MOLECULAR CHARACTERIZATION OF THE HAEMOLYSIN DETERMINANT OF *Vibrio cholerae* O1

Richard A. Alm, B.Sc. (Hons.) (Adelaide)

Department of Microbiology and Immunology

The University of Adelaide,

Adelaide, 5000

AUSTRALIA

A thesis submitted for the degree of Doctor of Philosopy.

November, 1989.

For my loving mother.

"We prefer to take our chance of cholera and the rest than be bullied into health. There is nothing a man hates so much as being cleaned against his will - it is a positive fact that many have died of a good washing, as much from the initiation of the nerves as from the exposure of the cuticle, no longer protected by dirt"

The Times

London. 1st August, 1854.

CONTENTS

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: Characterization of the *hly* locus of *V.cholerae* O1

Chapter 4: Genetic organization of the *hlyA* gene from Classical and El Tor strains of *Vibrio cholerae*

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 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.

Richard A. Alm

ABSTRACT

Vibrio cholerae O1, the aetiological agent of cholera in man, exists as two biotypes, Classical and El Tor. Both biotypes elaborate a range of extracellular proteins which have been implicated in pathogenesis. Strains of the El Tor biotype were originally distinguished on the basis of producing a soluble haemolysin that is excreted into the growth medium. Although Classical strains never produce this haemolysin, Southern hybridization demonstrates that they contain DNA essentially homologous to the entire gene. The structural gene (*hlyA*) has been cloned from both biotypes and the nucleotide sequence determined. Analysis of the nucleotide sequence revealed that the Classical strain 569B carries an 11 base pair deletion in *hlyA*. This would result in the production of a truncated protein product of 27 kDa ($HlyA^*$) which can be visualized in *E.coli* minicells. A oligodeoxynucleotide was synthesized that spans this deleted region and used to probe a range of Vibrio strains. DNA hybridization analysis implied that all *V.cholerae* strains of the Classical biotype were deleted in this region.

The haemolysin is excreted from *V.cholerae* as an 80 kDal protein whereas *E.coli* K-12 cells harbouring the cloned gene accumulate this protein in the periplasmic space, indicating that *E.coli* does not possess the complete machinery to allow effective excretion of the haemolysin. The HlyA protein is translated with a signal sequence, characteristic of extracellularly located proteins of Gram negative bacteria. Once the HlyA protein is excreted from *V.cholerae* it is readily cleaved initially to two peptides of 65 kDa and 15 kDa.

An antibiotic resistance cartridge was inserted into the N-terminal region of *hlyA* which inactivated both the 80 kDa haemolysin as well as $HlyA^*$. Isogenic $hlyA^+$ and *hlyA* mutants were constructed by the introduction of this mutation via conjugation and reciprocal recombination into the *V.cholerae* chromosome. These mutants were

used to assess the potential role of the haemolysin in pathogenesis. As well as being haemolytic, the HlyA protein was shown to be cytolytic for cultured human epithelial and mouse macrophage cells. A possible explanation as to why Classical strains of *V.cholerae* have retained the genetic information to encode HlyA* was suggested when it was observed that this N-terminal domain caused fluid accumulation in ligated ileal segments of rabbits suggesting that it functions as a second enterotoxin in *V.cholerae*.

Export of the haemolysin requires the translation of the HlyA protein at the surface of the cytoplasmic membrane and translocation across this membrane, the periplasmic space and the outer membrane. The mechanism by which this occurs is ill understood. A second adjacent gene , *hlyB* , has been shown to have a role in haemolysin production. Cell fractionation studies have localized the HlyB protein to the outer membrane. By site directed mutagenesis specific *hlyB* mutants have been constructed. These mutants are defective in the export of HlyA in early to mid-exponential phase growth. It is suggested that the HlyB protein is necessary for effective export of the HlyA protein in *V.cholerae* but is not sufficient for excretion in *E.coli* K-12.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Paul Manning for his excellent supervision and continued encouragement throughout this study.

I would also like to thank Drs. Mike Heuzenroeder, Sue Williams, Graham Mayrhofer, Peter Ey and Connor Thomas along with Garry Penney and Marg Quin for their assistance with aspects of my work. My thanks must also go to Chris Cursaro for his expertise in photography.

For his many helpful discussions and friendship over the years, I extend my sincerest thanks to Uwe Stroeher.

Finally, I wish to thank all the past and present occupants that have worked with me in Paul Manning's "Centre of the Scientific Universe".

LIST OF ABBREVIATIONS

A : adenine

aa : amino acid

ATP : adenosine 5'-triphosphate

BHI : brain heart infusion

bp : base pair

BSA : bovine serum albumin

CAT : chloramphenicol acetyltransferase

C : cytosine

Cm : chloramphenicol

cpm : counts per minute

CT : cholera toxin

DNA : deoxyribonucleic acid

DNase : deoxyribonuclease

dNTP : deoxyribonucleoside triphosphate

ddNTP : dideoxyribonucleoside triphosphate

DDT : dithiothreitol

EDTA : ethylene-diamine-tetra-acetic acid

Et Br : ethidium bromide

G : guanine

GM_1 : Galatosyl-N-acetyl-galactosaminyl-sialosyl-lactosyl ceramide

Gm : gentamycin

HA : haemagglutinin

Hly : haemolysin

IM : inner membrane

IPTG : isopropyl-Ã-D-thiogalactopyranoside

kb : kilobase pairs

kDa : kilodalton

Km : kanamycin

LB : Luria broth

LPS : lipopolysaccharide

LT : heat labile toxin

MFRHA : mannose-fucose resistant haemagglutinin

mRNA : messenger ribonucleic acid

NA : nutrient agar

NB : nutrient broth

nt : nucleotide

NTG : nitrosoguanidine

OD : optical density

ORF : open reading frame

PAGE : polyacrylamide gel electrophoresis

PBS : phosphate buffered saline

PEG : polyethylene glycol-6000

^{R} : resistant

RBC : red blood cell

RF : replicative form

RNA : ribonucleic acid

rpm : revolutions per minute

^{S} : sensitive

SD : Shine-Dalgarno

SDS : sodium dodecyl sulphate

SHA : soluble haemagglutinin

Sm : streptomycin

Sp : spectinomycin

T : thymine

Tc : tetracycline

TEMED : N,N,N',N'-tetramethyl-ethylene-diamine

Tn : transposon

Tris : Tris (hydroxymethyl) aminomethane

U : uracil

UV : ultraviolet

v/v : volume per volume

w/v : weight per volume

X-gal : N,N'-dimethyl formamide

Chapter 1

INTRODUCTION

1.1 *Vibrio cholerae*

Vibrio cholerae belongs to the genus Vibrio within the Vibrionacae family, which also include the genera Aeromonas, Photobacterium, Plesiomonas and Lucibacterium (Shewan and Veron, 1975; Bauman and Schubert, 1984). *Vibrio cholerae* was first described in 1854 as the aetiological agent of the diarrhoeal disease cholera by Pacini (Hugh, 1964). The transmission of cholera occurs via the faecal-oral route with ingestion of water contaminated with human faecal material being acknowledged as the major source.

Vibrio cholerae is a Gram negative, facultatively aerobic motile rod with a distinct curved morphology. It is usually 0.3-0.4 μm wide and 1.5-2.0 μm long with a single polar sheathed flagellum (Davis *et al.*, 1980). The organism prefers alkaline growth conditions and is extremely sensitive to acid.

1.1.1 Biotype Differentiation

V.cholerae can be subdivided into six O-groups based on the O-antigen of the lipopolysaccharide. Within *V.cholerae* O1, two biotypes, Classical and El Tor can be defined on the basis of a number of characteristics which are discussed below (Feeley, 1965). Since the turn of the 19th century, seven cholera pandemics have occurred. The first six pandemics were caused by Classical strains of *V.cholerae*. It was thought at first that *V.cholerae* of the El Tor biotype were non-pathogenic as they were

only associated with a mild diarrhoea. However, in 1962, the World Health Organization (WHO) defined the disease caused by these El Tor strains as true cholera. Furthermore, the current seventh pandemic which initiated in South-East Asia is due to the El Tor biotype, indicating that *V.cholerae* of both biotypes are pathogenic for man.

The two biotypes of *V.cholerae* O1 were originally differentiated by the capacity of the El Tor isolates to produce a soluble haemolysin for sheep erythrocytes. However, considerable variation in the haemolytic phenotype is now seen. Since El Tor isolates were not always haemolytic, alternative methods to differentiate Classical and El Tor *V.cholerae* were sought. It was noted that El Tor but not Classical strains could agglutinate chicken erythrocytes and were resistant to the antibiotic polymyxin B (Gan and Tjia, 1963; Roy *et al.*, 1965; Gangarosa *et al.*, 1967). However, exceptions to the above properties were not uncommon (Rizvi *et al.*, 1965; Pesigan *et al.*, 1967). The most reliable test available to date is the sensitivity of Classical *V.cholerae* strains to Mukerjee's group IV vibriophages, to which El Tor isolates are resistant (Monsur *et al.*, 1965; Mukerjee and Roy, 1961).

1.1.2 Serotype Differentiation

V.cholerae O1 strains of both biotypes can be subdivided further into three serotypes depending on the antigenic specificity of the heat-stable polysaccharide fraction of the bacterial lipopolysaccharide. These serotypes are Inaba, Ogawa and Hikojima and differentiation between them is based on the presence of three antigenic factors termed A, B and C. Strains of the Inaba serotype express the A and C antigens while the Ogawa serotype possess A, B and only trace amounts of C (Burrows *et al.*, 1946; Sakazaki and Tamura, 1971; Redmond *et al* ., 1973; Redmond, 1979) and the third rare serotypic subclass, Hikojima, has A, B and C antigens (Burrows *et al.*, 1946). However, the Hikojima serotype is extremely unstable and it has been speculated that such strains are in fact segregating diploids (Bhaskaran and Sinha, 1971).

Alternatively, Hikojima strains may represent the other subclasses undergoing seroconversion at an elevated frequency. Such serotype changes have been reported both in man (Gangarosa *et al.*, 1967) and in germ-free mice (Sack and Miller, 1969). It has been suggested by Ogg and co-workers (1978, 1979) that vibriophage CP-T1, by means of a lysogenic conversion, could be responsible for these seroconvertants. However, DNA hybridization experiments using cloned CP-T1 fragments as probes could not detect CP-T1 as a prophage in supposed lysogens indicating that seroconversion is not due to the presence of CP-T1 (Guidolin and Manning, 1985). The *rfb* region of *V.cholerae* which encodes the O-antigen side-chains of the lipopolysaccharides of the Inaba and Ogawa serotypes have been cloned and expressed in *E.coli* K-12 (Manning *et al.*, 1986). Detailed restriction analysis implies that the regions are extremely closely related and that only subtle changes are involved in serotype conversion (Ward *et al.*, 1987). These regions are currently being sequenced from both Inaba and Ogawa serotypes to ascertain the presence of any differences that may help in the understanding of serotype conversion (U. Stroeher, personal communication).

1.1.3 Pathogenesis

Cholera is an acute diarrhoeal disease of humans caused by the ingestion of contaminated food or drinking water. The induction of disease by *V.cholerae* O1 occurs after the organisms have passed through and survived the gastric acid barrier of the stomach and successfully entered the small intestine. Gastric acid provides a substantial barrier to infection by *V.cholerae* as the infectious dose can be lowered from 10^8 to 10^4 organisms if stomach acidity is neutralized by sodium bicarbonate prior to infection (Cash *et al.*, 1974a, 1974b; Nalin *et al.*, 1978). Colonization of the small intestine occurs via adherence to the mucosal epithelium, and is important as the vibrios must be able to compete with the normal intestinal flora as well as resist normal gut peristalsis (Dixon, 1960; Freter, 1974; Ofek and Beachey, 1980). However, the final

diarrhoeal response is multifactorial. Motility and chemotaxis are involved in penetration of the mucous layer of the small bowel (Jones *et al.*, 1976; Yancey *et al.*, 1978; Freter and O'Brien, 1981; Attridge and Rowley, 1983a). Proteases, neuraminidases and DNases are liberated to degrade the mucus and allow close association with the gut so that toxins liberated by the bacterium can be effectively delivered (Peterson *et al.*, 1972; Schneider and Parker, 1978; Pierce *et al.*, 1985). Therefore, colonization of the small intestine and the release and action of the enterotoxin(s) are two essential steps in the manifestation of a diseased state and a considerable effort has been given to understanding both of these processes.

1.1.4 Adhesion by *V.cholerae*

Motility, chemotaxis and extracellular protein production are factors that enhance *V.cholerae* colonization by permitting a close association with the intestinal epithelium. However, the nature of the actual structures involved in adherence have been the subject of speculation for a number of years. Surface structures such as lipopolysaccharide, flagellar sheath, outer membrane proteins, fimbriae and haemagglutinins have all been implicated as possible adherence factors (Jones *et al.*, 1976; Jones and Freter, 1976; Chitnis *et al.*, 1982; Hanne and Finkelstein, 1982; Attridge and Rowley, 1983a, 1983b; Kabir, 1983; Kabir and Showkat, 1983; Ehara *et al.*, 1986, 1987).

