. cholerae*. Thirdly, 569B is a poor colonizing strain (Taylor *et al.* 1988b) therefore it represents a sensitive means of assaying the significance of MFRHA.

6.12 Role of the MFRHA

Haemagglutination of RBC's has been considered to mimick the interaction of bacteria with the intestinal epithelium. Thus, what could be the role of the MFRHA in pathogenesis?. It must be remembered that haemagglutination is a means of identifying this protein and that its natural role *in vivo* remains uncertain, however, it does facilitate adherence to cultured HEp-2 epithelial cells. Since most adhesins at least in Gram negative bacteria have been associated with the production of pili, it seemed reasonable to draw the analogy that the MFRHA may normally be part of a pilus structure.

Though this is purely speculative there are a number of observations making it tempting to postulate such an arrangement.

1. Most fimbriae identified to date have been characterized by their haemagglutinating activity.

2. mRNA analysis seems to indicate that the genes for at least 3 proteins are under the same transcriptional control and result in a polycistronic messenger.

3. The sequence of the MFRHA indicates an unusual signal sequence which may be indicative of pilus related sequences.

4. There are a number of characteristics which are common amongst pilus sequences and are observed in the MFRHA gene sequence: the presence of two cysteine amino acids seperated by 28 residues and situated in the amino terminal portion of the protein, a pentultimate tyrosine, as well as a glycine residue situated 20 amino acids from the carboxy-terminus (van Die and Bergmans, 1984; Mooi and de Graaf, 1985; Rhen *et al.* 1985; Lindberg *et al.* 1986).

5. The MFRHA protein can not be seen in cell envelope preparations which

may indicate that like PapG, there is only one copy per pilus. The poor Shine-Delgarno is suggestive of low amounts of products. Purification of PapG has been attempted, but the protein could not be stabilized sufficiently to enable it to survive beyond membrane fractionation (Lindberg, 1987). Considerable effort has been made to purify the MFRHA protein but to date this has proved similarly fruitless.

6. The *Xba*I fragment hybridizes with multiple sequences on the chromosome, suggesting the presence of related genes. This may indicate that the *Xba*I fragment encodes a minor pilin (equivalent to PapE in the Pap pilus system) which hybridizes to the major or other minor pilin subunits elsewhere. In fact, the 15 kDal protein encoded by part of the *Xba*I fragment correlates well in size with that of PapE. The nucleotide sequence of the 0.7 kb *Xba*I fragment is currently being determined (C. Clark, personal communication).

6.13 Future prospects

The cloning and characterization of the genes involved in the expression of the MFRHA, described in this thesis, presents a basis for future research.

The role haemagglutinins play in pathogenesis is unknown. Because strains have multiple haemagglutinins being expressed it is extremely difficult to assign individual functions unless specific mutations are introduced into the chromosome and then evaluated. To do this it is necessary to clone the respective HA genes. This thesis reports the first cloning of a gene encoding a *V. cholerae* HA.

Although it has been shown that a mutation in the MFRHA and 15 kDal proteins may play an important role in the infectious process it is not known why. It is probably due to limiting the ability of the organism to colonize. Hence this thesis gives an indication that the MFRHA is an important virulence factor and lays the foundation for further examination of exactly how such a MFRHA$^-$ mutation is decreasing virulence.

Preliminary results seem to indicate that a MFRHA$^-$ strain may have vaccine potential. Of course it would be necessary to construct a MFRHA$^-$ construct in a

V. cholerae strain which has the toxin genes deleted. Alternatively, the MFRHA may itself be a protective antigen and so overproduction in a suitably attenuated *Salmonella* host may be another approach to a vaccine (Manning, 1988).

Although many attempts have been made to purify the MFRHA and raise antiserum all have failed. The difficulty lies with the fact that there is thought to be only one or two copies of the MFRHA per pilus, as supported by the presence of a poor Shine-Delgarno sequence. Therefore future developments could involve site-directed mutagenesis to construct a consensus Shine-Delgarno sequence. This will in conjuction with expression vectors increase protein production. It would also allow antiserum to be raised which could be use in immuno-gold electron micscropy to possibly prove that the MFRHA is a tip protein on a fimbrial structure.

If in fact the MFRHA gene is part of an operon or cluster of operons encoding a pilus, the role of the different genes in the biogenesis of the fimbriae could be studied by the introduction of individual mutations in each gene. The cosmid clones harbouring the MFRHA gene isolated here, may provide the additional genes associated with the corresponding pilus. A study of the phenotypes of the mutated pilin genes could contribute to the elucidation of the mechanism of fimbriae assembly. Whether the genetic and structural organizations will reflect that of the Pap or Tcp pilus remains to be seen.

Chapter 7

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