Bales and Lankford (1961) suggested that the interaction between *V.cholerae* and erythrocytes may mimic that of the organism with the intestinal epithelium and these tests are now employed as a means to examine adhesive properties. Hanne and Finkelstein (1982) studied both Classical and El Tor strains of *V.cholerae* O1 for their haemagglutinating ability. These workers have described four haemagglutinins (HA's) of which three are cell-associated and one is excreted into the culture supernatant. These HA's can be distinguished by their resistance or sensitivity patterns to various sugars, requirement for divalent cations and species of erythrocytes

upon which they act. The potential roles that these structures play in adhesion is still open to debate.

Fimbriae (pili) are the hair-like appendages that protrude into the external medium from the bacterium. They vary widely both in their dimensions, their number and their distribution on the organism (Sokatch, 1979). Fimbriae have been postulated as organelles that may mediate attachment and the first evidence for the presence of fimbriae on the surface of *V.cholerae* cells was reported by Tweedy *et al.* (1968) but could not be confirmed by subsequent investigations (Nelson *et al.*, 1976, 1977). It was Ehara *et al.* (1986) who first provided definite evidence for the production of fimbriae on pathogenic El Tor strains providing a strong correlation between colonization and fimbriae production. Pathogenic *V.cholerae* strains that colonized the intestinal epithelium were shown to produce fimbriae whereas non-pathogenic strains that failed to produce fimbriae were unable to effectively colonize the gut (Ehara *et al.*, 1986). Taylor and co-workers (1987a) isolated, by transposon mutagenesis of the *V.cholerae* genome, a mutant that demonstrated a marked decrease in the intestinal colonization of suckling mice. This insertion mutant was shown to lie in the gene that encodes the major subunit of a pilus structure of *V.cholerae*. This pilus was termed the Tcp pilus (toxin co-regulated pilus) as it was shown that its production was co-regulated with the cholera enterotoxin (CT) and the structural gene was subsequently named, *tcpA* (Taylor *et al.*, 1987a; Peterson *et al.*, 1988). Concomitant with the loss of this pilus was loss of the ability to haemagglutinate mouse erythrocytes in the presence of L-fucose, a sugar shown to inhibit the majority of the haemagglutinating ability of *V.cholerae* 0395 (Taylor *et al.*, 1987a). The Tcp^- strains showed a marked decrease in intestinal colonization which seems to indicate that this fimbrial structure may play an extremely important role in the pathogenesis of *V.cholerae* O1 in humans.

1.1.5 Cholera toxin production

Cholera enterotoxin (CT) is now recognized as one of the most important virulence factors produced by *V.cholerae* because it is primarily responsible for the severe diarrhoea associated with the disease (De, 1959; Finkelstein, 1973). CT has been studied extensively and is well characterized in terms of structure, function, biological activity and regulation (Van Heyningen, 1977; Holmgren, 1981; Mekalanos, 1985). Cholera enterotoxin is a heat-labile multimeric structure of 85 kDa. The molecule is composed of an A subunit (27 kDa) surrounded by a ring of five B subunits (11.6 kDa) (Lospalluto and Finkelstein, 1972; Ludwig *et al.*, 1986). Alone, both subunits have been shown to be non-toxic. The A subunit, although synthesized as a single polypeptide is cleaved to form two fragments A_1(22 kDa) and A_2(5 kDa) (Mekalanos *et al.*, 1979; Pearson and Mekalanos, 1982). In order for the A subunit to be active and toxic, this cleavage must occur and it has been suggested that the soluble haemagglutinin/protease is the enzyme responsible for this cleavage (Booth *et al.*, 1984). The B subunits possess a very high affinity for their cell-surface receptor, ganglioside GM_1, which allows the A chain to traverse the membrane and activate the target cells adenylate cyclase complex (Gill and Meren, 1978; Cassel and Pfeiffer, 1978).

CT and the heat-labile toxin (LT) of *E.coli* are both structurally, immunologically and genetically related (Moseley and Falkow, 1980; Spicer and Noble, 1982; Mekalanos *et al.*, 1983). The degree of DNA homology is such that by using probes directed against the *E.coli* heat-labile toxin, the cholera toxin genes could be identified in toxigenic *V.cholerae* strains, but not in non-toxigenic or environmental strains (Moseley and Falkow, 1980). The cholera toxin genes *ctxA* and *ctxB* were subsequently cloned and the nucleotide sequence determined (Pearson and Mekalanos, 1982; Mekalanos *et al.*, 1983; Lockman and Kaper, 1983; Lockman *et al.*, 1984). The genetic organization of both LT and cholera toxin is similar where the genes for the A and B subunits form an operon with the A cistron preceeding the B cistron. However,

the LT operon (*eltAB*) appears to be located on plasmids (Dallas *et al.*, 1979) whereas the cholera toxin operon has been shown to exist on the chromosome of both Classical and El Tor *V.cholerae* strains (Kaper *et al.*, 1981a; Pearson and Mekalanos, 1982; Mekalanos *et al.*, 1983).

V.cholerae strains of the Classical biotype all contain two copies of the cholera toxin operon (*ctxAB*) that are widely separated on the bacterial chromosome (Pearson and Mekalanos, 1982; Mekalanos *et al.*, 1983). Although it was originally thought that El Tor strains only had a single copy of the *ctxAB* operon (Moseley and Falkow, 1980; Kaper *et al.*, 1981a), a number of El Tor strains have subsequently been identified that carry *ctxAB* duplications (Mekalanos, 1983). Such strains have their copies arranged on large tandem repeats which are either 7 kb or 9.7 kb in length. This variation in size was accounted for by the presence of different numbers of copies of a 2.7 kb repetitive sequence (RS1) at the junction of these duplications (Mekalanos, 1983, 1985). Directly repeated copies of RS1 are found both upstream and downstream of the *ctxAB* operon and amplification of this region of DNA would now seem to be responsible for the hypertoxigenic phenotype of El Tor variants upon intestinal passage in rabbits (Mekalanos, 1983). This amplification (and deletion) is RecA dependent indicating that homologous recombination, presumably between the RS1 elements, is involved.

When the cloned *V.cholerae* O1 *ctxAB* operon was expressed in *E.coli* it was discovered that only 4% of the amount of toxin was made compared to that produced by the wild-type *V.cholerae* (Pearson and Mekalanos, 1982). Consequently, it was suggested that *E.coli* lacks a positive regulatory element that promotes efficient expression of the *ctxAB* operon in *V.cholerae*. Miller and Mekalanos (1984) cloned the positive regulatory gene (*toxR*) and showed it to increase expression of the toxin genes in *E.coli*. ToxR was thought to act on the *ctxAB* promoter rather than the structural genes as ToxR was unable to effect the expression of LT genes in *E.coli* (Miller and Mekalanos, 1984). The LT genes display significant homology with cholera toxin

structural genes but the LT promoter displays no significant homology with the *ctxAB* promoter (Mekalanos *et al.*, 1983). The nucleotide sequence of *toxR* was determined and the gene product is a 32.5 kDa transmembrane DNA-binding protein (Miller *et al.*, 1987). This protein activates transcription by binding to the tandemly repeated sequence TTTTGAT directly upstream from the *ctxAB* promoter (Miller *et al.*, 1987). Southern hybridization analysis has demonstrated that *toxR* is not only present in all toxigenic Classical and El Tor strains of *V.cholerae*, but also in non-toxigenic strains which lack the *ctxAB* operon.

A second regulatory gene designated *toxS* has now been identified and lies downstream from *toxR* (Peterson *et al.*, 1988; Miller *et al.*, 1989). The ToxS protein acts in *trans* with ToxR and is required for activation of expression of the *ctxAB* genes by ToxR (Miller *et al.*, 1989). Miller *et al.* (1989) demonstrated that ToxS does not directly activate the *toxR* promoter nor does it cause ToxR to activate its own promoter. Therefore, ToxS may act to modify ToxR in such a way as to make it competent for transcriptional activation of the *ctxAB* promoter. At least 12 different transcriptional units have been demonstrated to be regulated by this system (Taylor *et al.*, 1988). These include cholera toxin, the Tcp pilus involved in intestinal colonization, the *ompU* gene which encodes a 38 kDa outer membrane protein and the Acf colonization factor (Miller and Mekalanos, 1984; Taylor *et al.*, 1987a, 1987b; Miller, 1985 - cited by Taylor *et al.*, 1987a; Manning, 1988).

1.1.6 Molecular Approaches to live cholera vaccines

Considerable effort has been applied to the construction of a live oral vaccine that could induce a strong and lasting immunity against *V.cholerae* O1. Essentially, the two methods by which these vaccines are being constructed are:

(1) removal of the genes that encode virulence determinants, and

(2) expression of *V.cholerae* protective antigens in an avirulent but immunogenic host of another species.

Environmental isolates of *V.cholerae* O1 that are non-toxigenic and lack the *ctxAB* operon were evaluated as candidates for live oral cholera vaccines (Cash *et al.*, 1974b; Levine *et al.*, 1982). However, these isolates either colonized the gut poorly or failed to do so at all and provided no protection upon rechallenge of a virulent strain (Cash *et al.*, 1974b; Levine *et al.*, 1982). By nitrosoguanidine mutagenesis Honda and Finkelstein (1979a) isolated a mutant, Texas Star SR, in an El Tor strain which was defective in the production of the A subunit but could still produce the B subunit of cholera. The exact mutation in *ctxA* was not defined and theoretically reversion of this mutation to again produce an active toxin was a consideration. Furthermore, nitrosoguanidine mutagenesis induces multiple mutations, which remained unidentified in this strain. When Texas Star-SR was tested in volunteers, 24% of the recipients experienced mild diarrhoea (Levine *et al.*, 1984). The use of this prototype strain in human trials (Levine *et al.*, 1983) indicated that live attenuated *V.cholerae* strains as vaccine candidates had potential.

A normal cholera infection leads to the production of antitoxin antibodies, the majority of which are directed against the B subunit of the toxin (Holmgren, 1981). Several *V.cholerae* O1 vaccine strains have been produced using recombinant DNA techniques to precisely remove both the A and B subunits of the cholera enterotoxin, or just the A subunit (Kaper *et al.*, 1984a, 1984b; Mekalanos *et al.*, 1983). These strains, when tested in volunteers, were immunogenic and protected against rechallenge of wild type *V.cholerae* strains. However, over 50% of volunteers developed mild clinical diarrhoea, presumably due to other toxins that had not been removed or perhaps even the act of colonization itself (Levine *et al.*, 1988).

Both O1 and non-O1 serotypes of *V.cholerae* produce a variety of cytotoxins (Nishibuchi *et al.*, 1983; Madden *et al.*, 1984; O'Brien *et al.*, 1984; Sanyal *et al.*, 1984; Yamamoto *et al.*, 1986), some of which have been shown to stimulate secretion by the intestinal mucosa. Two potential candidates to explain this residual diarrhoea are the Shiga-like toxin (O'Brien *et al.*, 1984) and the El Tor haemolysin

(Yamamoto *et al.*, 1984, 1986; Ichinose *et al.*, 1987). Kaper *et al.* (1986) constructed a small deletion in the structural gene for the haemolysin, *hlyA*, but no relief from the mild diarrhoea was observed (Levine *et al.*, 1988).

A *ctxA* mutant in the Classical *V.cholerae* strain 569B that phenotypically does not produce the Shiga-like toxin causes significantly fewer diarrhoeal episodes in volunteers than *ctx* mutants which still produce the Shiga-like toxin (Levine *et al.*, 1988). This observation suggests that the Shiga-like toxin may be partially responsible for producing the mild diarrhoeal reactions seen in the recipients of live oral *V.cholerae* vaccine candidate strains.

The mild diarrhoea which is detected may also be due to the efficient colonization of the intestinal epithelium by *V.cholerae*. Smith and Linggood (1971) demonstrated that *E.coli* strains that contain K-88 fimbriae encoding plasmids cause mild diarrhoea in piglets in the absence of a plasmid encoding the enterotoxin. Conversely, the same strain carrying the plasmid which encodes the enterotoxin but lacking the K-88 fimbrial plasmid was unable to cause diarrhoea. These results suggest that efficient colonization of the intestine can lead to mild diarrhoea. If this is the case for *V.cholerae*, then the prospects for a live oral attenuated vaccine strain free from all side effects are not promising, since Pierce *et al.* (1988) have demonstrated that the ability to colonize the intestine is the major determinant of the immunizing efficiency of *V.cholerae*.

An alternative method of producing a live oral vaccine has been attempted by cloning protective *V.cholerae* antigens into an avirulent *Salmonella typhi* host (Manning, 1987, 1989a, 1989b). A problem arises in that one must identify not only immunogenic antigens of *V.cholerae* but also protective ones. Potential antigens that would be advantageous to have expressed and display protective immunity include the B subunit of cholera toxin (Curlin *et al.*, 1975; Svennerholm *et al.*, 1982), the Tcp pilus (Taylor *et al.*, 1988; Sharma *et al.*, 1988) and the O-antigen region of the lipopolysaccharide (Clements *et al.*, 1982; Manning *et al.*, 1986). Forrest *et al.* (1989)

orally immunized human volunteers with the live oral typhoid vaccine *S.typhi* Ty21a (Germainer and Fürer, 1975) carrying the *V.cholerae* lipopolysaccharide O-antigen genes. This resulted in significant levels of specific IgA in the gut. Whether one can express all the required antigens to produce an effective, long lived immune response into the avirulent host is yet to be evaluated, but may be the answer to an effective oral cholera vaccine.

1.2 Extracellular Proteins of *Vibrio cholerae*

V.cholerae O1 produces a wide range of proteins which are released into the extracellular medium and may contribute to intestinal colonization or pathogenesis. These include the cholera enterotoxin (CT) (see above), haemolysins, proteases, deoxyribonucleases, neuraminidase and haemagglutinins.

1.2.1 Neuraminidase

The structural gene, *nanH*, for the neuraminidase of *V.cholerae* O1 was cloned from a Classical strain (Galen *et al.*, 1987; Vimr *et al.*, 1988). The neuraminidase is capable of cleaving sialic acid residues from a wide range of glycoconjugates. Positive clones were identified by their ability to cleave a fluorogenic substrate. This enzyme has the ability to hydrolyse di- and tri-sialosyl gangliosides to GM_1 gangliosides. It has been suggested that this enzyme may play a role in pathogenesis by increasing the density of GM_1 receptors on intestinal cells to which the B subunit of cholera enterotoxin can bind. Kabir and co-workers (1984) have presented epidemiological and experimental evidence that the neuraminidase is associated with increased virulence of *V.cholerae*.

As predicted from its extracellular location, nucleotide sequence analysis of *nanH* reveals the presence of a potential signal peptide of 24 amino acids that is similar to the signal peptides found on other excreted proteins. The mature protein

predicted from the DNA sequence has a size of 75.9 kDa. When expressed in *E.coli* K-12, the *V.cholerae nanH* gene product was not excreted into the growth medium but most of the enzyme was retained in the periplasmic space.

By the introduction via recombination of an insertionally inactivated *nanH* gene back into the parent *V.cholerae* genome, the possible role of neuraminidase in the pathogenic mechanism of cholera was investigated. Surprisingly, however, it was found that the mutant with the inactivated *nanH* gene was unaffected in its virulence (Galen *et al.*, 1987).

1.2.2 Deoxyribonucleases (DNases)

The Dnases produced by *V.cholerae* O1 can be found both excreted into the extracellular medium or as part of a pool of active enzyme located in the periplasmic space (Young and Broadbent, 1985). It has been suggested that the DNases belong to a group of extracellular proteins that are excreted into the growth medium via a periplasmic intermediate. As a result of these extracellular DNases, *V.cholerae* has a limited ability to accept foreign plasmids (Focareta and Manning, 1987). This phenomenon has also been seen in *Serratia marcescens* by Timmis and Winkler (1973) who demonstrated that an extracellular DNase was implicated as the reason for poor efficiency of transformation.

The role of the extracellular DNases in the pathogenesis of cholera has not yet been clarified but it has been suggested that they may play a role in the colonization of the small intestine. Ferencz *et al.* (1980) found that DNA, possibly from intestinal mucous cells was a significant component of the mucous layer in the gut. Thus, the DNases of *V.cholerae* may aid colonization of the intestine by degradation of this DNA-rich mucous layer.

Structural genes encoding extracellular DNases have been cloned independently by two groups (Newland *et al.*, 1985; Focareta and Manning, 1987). Newland *et al.* (1985) designated their gene *xds* and subsequently localized the region

of DNA encoding the nuclease activity to 3.5 kb. A polypeptide of 100 kDa was produced in *E.coli* minicells. By transposon facilitated recombination, Newland and co-workers (1985) mapped the location of their *xds* gene between the *pro*-1 and *ile*-201 markers on the El Tor chromosome. In contrast to these findings, Focareta and Manning (1987) localized the gene for a 24 kDa DNase on a 1.2 kb DNA fragment. The chromosomal location of this gene has yet to be mapped. Although the DNase is found extracellularly in *V.cholerae*, cell fractionation studies have shown the protein to accumulate in the periplasmic space when cloned into *E.coli* K-12. After determining the nucleotide sequence, an ORF of 690 bp was found corresponding to a protein product of 26.4 kDa. This protein has a typical amino-terminal leader peptide of 18 amino acids. The size of the mature protein after cleavage of this signal sequence would be 24.2 kDa which is in excellent agreement with the size observed on SDS-polyacrylamide gel electrophoresis under non-denaturing conditions, where it was also possible to detect DNase activity (Focareta and Manning, 1987). The differences between the two DNases have been resolved by Southern hybridization analysis which show both DNases to be genetically unrelated (Focareta, 1989).

Deletion mutations were introduced into the *V.cholerae* chromosome in one, the other or both of these DNases (Focareta, 1989). It was found that the strains carrying either one of the single mutations were now able to be transformed, albeit with a low efficiency. However, the strain carrying both mutations gave a further 10-fold increase in the number of transformants, indicating that the inability of wild-type *V.cholerae* strains to be transformed with plasmid DNA was due to the presence of the two extracellular deoxyribonucleases (Focareta, 1989).

1.2.3 Soluble Haemagglutinin

The soluble haemagglutinin is present in all strains irrespective of biotype or serotype and found in cell-free supernatants (Finkelstein and Hanne, 1982; Hanne and Finkelstein, 1982). More recently, it has been referred to as the soluble

haemagglutinin/protease (SHA/protease) due to the proteolytic activity it exhibits (Finkelstein *et al.*, 1983; Booth *et al.*, 1983, 1984). This enzyme has the ability not only to agglutinate erythrocytes but also to hydrolyze fibronectin, ovomucin and lactoferrin (Finkelstein *et al.*, 1983). Booth *et al.* (1984) suggested that the SHA/protease was an endogenous enzyme of *V.cholerae* responsible for the cleavage of the A subunit of cholera enterotoxin which results in the activation of the toxin. When purified, un-nicked cholera toxin was incubated with SHA/protease it was converted to the nicked, active form. But if the SHA/protease was inhibited, then nicking was prevented (Booth *et al.*, 1984). This protein may also be involved in the adherence of *V.cholerae* bacterium to the intestinal epithelium, therefore representing an important virulence factor in the pathogenesis of *V.cholerae* (Finkelstein *et al.*, 1978).

1.2.4 Haemolysins

The production and release into the medium of a haemolysin for sheep erythrocytes was originally one of the criteria for differentiating between the Classical and El Tor biotypes of *V.cholerae* O1 (Gardner and Venkatraman, 1935). However, recently this has become an unreliable test as strains that produce no haemolysin but have other biochemical properties characteristic of the El Tor biotype have been isolated (de Moor, 1963). Furthermore, Richardson *et al.* (1986) have reported that Classical vibrios exhibit haemolytic activity when assayed using chicken and rabbit erythrocytes.

V.cholerae non-O1 clinical isolates that do not produce a cholera-like enterotoxin still induce diarrhoea. These strains have been shown to produce a haemolysin that has been implicated as a potential diarrhoeagenic factor (McIntyre and Feeley, 1965). The haemolysin produced by these non-O1 isolates was shown by Yamamoto *et al.* (1986) to be biologically, physiochemically and immunologically indistinguishable from the haemolysin produced by El Tor strains of *V.cholerae* O1.

The El Tor haemolysin has been purified and shown to be a 61-65 kDa protein (Yamamoto *et al.*, 1986). The structural gene, *hlyA*, that encodes this protein, has been cloned independently by two groups and a 80 kDa protein product identified (Manning *et al.*, 1984; Goldberg and Murphy, 1984). *E.coli* K-12 cells containing the cloned *V.cholerae hlyA* gene produce only small zones of haemolysis on agar plates containing sheep erythrocytes which is in stark contrast to the large zones seen with wild-type *V.cholerae* (Manning *et al.*, 1984; Mercurio and Manning, 1985). This is due to the fact that although the haemolysin is produced in *E.coli*, it is unable to be actively excreted into the extracellular medium but accumulates in the periplasmic space (Mecurio and Manning, 1985). This problem can be alleviated by introducing the cloned genes into a *tolA* mutant strain of *E.coli* which has a leaky outer membrane allowing release of periplasmic proteins (Bernstein *et al.*, 1972; Anderson *et al.*, 1979). The zones of haemolysis are then comparable to those detected in the *V.cholerae* parent. The same phenomenon has been observed when haemolysins and other extracellular proteins from a number of bacterial species were introduced into *E.coli* (Pearson and Mekalanos, 1982; Coleman *et al.*, 1983; Kehoe *et al.*, 1983; Focareta and Manning, 1987; Vimr *et al.*, 1988). Therefore, the inability of *E.coli* to actually excrete these proteins may be due to the absence of additional determinants required for release, as *E.coli* secretes these proteins into the periplasm but lacks the specific excretion mechanism to release soluble proteins into the extracellular medium. Furthermore, the presence of the 80 kDa protein in the *E.coli* periplasm compared to the 65 kDa extracellular form seen in *V.cholerae* seems to indicate that a major cleavage within HlyA occurs upon release from the periplasmic space.

When *V.cholerae* El Tor strains regardless of their haemolytic phenotype, Classical strains and *V.cholerae* non-O1 strains were analysed by Southern blot hybridization, all were shown to contain DNA homologous to the *hly* locus (Goldberg and Murphy, 1984; Brown and Manning, 1985). In addition, Richardson *et al.* (1986) have cloned two haemolytic determinants from the Classical *V.cholerae* strain 395. One

is identical to the El Tor *hlyA* structural gene while restriction analysis and Southern hybridization have shown the second determinant to be unique. Goldberg and Murphy (1985) have analysed the *hly* loci from a non-haemolytic El Tor strain and a haemolytic revertant as well as from a non-haemolytic Classical strain using restriction endonucleases. They described a deletion of approximately 20 bp in the *hly* locus of the Classical 569B strain with respect to the El Tor *hlyA* gene. Furthermore, the *hlyA* genes of a haemolytic and non-haemolytic El Tor strain were proposed to differ by a 10-15 base pair insertion (Goldberg and Murphy, 1985).

Other genes involved in haemolysin production have also been identified. Manning *et al.* (1984) have identified a neighbouring gene, *hlyB* and postulated that it plays a role in haemolysin production as Tn*1725* insertions in *hlyB* cause a decrease in haemolytic activity. Furthermore, a gene that may be resposible for the regulation of *hlyA* was found tightly linked to *toxR* on the chromosome, and designated *hlyR* (von Mechow *et al.*, 1985).

Despite numerous studies on the epidemiology of *V.cholerae* haemolysin, the exact role of the El Tor haemolysin in the disease process has not been elucidated. The haemolysin purified by Honda and Finkelstein (1979b) was cytotoxic, cardiotoxic and rapidly lethal for mice but was only 20 kDa in size. Furthermore, it was noted that cholera patients, infected with El Tor strains, were afflicted with dysentery, which suggests that the integrity of the intestinal epithelium had been destroyed, perhaps by the haemolysin (Vasil *et al.*, 1974). In addition, non-toxigenic El Tor strains that produce a diarrhoeagenic factor correlating with the haemolysin have been isolated (Mekalanos *et al.*, 1983; Sanyal *et al.*, 1983; Ichinose *et al.*, 1987). McCardell *et al.* (1985) and Spira *et al.* (1986) have described a cytolysin that is identical to the El Tor haemolysin and is produced by non-O1 and O1 strains of *V.cholerae*. This cytolysin causes fluid accumulation in the rabbit gut as well as being cytotoxic for Chinese hampster ovary (CHO) and Y-1 adrenal cells.

1.3 Haemolysins from non-cholera *Vibrios*

1.3.1 *Vibrio damsela* cytolysin

Vibrio damsela is a halophilic organism that causes skin ulcers and fatal disease in temperate-water damsel fish and wound infections in humans (Love *et al.*, 1981; Morris *et al.*, 1982). Kreger (1984) has described a correlation between the ability of this organism to cause disease in mice and the production *in vitro* of large amounts of a cytolytic toxin. This toxin has a size of 57 kDa and is antigenically distinct from cytolysins isolated from other Vibrionaceae. This extracellular cytolysin is active against mouse erythrocytes and is found in the growth medium in amounts that vary widely between *V.damsela* strains. This toxin was shown to be lethal for mice after intraperitoneal, intravenous and subcutaneous administration (Kreger, 1984). Furthermore, subcutaneous injection caused grossly observable changes similar to those observed during lethal experimental infection caused by subcutaneous administration of *V.damsela* (Kreger, 1984). The role of this toxin in the pathogenesis of the disease caused by *V.damsela* in its natural hosts is still under investigation.

1.3.2 *Vibrio fluvialis* cytolysin

Vibrio fluvialis is a halophilic bacterium that has been isolated from marine environments as well as from humans exhibiting severe diarrhoeal disease implicating it as a potential enteric pathogen for humans (Blake *et al.*, 1980). Studies with culture supernatants of *V.fluvialis* reveal that it elaborates a wide range of extracellular proteins. These include haemolysins/cytolysins, proteases, non-haemolytic cytolysins and a factor which causes CHO cell elongation similar to cholera enterotoxin (Lockwood *et al.*, 1982). Wall *et al.* (1984) noted that although the culture supernatants possessed haemolytic and cell-killing activities, they were not separable by gel filtration. Furthermore, although this cytolysin has not yet been proven to be involved in the pathogenesis of *V.fluvialis* in humans, the presence of blood in the diarrhoea of patients suggests tissue destruction of the intestine.

1.3.3 *Vibrio vulnificus* cytolysin

Vibrio vulnificus, associated with wound infections, septicaemia, meningitis and pneumonia was found to elaborate heat-labile extracellular toxins in large amounts (Poole and Oliver, 1978; Blake *et al.*, 1979; Kelly and Avery, 1980; Kreger and Lockwood, 1981). The bacteriologically sterile culture supernatants of mid-exponential phase cultures of a virulent isolate of *V.vulnificus* possessed haemolytic activity against mammalian erythrocytes, cytotoxic activity for CHO cells, lethal activity for mice and vascular permeability activity in guinea pig skin (Kreger and Lockwood, 1981). In addition, these four toxic activities could not be separated by gel filtration and the specific activity of the toxins was directly proportional to the virulence of the strain (Kreger and Lockwood, 1981). A monoclonal antibody raised against the haemolysin which completely neutralized the haemolytic activity also abolished the lethal activity for mice (Okada *et al.*, 1987). Thus, since the monoclonal antibody has specificity for a single antigenic epitope, it has been suggested that the haemolysin is identical to the lethal toxin present in the extracellular growth medium of *V.vulnificus*. The gene that encodes this cytolysin was cloned into *E.coli* K-12, and when used as a DNA probe, homologous gene sequences were detected in all *V.vulnificus* strains but were absent in strains tested from 29 other bacterial species including both O1 and non-O1 serotypes of *V.cholerae* (Wright *et al.*, 1985).

The activity of the *V.vulnificus* haemolysin involves a temperature-independent binding step and a temperature-dependent cell degradation step. The haemolytic activity can be inhibited by the addition of divalent cations which act to stabilize the erythrocyte membrane and by cholesterol, which suggests that cholesterol constitutes the haemolysin binding site on the erythrocyte membrane (Shinoda *et al.*, 1985). This cholesterol-binding activity was utilized by Yamanaka *et al.* (1987) to prepare specific anti-haemolysin antiserum by immunizing experimental animals with haemolysin-bound liposomes.

1.3.4 *Vibrio metschnikovii* cytolysin

Vibrio metschnikovii has recently been implicated as a cause of diarrhoea in humans and the organism is found widely distributed throughout rivers, seafood and sewerage (Lee *et al.*, 1978). Miyake and co-workers (1988) have purified an extracellular cytolysin from *V.metschnikovii.* The purified cytolysin is a heat-labile protein with a molecular size of 50 kDa. The protein was active against calf erythrocytes and this lysis could be inhibited by divalent cations (Miyake *et al.*, 1988). Biological activities of this cytolysin include fluid accumulation in the intestines of infant mice and increased vascular permeability of rabbit skin. Furthermore, the cytolysin lysed cultured Vero and CHO cells (Miyake *et al.*, 1988). All of these biological activities were neutralized by the addition of anti-cytolysin antibody. This cytolysin possesses no immunological cross reactivity with haemolysins from other *Vibrio* species including both O1 and non-O1 serotypes of *V.cholerae, V.vulnificus* and *V.fluvialis* indicating that it is a new haemolysin (Miyake *et al.*, 1988).

1.3.5 Kanagawa phenomenon of *Vibrio parahaemolyticus*

V.parahaemolyticus is a major cause of diarrhoea and gastroenteritis that is associated with seafood consumption (Blake *et al.*, 1980). A number of extracellular virulence factors have been described including a thermostable direct haemolysin (TDH), a Chinese hampster ovary cell elongation factor and others involved in cytotoxicity, adherence and invasiveness (Honda *et al.*, 1976a; Blake *et al.*, 1980). This organism can be classified based on the expression of TDH which can be detected on a special blood agar medium (Wagatsuma medium). The haemolytic activity on Wagatsuma medium is termed the Kanagawa phenomenon, and a Kanagawa phenomenon positive phenotype is closely associated with pathogenicity in humans, as 96.5% of clinical isolates are Kanagawa phenomenon positive whereas 99% of environmental isolates are Kanagawa phenomenon negative (Miyamoto *et al.*, 1969; Takeda, 1983; Honda *et al.*, 1976a). This virulence factor has received the most

attention (Sakurai *et al.*, 1973; Miyamoto *et al.*, 1980).

The lethal toxin from a culture supernatant of a Kanagawa phenomenon positive *V.parahaemolyticus* strain was purified extensively and was demonstrated to be identical to the TDH (Honda *et al.*, 1976b). The lethal effect on mice was extremely rapid; a dose of 5 μg kill the animals within 60 secs when injected intravenously (Honda *et al.*, 1976b). Honda *et al.* (1976c) have described marked cardiotoxic activity for mice and rats associated with the TDH. The lethal toxin (TDH) primarily affects the heart, and thus explains why intraperitoneal injection was less effective than administration via the intravenous route (Honda *et al.*, 1976b). When administered intraperitoneally, the toxin must enter the blood stream before affecting the heart, where the rhythmic heart beat is significantly disturbed (Honda *et al.*, 1976c). Furthermore, it has been reported that the TDH possesses enteropathogenic activity giving a positive reaction in the rabbit ileal loop assay and causing diarrhoea in monkeys (Zen-Yoji *et al.*, 1974).

The gene encoding the TDH has been cloned into *E.coli* K-12 and the nucleotide sequence determined (Kaper *et al.*, 1984c; Nishibuchi and Kaper, 1985). The structural gene, *tdh*, encodes a 18.5 kDa mature protein with a 24 amino acid signal peptide. When the *tdh* gene was expressed in *E.coli*, TDH activity was detected in cell lysates. In contrast, the TDH was produced extracellularly, like the parent strain when introduced into in a Kanagawa phenomenon negative strain of *V.parahaemolyticus* (Nishibuchi and Kaper, 1985). When used as a DNA probe, the *tdh* gene detected no homologous DNA with Kanagawa phenomenon negative strains, and failed to correlate completely with the Kanagawa phenomenon positive reaction (Kaper *et al.*, 1984c). From the sequence of *tdh* Nishibuchi *et al.* (1985) further developed synthetic oligodeoxyribonucleotide probes of 19 to 21 nt to provide a more suitable method for the definition of Kanagawa-phenomenon positive strains from those that are only weakly positive. These workers tested 121 *Vibrio* strains other than *V.parahaemolyticus*, with the *tdh* gene probe, and only *V.hollisae* reacted positively.

V.hollisae reacted with the probe under low stringency suggesting that it possesses *tdh*-related sequences (Nishibuchi *et al.*, 1985).

A clinical isolate of *V.parahaemolyticus* that was assessed to be Kanagawa phenomenon negative by conventional assays such as the Wagatsuma agar test, a modified Elek method and a DNA colony hybridization test was still haemolytic (Honda *et al.*, 1988). DNA homology was slight but the haemolytic determinant, found in the extracellular medium was shown to be immunologically related but not identical to the TDH (Honda *et al.*, 1988). It appears to be composed of two subunits and exists as a dimer like the TDH but it differs in its haemolytic activity on erythrocytes from various animal sources (Honda *et al.*, 1988).

1.4 Haemolytic determinants of other Gram negative bacteria

Synthesis of haemolysins has been reported throughout a broad spectrum of Gram negative species. The isolation of the genes and their products in conjunction with studies in animal models has helped elucidate the contribution of haemolysins to pathogenesis. Haemolysins have been demonstrated to play an important role in the virulence of many bacterial infections. Furthermore, haemolytic strains of *Pseudomonas aeruginosa* and *E.coli* are usually associated with clinical disease, but rarely isolated from the environment (Al-Dujail and Harris, 1975; Waalwijk *et al.*, 1982).

1.4.1 *Escherichia coli* haemolysin

The best studied haemolysin with respect to genetics, excretion and regulation is the α-haemolysin of *Escherichia coli*. *E.coli* associated with extra-intestinal infections in humans commonly excrete this α-haemolysin (Hughes *et al.*, 1985; Minshew *et al.*, 1978), although pathogenic isolates of *E.coli* from animal intestinal origin also possess a haemolytic phenotype (Smith, 1963). The genetic locus resposible for the synthesis and excretion of the α-haemolysin is found either on large

transmissible plasmids for animal infections or in the bacterial genome in the case of human infections (Goebel *et al.*, 1974; Minshew *et al.*, 1978; De la Cruz *et al.*, 1980; Müller *et al.*, 1983).

It was demonstrated by Waalwijk *et al.* (1982) that the introduction of a plasmid bearing the haemolysin genes of *E.coli* into a non-haemolytic strain resulted in a marked increase in the virulence of the strain in mice, strongly suggesting that the haemolysin contributes to the pathogenicity of *E.coli.* DNA-DNA hybridisation analysis has demonstrated that the plasmid encoded haemolysin determinants display considerable homology with the chromosomal determinants, which suggest a common origin (De la Cruz *et al.*, 1980). A 1.9 kb insertion sequence (IS*91*) was found on a plasmid carrying the haemolysin locus which was subsequently shown to be present on other plasmids (Zabala, 1982; De la Cruz, 1983). Zabala *et al.* (1984) demonstrated that the haemolysin determinant possesses some of the characteristics of a transposable element suggesting that it may have spread by illegitimate recombination or transposition events aided by this insertion element.

A more precise analysis of the role of the haemolysin in the pathogenesis of disease has been examined. The production of haemolysin by *E.coli* was demonstrated to induce release of histamine from rat mast cells and leukotrienes from human polymorphonuclear leukocytes (Scheffer *et al.*, 1985; König *et al.*, 1986). These mediators cause enhancement of vascular permeability, chemotaxis, aggregation of granulocytes and other inflammatory disease processes (Scheffer *et al.*, 1985). The level of leukotriene production was directly dependent on the amount of haemolytic activity in the culture supernatant. Bacterial strains that could produce the haemolysin but failed to excrete it as well as *hly* mutants failed to release these inflammatory mediators (König *et al.*, 1986).

Haemolysin production has also been suggested to facilitate acquisition of essential iron from the environment. Iron is required by the bacterium to establish and maintain infections, and the haemoglobin present in erythrocytes represents a rich iron source. Lebek and Gruenig (1985) demonstrated that upon the addition of extra iron ($FeCl_3$), a significant reduction in haemolytic activity was detected. Furthermore, haemolytic activity could be greatly increased in some isolates by reducing the Fe^{3+} concentration by the addition of iron chelators. These workers suggested that haemolysin excretion in *E.coli* is directly related to bacterial iron metabolism, however, excretion may be differentially regulated among *E.coli* strains.

The haemolysin determinant is highly conserved and four genes, *hlyA*, *hlyB*, *hlyC* and *hlyD* have been identified as necessary for the synthesis and release of active haemolysin into the culture medium (Wagner *et al.*, 1983; Mackman *et al.*, 1986). Complementation analysis between specific deletion mutants have aided in defining the function of these four genes.

The *hlyA* gene product, HlyA is the haemolysin proper and exists as a 110 kDa polypeptide (Felmlee *et al.*, 1985a). The product of *hlyC*, a 20 kDa cytoplasmic protein is required to activate HlyA (Nicaud *et al.*, 1985). It has been suggested that HlyC acts by post-translationally modifying HlyA and this does not involve extensive glycosylation or phosphorylation (Mackman *et al.*, 1986). Nicaud *et al.* (1985) showed conclusively that HlyA can be excreted efficiently, although as an inactive polypeptide, to the external medium in the absence of the *hlyC* gene product. Hence HlyC plays no part in either the synthesis or excretion of HlyA (Nicaud *et al.*, 1985). Excretion of the α-haemolysin across both the cytoplasmic and outer membranes occurs via a mechanism different from the leader-peptide dependent pathway described for other membrane-translocated proteins in *Escherichia coli* (Randall and Hardy, 1984; Pugsley and Schwartz, 1985). The extracellular release of the *E.coli* haemolysin represents an intriguing cellular event. It appears analogous to the example in eukaryotic cells where ovalbumin is excreted without the removal of a leader/signal peptide (Palmiter *et al.*,

1978). Furthermore, *E.coli* efficiently secretes recombinant-derived ovalbumin (Fraser and Bruce, 1978) across the cytoplasmic membrane suggesting that the presence of a cleavable signal peptide is not an absolute requirement in Gram negative bacteria. HlyA is released from the cell without any N-terminal processing indicative of signal peptide cleavage, with only the formylated methionine missing (Felmlee *et al.*, 1985a). The passage of HlyA from the cytoplasm across both membranes and its release from the cell surface requires the function of two further cistrons, *hlyB* and *hlyD* which complete the *hly* determinant (Wagner *et al.*, 1983).

Analysis in *E.coli* minicells of the DNA containing the *hlyB* cistron reveals two membrane-bound protein products of 77 kDa and 46 kDa (Felmlee *et al.*, 1985b). Nucleotide sequence analysis of this cistron indicates that *hlyB* would encode a peptide of 79.9 kDa (Felmlee *et al.*, 1985b). Either the 46 kDa peptide is a proteolytic cleavage product of HlyB or alternatively there may be a second internal start codon within the ORF encoding HlyB. It was noted that an internal methionine codon preceeded by a potential ribosome binding site existed and this would give rise to a translated product of 46 kDa (Felmlee *et al.*, 1985b). Furthermore, the first 21 amino acids of this potential product possesses a signal-like sequence which would enable it to become membrane bound (Felmlee *et al.*, 1985b). It is not yet precisely known whether the haemolysin transport function assigned to the *hlyB* gene product is carried out by either or both of the 77 kDa and 46 kDa protein products. The *hlyD* cistron encodes a 53 kDa protein which is inserted primarily in the inner membrane (Mackman *et al.*, 1986).

Cellular fractionation indicated that during normal excretion, haemolytic activity does not accumulate in the periplasm. Furthermore, upon the removal of *hlyB* or *hlyD*, haemolytic activity, hence HlyA does not accumulate in the periplasm (Gray *et al.*, 1986; Oropeza-Wekerle *et al.*, 1989). Thus, the lack of strong evidence for a periplasmic intermediate of HlyA has led to a number of models being proposed for the excretion of the haemolysin (Mackman *et al.*, 1986). These include the passage of

HlyA protein directly to the extracellular space through specific export sites or pores formed by HlyB and HlyD from the cytoplasmic membrane to the outer membrane, traversing the periplasm. Alternatively, the inner membrane localized export proteins HlyB and HlyD interact with HlyA, followed by the concomitant budding of a vesicle which fuses to the outer membrane and then everting to release HlyA into the medium. These models both require a specific interaction between HlyA and either one or both of the export proteins. The models suggested by Mackman *et al.* (1986) do not differentiate between the roles that HlyB and HlyD play in excretion nor whether both together form a complex that is recognised by HlyA. Although the excretion of HlyA does not involve any N-terminal processing of a signal peptide, Gray *et al.* (1986) implicated the carboxy-terminal region of the protein as essential for effective excretion of HlyA. These workers demonstrated that upon removal of 27 aa's from the carboxy-terminal end of the protein, although the remaining polypeptide retained its haemolytic activity, its excretion to the extracellular medium was inhibited. Furthermore, a 23 kDa polypeptide representing the C-terminal portion of HlyA which does not contain a classical signal sequence is excreted with the same efficiency as the entire HlyA protein provided the HlyB and HlyD export functions are also present (Nicaud *et al.*, 1986). This supports an excretion mechanism in which the C-terminal portion of HlyA contains a specific topogenic sequence which interacts with the secretion machinery located in the inner membrane allowing it to be then directly transferred to the medium.

Gray *et al.* (1986) found two blocks of conserved sequences at the C-terminal end of HlyB which correspond to an ATP-binding site similar to those found in a number of *E.coli* and *Salmonella typhimurium* polypeptides involved in cytoplasmic membrane transport systems (Higgins *et al.*, 1985). Thus, a possible role for HlyB could be as a transducer to provide energy for translocation of HlyA through a proteinaceous pore generated by HlyD (Gray *et al.*, 1986)

1.4.2 Haemolysins of *Aeromonas* species

The Gram negative enteropathogen *Aeromonas hydrophila*, associated with foodborne disease in humans elaborates a plethora of extracellular determinants. These include an enterotoxin, proteases and two haemolysins, one of which is cytotoxic (Kaper *et al.*, 1981b; Ljungh and Wadstrom, 1983; Rahim *et al.*, 1984; Chakraborty *et al.*, 1984). Although the roles of these exotoxins in the pathogenesis of *Aeromonas* infection is poorly understood, the cytotoxic haemolysin (aerolysin) has been strongly implicated as an essential virulence factor (Donta and Haddow, 1978; Kaper *et al.*, 1981b; Burke *et al.*, 1982). *A.hydrophila* isolated from a patient suffering diarrhoea produces a haemolysin that is a cytotoxic enterotoxin which causes fluid accumulation in infant mouse intestines and rabbit ileal loops as well as eliciting cytotoxic effects on cultured cells (Asao *et al.*, 1984). However, a second haemolysin isolated from *A.hydrophila* strain CA-11 is shown to be antigenically similar to the aerolysin. Although it is also a cytotoxic enterotoxin, the specific biological activities vary from those of the aerolysin. The significance, if any, of these two haemolytic determinants which are immunologically and biologically similar is yet to be understood.

The aerolysin has been purified, shown to have a size of 57 kDa and is toxic for both rat and human erythrocytes and Vero culture cell lines (Howard and Buckley, 1982, 1985; Asao *et al.*, 1984, 1986). The structural gene for the aerolysin, *aerA*, has been cloned and expressed in *E.coli*, and although normal processing involving removal of its signal sequence occurred, no haemolytic activity was detected in culture supernatants of growing cultures (Howard and Buckley, 1986; Chakraborty *et al.*, 1986). This suggests that the aerolysin is not actively excreted in *E.coli* (Chakraborty *et al.*, 1986). The work of Wong *et al.* (1989) demonstrated that in contrast to *E.coli*, *A.salmonicida* can export *A.hydrophila* aerolysin and phospholipase to the extracellular medium without cell death or disruption. It was also shown by these workers that a periplasmic intermediate is part of the normal route of export in *Aeromonas* species.

Adjacent genes both upstream and downstream of *aerA* were shown to be involved in controlling the expression and activity of the structural gene. These genes have been designated *aerB* and *aerC* (Chakraborty *et al.*, 1986). Reduced haemolytic activity was seen with plasmids lacking *aerB*, but this was not as a result of the loss of transport function. The upstream gene, *aerC* is postulated to act as a regulatory gene involved in modulating the expression of aerolysin (Chakraborty *et al.*, 1986).

1.4.3 *Pseudomonas aeruginosa* haemolysin

P.aeruiginosa strains produce two products which display haemolytic activity, a heat-stable glycolipid and a heat-labile phospholipase C (PLC). The gene for the extracellular PLC has been cloned into *E.coli*, and the resultant expression and transport was poor with the enzyme remaining cell-associated (Vasil *et al.*, 1982; Coleman *et al.*, 1983). The PLC gene has been sequenced, and shown to possess a 38 aa N-terminal signal sequence, removal of which results in a mature product of 78.2 kDa (Pritchard and Vasil, 1986).

The role in pathogenesis of both of these determinants is unclear, although the PLC has been implicated as a major factor in lung and urinary tract infections (Kurioka and Liu, 1967; Berka *et al.*, 1981). In contrast, the heat-stable glycolipid determinant has been suggested to have a role in ocular and pulmonary infections (Liu, 1979).

1.4.4 Haemolysin of *Serratia marcescens*

Serratia marcescens, a Gram negative opportunistic pathogen causes bacteraemia, keratitis and infections of the urinary and respiratory tract (Maki *et al.*, 1973). The virulence determinants of this organism are still being elucidated, however, a cell-bound haemolysin which causes the release of inflammatory mediators has been strongly implicated (König *et al.*, 1987). It has been demonstrated that haemolytic isolates induce the release of leukotrienes C_4 and B_4 from polymorphonuclear

leukocytes and histamine from rat mast cells (König *et al.*, 1987). By stimulating the release of these compounds the haemolysin could increase vascular permeability, oedema formation and granulocyte accumulation, all factors which are characteristic of *Serratia* infections.

In contrast to other bacterial haemolysins, the *Serratia* haemolysin is membrane associated and haemolytic activity is not found in the extracellular growth medium (Braun *et al.*, 1985). Poole *et al.* (1988) have cloned and subsequently sequenced the 7.3 kb DNA fragment which encodes haemolytic activity from *S.marcescens*. Two open reading frames, *shlA* and *shlB* were identified. The haemolysin proper is encoded by *shlA* and is expressed as a 165 kDa polypeptide, whereas the *shlB* gene product is 61.9 kDa (Poole *et al.*, 1988). Signal sequences are present at the amino-termini of both proteins as is expected with their outer membrane location. Both *shlA* and *shlB* are essential for haemolytic activity (Braun *et al.*, 1987), and although ShlA has been shown to interact with the erythrocyte membrane, this interaction, the lysis of the erythrocyte and an active ShlA protein depend upon a functional *shlB* gene product (Poole *et al.*, 1988). The inactive ShlA protein produced in a *shlB* mutant strain remains internalized in the bacterial cell and exists in a different conformational state than the active ShlA. The active ShlA haemolysin firmly integrates into the erythrocyte membrane, forms a transmembrane channel and can only be released under harsh conditions that also dissolved other erythrocyte membrane proteins (Schiebel and Braun, 1989). Lysis of the erythrocyte can be progressively prevented by saccharides of increasing molecular weight and Dextran 4 (4 kDa) totally inhibited lysis (Braun *et al.*, 1987) Prevention of cell lysis by the saccharides was due to counterbalancing the osmotic stress and not by preventing channel formation in the erythrocyte membrane (Braun *et al.*, 1987).

Specifically constructed deletion mutants were used to determine the domains necessary for lytic activity, integration into the erythrocyte membrane and association with ShlB. Removal of aa's from the C-terminal end reduced haemolytic

activity, but a 37% deletion failed to completely abolish haemolytic activity. However, elimination of 54% of the gene resulted in a non-haemolytic phenotype even though this truncated product was still able to associate with the erythrocyte membrane (Poole *et al.*, 1988). An inactive polypeptide of 261 aa's from the N-terminus was the smallest protein that could still be excreted (V. Braun, personal communication). Therefore, the data suggest that the N-terminal region is the target for ShlB association as well as conferring the ability to associate with the erythrocyte, whereas the C-terminal end contains at least some of the channel-forming ability which results in lysis of the erythrocyte (Poole *et al.*, 1988; V. Braun, personal communication). The ShlA and ShlB polypeptides are transported across the cytoplasmic membrane in a signal-sequence dependent fashion, however, passage of ShlA across the outer membrane so that contact can be made with the erythrocyte requires a functional ShlB protein. ShlA is made active by ShlB either at the onset of excretion or during translocation. Once associated with the erythrocyte, each ShlA protein integrates and forms a small water-filled channel through which water and solutes up to approximately 1000 Da can diffuse.

The haemolysin may also play a role in pathogenesis by providing the bacterium with essential iron. The ability to establish infections depends somewhat on the ability to acquire iron, and the haem-bound iron present in the erythrocyte would represent an additional iron source. Expression of the *shlB,A* locus has been examined under varying conditions of iron stress. Production of iron regulated proteins such as iron-specific chelators, siderophores, and membrane associated receptors is enhanced as a result of iron limitation. This increase is mediated in *E.coli* by the product of the *fur* locus (Hantke, 1982). Poole and Braun (1988) demonstrated that restricting the availability of iron resulted in a marked increase in the haemolytic activity. The site at which this iron regulation acts is located upstream of the *shlB,A* locus where a nucleotide sequence which exhibits some homology with the "Fur-consensus" sequences of *E.coli* can be seen (Poole and Braun, 1988).

1.5 Haemolysins of Gram positive bacteria

Synthesis of haemolysins has been reported throughout a spectrum of Gram positive bacterial species. The haemolysins of the Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes*, are known to be vital in the establishment and maintenance of infections. With the exception of α-toxin of *Staphylococcus aureus*, all genetically analyzed Gram positive haemolysins are sulfhydrl-activated (SH-activated) cytolysins. These haemolysins share common biochemical properties such as sensitivity to oxygen and binding to cholesterol which is the assumed membrane receptor for these cytolytic proteins. Furthermore, these proteins display immunological cross-reactivity. The problems with excretion seen for Gram negative bacteria are also not encountered as there is no outer membrane to cross.

1.5.1 *Staphylococcus aureus* haemolysin

S.aureus produces a range of extracellular products which have been considered as potential virulence factors (Easmon and Adlam, 1984; Jonsson *et al.*, 1985). The α-haemolysin has been implicated as the most important product in causing tissue damage and in establishing and maintaining infections (Freer and Arbuthnott, 1983). Kehoe *et al.* (1983) cloned the *S.aureus* α-haemolysin gene into *E.coli*. The low amounts of haemolysin produced were not excreted into the culture supernatant but remained associated with the cell. When this clone was introduced into *Bacillus subtilis*, active α-haemolysin was detected in culture supernatants of growing cultures, where immunoprecipitation with anti-α-haemolysin antiserum identified proteins of 33 kDa and 34 kDa (Fairweather *et al.*, 1983).

1.5.2 Haemolysins of *Streptococcus* species

The haemolysins from the human pathogens *S.pyogenes* (Streptolysin O) and *S.pneumoniae* (Pneumolysin) have been cloned (Kehoe and Timmis. 1984; Paton *et al.*, 1986). Streptolysin O possesses tissue-damaging and cytolytic properties and inhibits chemotaxis of human neutrophils suggesting that it plays a role in the pathogenesis of streptococcal infections (Andersen and Van Epps, 1972; Van Epps and Andersen, 1974; Alouf, 1980). Two forms of streptolysin O are detected in *E.coli*, with molecular weights of 68 kDa and 61 kDa. Two polypeptides of size 69 kDa and 60 kDa can be identified in culture supernatants of *S.pyogenes*, suggesting that a similar proteolytic cleavage occurs in both organisms (Kehoe and Timmis, 1984). However, studies by Suzuki *et al.* (1988) have demonstrated that the haemolytic determinant has a molecular size of 64 kDa and suffers no proteolytic cleavage.

When the cloned pneumolysin gene was expressed in *E.coli*, active pneumolysin accumulated in the cytoplasm (Paton *et al.*, 1986). Pneumolysin, unlike the other thiol-activated toxins which are excreted by their producing bacteria, is found primarily in the pneumococcal cytoplasm (Johnson, 1977). The cloned pneumolysin gene directs the production of two polypeptides (56 kDa and 53 kDa) which are precipitated by antiserum directed to streptolysin O (Walker *et al.*, 1987). Analysis of the predicted aa sequence from the determined nt sequence revealed no obvious signal sequence associated with the N-terminal domain of the pneumolysin (Walker *et al.*, 1987).

Paton *et al.* (1983) demonstrated that mice immunized with a purified pneumolysin preparation survived significantly longer than control mice when challenged with a virulent strain of *S.pneumoniae*. This toxin also inhibits migration of human polymorphonuclear leukocytes, and therefore has the potential to inhibit phagocytic clearance of invading pneumococci as well as interfering with the establishment of a humoral immune response (Paton and Ferrante, 1983).

The genes encoding these determinants share aa and DNA sequence homology with each other and also with the perfringolysin O gene isolated from *Clostridium perfringens* (Tweten, 1988). Isolation and characterization of well-defined specific mutations within the coding sequences of these three determinants would permit more complete studies on structure, mode of-action, synthesis and release of the toxins as well as their exact contribution to the pathogenesis of the respective bacterial infections.

1.5.3 *Listeria monocytogenes* haemolysin

The haemolysin from *L.monocytogenes* is a determinant associated with the pathogenesis of this organism in humans and animals. Only haemolytic strains are isolated from patients suffering listeriosis, and these are the only strains which are virulent and possess the ability to invade host cells (Groves and Welshimer, 1977; Vicente *et al.*, 1985; Kathariou *et al.*, 1987; Portnoy *et al.*, 1988). A transposon insertion mutant which resulted in a non-haemolytic phenotype was avirulent and spontaneous loss of the transposon resulted in the recovery of the Hly^+ phenotype and also restored virulence (Gaillard *et al.*, 1986). Portnoy *et al.* (1988) describes three Hly^- avirulent mutants that upon reverting to a Hly^+ phenotype regained the ability to multiply in infected tissue cells. Mutants lacking haemolytic activity were unable to multiply in murine macrophages and fibroblasts and some but not all human cell lines (Gaillard *et al.*, 1987; Portnoy *et al.*, 1988)

The gene that encodes this haemolysin (Listeriolysin O) has been cloned and expressed in *E.coli* and its nucleotide sequence determined (Vicente *et al.*, 1985; Mengaud *et al.*, 1988). The predicted aa sequence revealed a putative 25 aa signal sequence, the cleavage of which would result in a mature excreted product of 58 kDa (Mengaud *et al.*, 1988). The protein sequence displays a high degree of homology with those of streptolysin O and pneumolysin, including the region surrounding the unique cysteine residue which is thought to be vital for lytic activity. DNA hybridization

analysis revealed that all *L.monocytogenes* strains possessed DNA homologous to the listeriolysin O gene, even the phenotypically non-haemolytic strains but the reason for this has yet to be determined (Mengaud *et al.*, 1988). Furthermore, no homology was detected with any other Listeria species, which may prove extremely useful in the development of a *L.monocytogenes* specific probe for accurate and rapid identification of this bacterium. Recently however, using DNA probes that encoded the listeriolysin and surrounding sequences, Leimeister-Wächter and Chakraborty (1989) detected homologous listeriolysin genes in the species *L.ivanovii* and *L.seeligeri* as well as in *L.monocytogenes.* Sequences unique to *L.monocytogenes* were found to be located downstream of the listeriolysin gene (Leimeister-Wächter and Chakraborty, 1989).

There is now evidence for a second haemolytic determinant present in a high percentage of pathogenic *L.monocytogenes* strains, a β-listeriolysin which has been implicated in human listerial infections (Parrisius *et al.*, 1986). When the cloned β-listeriolysin gene or synthetic oligonucleotides constructed from the determined nucleotide sequence of this gene are used as probes against *Listeria* species, only those β-haemolytic *L.monocytogenes* strains reacted. The synthetic probe could also detect *L.monocytogenes* in artificially spoiled food indicating its potential use as a detection mechanism for *L.monocytogenes* (Datta *et al.*, 1987, 1988). The relationship between the two forms of listeriolysin and their individual importance in human disease is not yet clarified.

1.6 Aims of this thesis

V.cholerae O1 is a member of a select group of Gram negative bacteria that possess the ability to excrete a large range of proteins out of the cell into the extracellular environment. Much of the work on this organism has centered on these extracellular products, particularly cholera toxin, and their contribution to the pathogenesis of disease. Virulence is multi-factorial, and the El Tor haemolysin although apparently not essential is thought to play a role in pathogenesis.

The aims of this study are to characterize the El Tor haemolysin at the protein and DNA level, and to determine the relevance of the non-haemolytic phenotype in Classical strains which still possess the *hly* genes. By site directed mutagenesis of the cloned gene, specific mutants will be constructed to determine the role that this haemolysin plays in pathogenesis. - In an attempt to gain a better understanding of the role of accessory proteins in the secretion\excretion of the haemolysin, the genes flanking *hlyA* will be examined.

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 routinely grown in Brain Heart Infusion (BHI) (Difco) prepared as directed by the manufacturers, or Luria broth. Luria broth (LB) and 2 x 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. Blood agar is NA which is supplemented with sheep erythrocytes to a final concentration of 5% prior to use. CFA agar was as described elsewhere (Evans *et al.*, 1977). 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. Casein agar was according to Lennette *et al.* (1980).

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 cultures 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. 2'-2'-dipyridyl was from Sigma. Caesium chloride (Cabot) was technical grade. Ethylene-diamine-tetra-acetic-acid, disodium salt (EDTA) was Analar analytical grade from Ajax Chemicals.

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 bis-acrylamide and urea (BRL).

The four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and their corresponding dideoxy-ribonucleotide 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. X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) and IPTG (isopropyl-β-D-thiogalacto-pyranoside) 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). The -35 sequencing primer was obtained from New England Biolabs. [^{35}S]-Methionine (1,270 Ci/mMole), [^{35}S]-dATP (>1000 Ci/mMole) and Sequenase was purchased from Amersham. Phosphorylated *Mlu*I and *Eco*RI linkers (8-mers) were purchased from New England

Biolabs, Inc., Beverley, Mass. Linkers were obtained in a lypholized form and resuspended in 0.1 ml of TE buffer, pH 8.0 and stored frozen at -20°C.

2.3 Enzymes

Deoxyribonuclease I (DNase I) and lysozyme were obtained from Sigma. Pronase was from Boehringer-Mannheim.

Restriction endonucleases *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Mlu*I, *Nru*I, *Pst*I, *Sau*3A and *Taq*I were purchased from either Boehringer-Mannheim, New England Biolabs, Pharmacia or Amersham.

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).

2.3.1 Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides (oligos) were synthesized using reagents purchased from Applied Biosystems or Ajax Chemicals (acetonitrile). Synthesis was performed on an Applied Biosystems 381A DNA synthesizer. Oligos were routinely of a purity that no further purification was required.

2.4 Maintenance of bacterial strains

For long time storage, 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 overnight at the appropriate temperature. The other half was streaked onto two nutrient agar plates and incubated overnight at the appropriate growth temperature. 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 as a suspension of freshly grown bacteria in glycerol (32% v/v) and peptone (0.6% w/v) at -70°C. Fresh cultures from

glycerols were prepared by streaking a loopful of the glycerol suspension onto a nutrient agar plate (with or without antibiotic as appropriate) followed by incubation overnight 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 x 4 in. freeze drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilized in a freeze drier. After the vaccuum 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°C.

2.5 Bacterial strains and plasmids

The *Vibrio cholerae* and other strains used are listed in Table 2.1. Strains of the El Tor biotype were distinguished from the Classical biotype by resistance to the antibiotic polymyxin B (50 units/ml) and sensitivity to biotype specific typing phages. Table 2.2 describes the *Escherichia coli* K-12 strains used in this study.

The plasmids and phage cloning vectors which were used in this study are listed in Table 2.3.

2.6 Transformation procedure

Transformation was performed essentially according to 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 shaken 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 h. The culture was then plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

2.7 Iron Starvation

Overnight cultures grown in LB (2 ml) were centrifuged and the cells resuspended in 20 ml fresh LB containing 0.2 mM, 0.3 mM or 0.5 mM 2'-2'-dipyridyl. They were incubated at 37°C with aeration to an OD_{650} of 0.6. The cultures were then tested in a liquid haemolysis assay (as described in Section 2.15)

2.8 Bacterial Conjugation

Overnight broth cultures grown in BHI or LB were diluted 1 in 20 and grown to early exponential phase with slow agitation. Donor and recipient bacteria were mixed at a ratio of 1:1 and the cells pelleted by centrifugation (5000 rpm, 5 min, bench centrifuge). The pellet was gently resuspended in 200 μl of broth and spread onto a nitrocellulose membrane filter (0.45 μm, type HA, Millipore Corp.) on a NA plate. This plate was incubated for 6-16 h at 37°C. The cells were then resuspended in 10 ml NB and samples plated onto selective agar and incubated overnight at 37°C.

2.9 DNA extraction procedures

2.9.1 Plasmid DNA isolation

Plasmid DNA was isolated by one of the four 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 shaken overnight culture were pelleted (this yielded about 10^9 cells) in an Eppendorf 5414 centrifuge for 30 sec, 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 sec 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 0.25M 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 (5M 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 isoamyl alcohol. CsCl was then removed by dialysis overnight against three changes of 2 litres TE at 4°C. DNA was stored at 4°C.

Method 4: Small scale plasmid purification was performed by the three step alkali lysis method using a modification of Garger *et al.* (1983). Overnight bacterial cultures (1.5 ml) were transferred to a microfuge tube and harvested by centrifugation (45 sec, Eppendorf 5414), and resuspended in 0.1 ml solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). The addition of 0.2 ml solution 2 (0.2 M NaOH, 1% (w/v) SDS) followed by a 5 min incubation on ice resulted in cell lysis. After the addition of 0.15 ml solution 3 (5M potassium acetate, pH 4.8) and a 5 min incubation on ice, protein, chromosomal DNA and high molecular weight RNA were collected by centrifugation (90 sec, Eppendorf 5414). The supernatant was transferred to a fresh tube and extracted once with TE-saturated phenol and once with diethyl ether. Plasmid DNA was precipitated by the addition of 2x volume 100% ethanol and a 2 min incubation at room temperature. The DNA was collected by centrifugation (15 min, Eppendorf 5414), washed with 70% (v/v) ethanol and dried *in vacuo.* The pellet was resuspended in 40 μl 1x TE.

2.9.2 Preparation of *V.cholerae* or *E.coli* genomic DNA

Whole genomic DNA from either *V.cholerae* or *E.coli* was prepared according to Manning *et al.* (1986). Cells from a 20 ml shaken overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl). The pellet was then resuspended in 2 ml 25% sucrose, 50 mM Tris-HCl, pH 8.0 and 1 ml lysozyme (10 mg/ml in 0.25 M EDTA, pH 8.0) was added and the mixture incubated on ice for 20 min. TE buffer (0.75 ml) and 0.25 ml lysis solution (5% (w/v) sarkosyl, 50 mM Tris-HCl, 0.25M 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 three extractions with TE saturated phenol and two with diethyl ether. The genomic DNA was then transferred to dialysis tubing and dialysed against 5 litres 1x TE buffer for 16 h with one change.

2.10 Analysis and manipulation of DNA

2.10.1 DNA quantitation

The concentration of DNA in solutions 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).

2.10.2 Restriction endonuclease digestion of DNA

Cleavage reactions with the restriction enzymes *Hin*dIII, *Bam*HI, *Eco*RI, *Pst*I and *Cla*I were performed using SPK buffer (10x: 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 h. 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, 0.1 mg/ml RNase A) was added.

2.10.3 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 100V for 4-5 h 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 using 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 the following method. 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 (1 g/ml) and then phenol:chloroform (1 g/ml). Residual phenol was removed with chloroform and the DNA precipitated with two volumes of ethanol and one tenth volume of 3M sodium acetate, pH 5.0. DNA was collected by centrifugation (15 min, Eppendorf 5414), washed once with 70% (v/v) ethanol and dried *in vacuo* before being resuspended in 1x TE buffer.

2.10.4 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 differ from those published (Ratcliff *et al.*, 1979) and were calculated with the program DNAFRAG (Rood and Gawthorne, 1984) using bacteriophage lambda and plasmid pBR322 as standards. The sizes (kilobases, kb) used were: 8.37; 7.2; 6.05; 4.90; 3.55; 2.63; 1.73; 1.61; 1.29; 1.19; 0.99; 0.86; 0.63; 0.48; 0.38.

2.10.5 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 (Boehringer-Mannheim) for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to a final concentration of 3 mM followed by heating at 65°C for 10 min. The reaction mix was then extracted twice with hot 56°C 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 3M 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.

2.10.6 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 10x 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.

2.10.7 Ligation of Linkers to blunt DNA ends

Phosphorylated linkers were ligated to blunt ends generated by Klenow fragment of *E.coli* DNA polymerase I 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 1x linker-kinase buffer (Maniatis *et al.*, 1982).

2.10.8 Nick translation

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 10x nick translation buffer (500 mM Tris-HCl, pH 7.2, 100 mM $MgCl_2$, 1 mM DTT, 500 μg/ml BSA) 1 μl each of 2 mM dATP, dGTP, dTTP. DNA (1 μg) and DNase (10 mg/ml; 1 μl) 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 h. [^{32}P]-labelled DNA was separated from unincorporated label by centrifugation through a mini-column of Sepharose CL-6B.

2.10.9 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 h 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), 5x Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% fraction V BSA) 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 h at 44°C.

Filters were washed twice with shaking at 37°C for 30 min in 2x SSC, containing 0.1% (w/v) SDS. This was followed by two further washes in 0.1x 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.

2.10.10 Kinasing single stranded DNA

Single stranded DNA (primers) were kinased using gamma-[^{32}P]-dATP. The reaction mix consisted of 100 mM DTT, 1 μl 10x kinase buffer, 3 units of polynucleotide kinase, 10 μl of gamma-[^{32}P] and 60 μg of primer. This reaction mix was

made up to 10 μl in water and incubated at 37°C for 30min. The 10X kinase buffer consisted of 500 mM Tris pH7.4 and 100 mM $MgCl_2$.

2.10.11 Dot Blots with single stranded DNA

Strains to be tested were grown up overnight on agar plates. These colonies were transferred to nitrocellulose discs (Schleicher and Schüll) and the colonies lysed by the following method. The nitrocellulose disc was placed colonies up, on Whatman 3MM paper soaked in 0.5 M NaOH and allowed to stand for 5 min. The discs were then subsequently treated for 5 min incubations by placing them sequentially on Whatman 3MM paper soaked in the following solutions: 0.1 M NaOH, 1.5 M NaCl; 1M Tris-HCl pH 7.5 (x2); 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl. The discs were then air dried, followed by baking under vaccum between Whatman 3MM sheets for 2 h at 80°C. All bacterial debris was removed by a 2 hour wash at 65°C in 3x SSC plus 0.2% SDS. Prior to hybridization, nitrocellulose discs were incubated for 4-5 h at 42°C in 15 ml of pre-hybridization solution containing 56.25 ml 4 M NaCl, 22.5 ml 1 M Tris-HCl pH 7.6, 9 ml 0.25 M EDTA, 25 ml 50x Denhardt's reagent, 2.5 ml denatured Herring Sperm DNA made to a final volume of 250 ml and a final concentration of 0.2% SDS. Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-hybridization but with the exclusion of Herring Sperm DNA). The kinased single-stranded probe was added, and hybridization allowed to occur for 16-24 h at 42°C. Filters were removed and washed twice for 30 min in 6x SSC. After air drying (15-20 min at room temperature), the discs were placed for autoradiography at -70°C with intensifying screens.

2.11 Protein analysis

2.11.1 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 in minimal medium to degrade long lived mRNAs corresponding to chromosomally encoded genes, 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 1x sample buffer (Lugtenberg *et al.*, 1975) and analysed by SDS-PAGE. Fractionation of minicells and extraction with Sarkosyl was according to Achtman *et al.* (1979).

2.11.2 Ammonium Sulphate precipitation

Bacterial supernatants and the periplasmic contents of cells were dispensed and various amounts of solid ammonium sulphate added. Once dissolved, the solution was left for 16 h on ice at 4°C. The precipitate was collected by centrifugation (15,000 rpm, 20 min, 4°C) and resuspended in 1 ml water. The supernatants were dialysed for 16 h at 4°C against water. Samples were mixed with an equal volume of 2x sample buffer (Lugtenberg *et al.*, 1975), heated to 100°C for 3 min and analysed by SDS-PAGE.

2.11.3 SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was usually performed on 11-20% polyacrylamide gradients 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 h. Proteins were stained 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 h.

Size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa).

2.11.4 Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 h 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 h at -70°C, using intensifying screens.

2.11.5 Cell fractionation

The cell fractionation procedure was a modification of that described by Osborn *et al.* (1972). Cells were grown in BHI to mid exponential phase at 37°C (50 ml, OD_{650} of 0.6). Cells were pelleted in a Sorvall SS-34 rotor, (10,000 rpm, 10 min, 4°C) and resuspended in 1 ml of 20% sucrose, 30 mM Tris-HCl pH 8.1, transferred to SM-24 tubes and chilled on ice. Cells were converted to sphaeroplasts with 0.1 ml of 1 mg/ml lysozyme in 0.1 M EDTA pH 7.3 for 30 min on ice. Cells were centrifuged as above and the supernatant collected (periplasmic fraction). The cell pellet was frozen in an ethanol dry ice bath for 30 min, thawed and dispersed vigorously in 3 ml 3 mM EDTA pH 7.3. Cells were lysed by 60 x 1 sec. bursts with a Branson Ultrasonifier. Unlysed cells and large cell debris were removed by low speed centrifugation (5,000 rpm, 5 min, 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000 rpm in a 50Ti rotor for 60 min at 4°C in a Beckman L8-80 ultracentrifuge. The supernatant (cytoplasmic fraction) was collected and the membrane pellet was resuspended in 25% sucrose, 10 mM Tris-HCl pH 7.8, 1 mM EDTA. The separation of the outer and inner membrane fractions was performed by using a step gradient between 55% and 30% sucrose. The membrane bands were collected, pelleted by centrifugation and suspended in 0.1 ml of 10 mM sodium phosphate buffer pH 6.8.

2.11.6 Whole cell preparation

1 ml of an overnight culture ($1x10^9$ cells) was placed in a microfuge tube and the cells were collected by centrifugation (30 sec, Eppendorf 5414). The cell pellet was resuspended in 100 µl of 1x 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°C for future use.

2.11.7 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 and transferred to nitrocellulose (Schleicher and Schüll) at 200 mA for 2 h 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. The antiserum was diluted 1/1000 in TTBS, 0.02% (w/v) skim milk powder and incubated with gentle agitation at room temperature for 2-16 h. The antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. Bound antibody was detected using an anti-antibody coupled with horseradish peroxidase and peroxidase substrate. This was accomplished by incubating the filter for 2-16 h (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 filter 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). The antigen-antibody complexes were then visualized using peroxidase substrate (9.9 mg 4-chloro-1-napthol dissolved in 3.3 ml -20°C methanol added to 16.5 ml TBS containing 15 µl hydrogen peroxide) was added and allowed to incubate for 10-15 min with shaking, as described by Hawkes *et al.* (1982).

2.12 M13 cloning and sequencing procedures

2.12.1 Preparation of M13 replicative form (RF) DNA.

Fresh 2 x 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 h. Bacterial cells were removed by centrifugation (5,000 rpm, 10 min, bench centrifuge) and the supernatant added to 1 litre NB containing 10 ml of a shaken overnight culture of JM101. Following incubation for 14 hour at 37°C with shaking, replicative form (RF) DNA was prepared as described above for plasmid DNA purification.

2.12.2 Cloning with M13mp18 and M13mp19

The M13 vectors, M13mp18 and M13mp19 (Messing and Vieira, 1982; Vieira and Messing, 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.

2.12.3 Transfection of JM101

Strain JM101 was made competent for transformation/transfection as described in section 2.6. 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.

2.12.4 Generation of stepwise deletions

Deletions of increasing size were made in the *Pst*I - *Eco*RI fragment containing the *hlyA* gene using M13mp18 and M13mp19 harbouring this fragment. The method employed to construct these overlapping deletions was the "Cyclone System" (#77200) available from Integrated Sciences.

Once a single stranded template had been purified, a short synthetic oligodeoxynucleotide was annealed to the 3' end of the cloned insert at the end of the M13 polylinker generating a restriction site (either *Hin*dIII for M13mp18 or *Eco*RI for M13mp19). Once the DNA had been digested by the corresponding enzyme, single-stranded 3' to 5' exonuclease digestion was performed by T4 DNA polymerase for various lengths of time depending on the size of deletion required. After heat-inactivation, the deletion products were tailed with dA's (M13mp18) or dG's (M13mp19) using terminal deoxyribonucleotidyl transferase (TdT) thus producing a short homopolymer tail at the 3' end. Fresh oligodeoxynucleotide was annealed to the deletion products joining the two ends of the molecule. The remaining nick was sealed with T4 DNA ligase. The product was then used to transfect competent *E.coli* JM101 cells and individual plaques picked and screened for the size of insert. A suitable set of deleted clones were then subjected to DNA sequencing.

2.12.5 Screening M13 vectors for inserts

White plaques were picked from X-gal, IPTG plates with sterile toothpicks and added to 1 ml 2 x TY broth in microfuge tubes containing a 1:100 dilution of an overnight culture of JM101. These tubes were incubated for 5 h at 37°C. The cells were pelleted by centrifugation (30 sec, Eppendorf 5414) and 0.1 ml of supernatant was used to inoculate 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 or the

miniprep method (section 2.9.1). After restriction enzyme digestion, DNA was examined on 1% (w/v) agarose gels.

2.12.6 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 toothpicks to inoculate 2 ml 2x TY broth containing 20 µl of an overnight culture of JM101. After vigorous shaking at 37°C for 6 h, the culture was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was transferred to clean tubes and recentrifuged for 5 min. Two methods of lysing phage and collecting single stranded phage were employed. They are as follows:

Method 1 : 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 resuspended 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 6 µl of 3 M sodium acetate pH 5.0 and 400 µl absolute ethanol was added. 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.

Method 2 : 1 ml of the supernatant was added to a microfuge tube containing 250 μl of a 20% PEG/3.5 M ammonium acetate solution. This was vortexed well and incubated on ice for 30 min. The phage was collected by centrifugation (15 min, Eppendorf 5414) and all the supernatant carefully removed. The pellet was dissolved in 100 μl 1x TE buffer. 50 μl redistilled phenol was added and the tube vortexed for 2 min followed by incubation at room temperature for 5 min. 50 μl chloroform was then added, vortexed for 2 min, spun in a microfuge for 5 min and the upper aqueous phase was transferred to a fresh tube. The phenol/chloroform with 100 μl 1x TE buffer, spun as before and the aqueous phases combined. The whole was then extracted with an equal volume phenol/chloroform (three more times). This was then extracted with chloroform and 250 μl of the supernatant transferred to a microfuge tube containing 125 μl 7.5 M ammonium acetate. After addition of 0.75 ml 95% ethanol the tubes were stored overnight at -20°C. The DNA was collected by centrifugation (15 min, Eppendorf 5414), and the pellet washed twice with 95% ethanol. The pellet was dried *in vacuo* before resuspending DNA in 20 μl distilled water.

2.12.7 Dideoxy sequencing protocol with Klenow fragment

The method was 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°(μl)	C°(μl)	G°(μl)	T°(μl)
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
10x TE buffer	10	10	10	10

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 μl	7 μl	7 μl	7 μl
ddNTP	14 μl	14 μl	14 μl	14 μl

The mixes 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 15 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 sec immediately prior to loading.

2.12.8 Dideoxy sequencing protocol with Sequenase

The dideoxy chain termination preocedure of Sanger *et al.* (1977) was modified to encompass the use of Sequenase (modified T7 DNA polymerase) in place of Klenow (Tabor and Richardson, 1987). All reagents were stored at -20°C. Two types of labelling and termination mixes were used, namely the dGTP mixes and the dITP mixes. The contents of the dGTP mixes are as follows :

Labelling Mix (dGTP):	7.5 μM dGTP, dCTP and dTTP
ddG Termination Mix (dGTP):	80 μM dNTP, 8 μM ddGTP, 50 mM NaCl
ddA Termination Mix (dGTP):	80 μM dNTP, 8 μM ddATP, 50 mM NaCl
ddC Termination Mix (dGTP):	80 μM dNTP, 8 μM ddCTP, 50 mM NaCl
ddT Termination Mix (dGTP):	80 μM dNTP, 8 μM ddTTP, 50 mM NaCl

The dITP mixes were used to reduce gel artifacts due to secondary structures in DNA synthesized in the sequencing reaction (Barnes *et al.*, 1983; Gough and Murray, 1983). The dITP mixes were as follows :

Labelling Mix (dITP):	15 μM dITP, 7.5 μM dCTP,
	7.05 μM dTTP
ddG Termination Mix (dITP):	160 μM dITP, 80 μM dATP, dCTP
	dTTP, 1.6 μM ddGTP, 50 mM NaCl
ddA Termination Mix (dITP):	160 μM dITP, 80 μM dATP, dCTP
	dTTP, 8 μM ddATP, 50 mM NaCl
ddC Termination Mix (dITP):	160 μM dITP, 80 μM dATP, dCTP
	dTTP, 8 μM ddCTP, 50 mM NaCl
ddT Termination Mix (dITP):	160 10Hμ M dITP, 80 μM dATP, dCTP
	dTTP, 8 μM ddTTP, 50 mM NaCl

Normally the labelling mix was diluted 1:5 with water to obtain the working concentration, however, to read long sequences in a single reaction, a dilution of 4:5 was used. The synthetic primer was annealed to the template achieved by incubating 7 μl template (5-10 nM), 1 μl primer (500 nM) and 2 μl 5x Sequenase buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The mixture was heated in a metal block at 65°C for 3 minutes and then the block containing the tubes was allowed to cool to room temperature. To the annealed mixture, 2 μl of the appropriately diluted labelling mix, 1 μl DTT (0.1 M), 0.5 μl [α-^{35}S]-dATP (1000 Ci/mmol) and 2 μl of diluted Sequenase (1:8 dilution in 1x TE buffer) was added, spun, mixed, resuspended and then incubated for 5 minutes at room temperature. 3.5 μl of this mix was then aliquoted into four microfuge tubes, prewarmed to 37°C, labelled A, C, G and T, each containing 2.5 μl of the corresponding termination mix, then spun briefly to start the termination reaction. After 5 minutes at 37°C, 4 μl Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each of the reactions. Reaction mixes were heated to 100°C for 2 min and immediately 1.2 μl loaded onto the sequencing gel. For re-running, these samples were kept at -20°C for up to 2 weeks and heated to 100°C for 3 min prior to loading.

2.12.9 Double stranded sequencing

Plasmid DNA (2-4 μg/ml) was diluted to a volume of 18 μl with water. The DNA was denatured by the addition of 2 μl of 2 M NaOH and incubated for 5 min at RT. To this mix, 8 μl of 5M ammonium acetate pH 7.5 was added with 100 μl of 100% ethanol to precipitate the DNA. The supernatant was removed and the pellet washed in 70% ethanol. The pellet was dried *in vacuo* and dissolved in 7 μl of water. 2 μl of sequencing buffer (1x TM) and 1 μl of primer (0.5 pmol) was added to the DNA and heated to 37°C for 20 min. The labelling and termination reactions were run exactly as described for M13 single stranded template DNA.

2.12.10 DNA sequencing gels

Polyacrylamide gels for DNA sequencing were prepared using glass plates 33 x 39.4 cm and 33 x 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, 8M urea in 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA, pH 8.3), 420 μl 25% ammonium persulphate 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, 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 1x 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 at a constant voltage (700 V) for 15 min, and then increased to 1200 V (33 mA). After 4 h 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 and 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 2.0 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 h.

2.12.11 Analysis of DNA sequences

Sequencing data was analysed using the following computer programs: 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.

2.13 Animal experiments

2.13.1 Infant mouse cholera model

This method was first described by Ujiiye *et al.* (1968) and adapted by Attridge and Rowley (1983a) to assess the virulence of *V.cholerae* strains. Five to six day old infant mice (weight, 2.4-2.7 g) were taken from their mothers about 6 hr before use, to permit the emptying of stomach contents. Infant mice in groups of 8 were given 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. The number of mice surviving within each group was noted up to 48 h after challenge. The mice to be examined histologically had their intestines removed and fixed in phosphate-buffered formalin. Thin sections of tissue were cut, mounted on slides and stained with haematoxylin and eosin and examined under an Olympus BH-2 light microscope.

2.13.2 Rabbit Ileal loops

Enteropathogenicity in ileal loops of adult rabbits was carried out essentially as described by Sinha and Srivastava (1978). Bacterial strains were grown to mid exponential phase in LB and 1 ml samples were injected into closed ileal loops of adult rabbits. Autopsies were performed at various times after inoculation. The

amount of fluid accumulated within the loops was measured. Viable counts were performed on NA to determine the inoculum injected into each loop. The rabbits were deprived of food for 24 h and of water 12 h before the experiments. The loops to be examined histologically were fixed, mounted and examined as for the infant mice intestines.

2.14 *In vitro* Cytotoxicity experiments

2.14.1 Cytotoxicity for HEp-2 cells

Cytotoxicity for cultured human epithelial cells (HEp-2) was assayed as follows. Bacterial strains were grown overnight at 25°C on CFA agar and a loopful resuspended in 200 µl phosphate-buffered saline (PBS) to a density of 10^6 cells/ml. HEp-2 cell monolayers were washed with PBS and then incubated with 50 µl of the bacterial suspension for 15 minutes at 37°C. The monolayers were then washed with PBS and the cells fixed with -20°C methanol (2 min). The monolayers were examined by light microscopy after staining with Giemsa stain for 5 minutes.

2.14.2 ^{51}Cr release assay

The ^{51}Cr release *in vitro* cytotoxicity assay used was a variation of Brunner *et al.* (1968) which is essentially similar to Thorn *et al.* (1974). P815 cells labelled with [^{51}Cr]-chromate were used as the target cells. The assay was simplified and scaled down using plastic tissue culture trays (Flow Laboratories), each with 96 flat-bottomed wells (approximately 0.3 cm^2 in area and 0.3 ml in capacity). Dilutions of suspensions of bacterial cells or culture supernatants were mixed with target cells and dispensed into the trays so that each well contained 10^4 [^{51}Cr]-labelled P815 cells. Each experiment also included wells containing target cells only. The trays were then covered and incubated at 37°C for 4 h in a CO_2 incubator (100% humidity, 5% CO_2 in air). Quadruplicate wells were assayed for ^{51}Cr release by removing 100 µl from each well using an automatic pipette. The total releasable ^{51}Cr, usually about 80% of the incorporated label was determined by mixing a 1 ml sample of ^{51}Cr-P815 cells with 4

drops of chloroform. The results were expressed as the percentage cytolysis which was defined by the equation:

$$\% \text{ cytolysis} = \frac{(\text{experimental } ^{51}\text{Cr release - S.R.}) \times 100}{(\text{total releasable } ^{51}\text{Cr - S.R.})}$$

where S.R. is the spontaneous ^{51}Cr release.

2.15 Liquid Haemolysis assay

Bacterial cultures at various stages of growth in LB were tested as follows: cells were collected by centrifugation (45 sec, Eppendorf 5414) and the supernatant transferred to a fresh tube and kept for analysis. The cells were resuspended in fresh Luria broth and subjected to sonication (60 x 1 second pulses, Branson Ultrasonifier) on ice. The cell contents, extracellular growth medium and whole bacterial cultures from the various stages of growth were subjected to analysis. These samples were mixed with an equal volume of 5% washed sheep red blood cells in PBS and incubated at 37°C. 300 μl was taken at each designated time point and spun in a microfuge for 45 sec to pellet the erythrocyte and bacterial cells. The supernatant (200 μl) was then removed and placed in a 96-well tissue culture tray (Flow Laboratories). This was subsequently diluted two-fold across the tray. Samples were read at 414nm on a ELISA reader (Titertek Multiscan, Flow Laboratories) to determine the amount of haemoglobin released. The total releasable haemoglobin was determined by the addition of 2 drops 10% SDS to a 1:1 mixture of 5% sheep erythrocytes and LB.

Table 2.1 : Vibrionaceae strains

Strain	Biotype/Serotype	Genotype/ Phenotype	Source
V.cholerae			
O17	El Tor, Ogawa	Sm^R, Hly^+	K.Bhaskaran
MAK757	El Tor, Ogawa	*leu*, *trp*, Hly^-	D.C.Deb
H-1	El Tor, Ogawa	Hly^-	D.Sharma
1621	El Tor, Ogawa	Hly^+	J.E.Ogg
Ph8	El Tor, Ogawa	Hly^+	D.Rowley
RV79	El Tor, Ogawa	*leu*, *trp*, Hly^+	J.Mekalanos
NSW14	El Tor, Ogawa	Hly^+	P.Desmarchelier
M791	El Tor, Ogawa	RV79 VcA1::*arg*	J.Mekalanos
26-3	El Tor, Ogawa	Hly^+	R.Finkelstein
3083	El Tor, Ogawa	Hly^+	R.Finkelstein
909	El Tor, Ogawa	*his-2*, Hly^+	J.E.Ogg
T51	El Tor, Ogawa	Hly^+	D.Rowley
1074-78	El Tor, Ogawa	Tox^-,Hly^+	S.Attridge
1196-78	El Tor, Ogawa	Tox^-, Hly^+	S.Attridge
N16961	El Tor, Inaba	Hly^+	J.Kaper
JBK70	El Tor, Inaba	*ctxA*, Hly^+	J.Kaper
Ph9	El Tor, Inaba	Hly^+	D.Rowley
B149	El Tor, Inaba	Hly^+	P.Desmarchelier
KB5	Classical, Ogawa	Hly^-	D.Rowley
RV69	Classical, Ogawa	P^+, *arg-1*, *his-1* *ilv-1*, Hly^-	C.Parker
029	Classical, Ogawa	Hly^-	D.Rowley
903	Classical, Ogawa	*met*, Hly^-	J.E.Ogg

Strain	Biotype/Serotype	Genotype/ Phenotype	Source
569B	Classical, Inaba	Sm^R, nonmotile, Hly^-	K.Bhaskaran
CA401	Classical, Inaba	Hly^-	C.Parker
569B-165	Classical, Inaba	Hly^-	D.Rowley
V.mimicus		Hly^+	D.Sharma
V.fluvialis		Hly^+	D.Sharma
V.parahaemolyticus 1256/85		Hly^+	IMVS, Adelaide
V.cholerae non-O1 #52		Hly^+	P.Desmarchelier
V.cholerae non-O1 #59		Hly^+	P.Desmarchelier
V.cholerae non-O1 #65		Hly^+	P.Desmarchelier
V.cholerae non-O1 #67		Hly^+	P.Desmarchelier
Pasturella multicida		Hly^+	IMVS, Adelaide
Aeromonas sobria A191		Hly^+	M.Atkinson
Aeromonas caviae A321		Hly^+	M.Atkinson
Aeromonas hydrophila AE-18		Hly^+	M.Atkinson

Table 2.2 : *Escherichia coli* K-12 strains

Strain	Genotype/Phenotype	Source
DH1	F$^-$, *gyrA*-96, *recA*-1, *relA*-1, *endA*-1, *thi*-1, *hsdR*-17, *supE*-44, lambda$^-$	B.Bachmann
E851	*tolA* mutant of DH1	T.Focareta
DS410	F$^-$, *minA*, *minB*, *rpsL*	D.Sherratt
JM101	F'[*traD*-36, *proA,B*, *lacI*^{q}, *lacZ*, ∆M15], *supE*, *thi*-1, ∆[*lac-proA,B*]	A.Sivaprasad
LE392	F$^-$, *supF*, *supE*, *hsdR*, *galK*, *trpR*, *metB*, *lacY*	L.Enquist
S17-1	RP4-2-Tc::Mu-Km::Tn7/ *pro*, *hsdR*	U.Priefer
SM10	RP4-2-Tc::Mu *thi*, *thr*, *leu*, *supE*	U.Priefer
GC1	F$^-$, *hsdR*	T.Meyer

Table 2.3 : Plasmids and cloning vehicles

Plasmid/Phage	Antibiotic Marker	Reference
pBR322	Ap, Tc	Bolivar *et al.* (1977)
pBR325	Ap, Tc, Cm	Prentki *et al.* (1981)
pGB2	Sp, Sm	Churchward *et al.* (1984)
pME305	Tc	Rella *et al.* (1985)
pPH1JI	Gm, Sp, Sm	Beringer *et al.* (1978)
pPM431	Tc	Manning *et al.* (1984)
pPM698	Tc, Cm	Manning *et al.* (1984)
pPM657	Tc, Cm	Manning *et al.* (1984)
pPM610	Tc, Cm	Manning *et al.* (1984)
pPM685	Tc, Cm	Manning *et al.* (1984)
pPM642	Tc, Cm	Manning *et al.* (1984)
pPM1287	Tc	T.Focareta (unpublished)
pRK290	Tc	Ditta *et al.* (1980)
pSUP201-1	Ap, Cm	Simon *et al.* (1983)
pSUP205	Cm, Tc	Simon *et al.* (1983)
J225	Ap, Km	J.Hackett (unpublished)
M13mp18		Messing & Vieira (1982)
M13mp19		Messing & Vieira (1982)

CHAPTER 3

CHARACTERIZATION OF THE *hly* LOCUS OF *V.cholerae* O1

3.1 Introduction

Strains of *V.cholerae* O1, the aetiological agent of cholera in man, can be subdivided into two biotypes : Classical and El Tor. Haemolytic activity due to the production of a soluble haemolysin commonly has been associated with the El Tor but not the Classical biotype. Furthermore, El Tor strains have been isolated which display a non-haemolytic phenotype (Barrett and Blake, 1981). Analysis of non-haemolytic strains of both biotypes by Southern hybridization demonstrates that DNA homologous to the El Tor haemolysin structural gene and surrounding region is present in isolates of both biotypes irrespective of haemolytic phenotype (Goldberg and Murphy, 1985; Brown and Manning, 1985).

The genetic locus determining production of the El Tor haemolysin has been cloned and a size of 80 kDa for the mature product of the structural gene, *hlyA*, has been determined in *E.coli* K-12 (Manning *et al.*, 1984; Goldberg and Murphy, 1984, 1985). Mecurio and Manning (1985) demonstrated that the protein remains in the periplasm in *E.coli* whereas the bulk of the haemolytic activity is extracellular in *V.cholerae*. However, Yamamoto *et al.* (1986) purified the extracellular product from *V.cholerae* and showed it to have a size of approximately 65 kDa, suggesting that a significant portion is cleaved from the protein upon release from *V.cholerae*. Whether this cleavage is necessary for the release of the haemolysin from *V.cholerae* is unknown, but it is clear that *E.coli* K-12 cannot export either this protein or several other

extracellular proteins of *V.cholerae* beyond the periplasm (Pearson and Mekalanos, 1982; Newland *et al.*, 1985; Focareta and Manning, 1987; Vimr *et al.*, 1988).

A detailed molecular and genetic analysis of the haemolysin is needed in order to understand the possible role that this protein may play in the pathogenic process of *V.cholerae*. This chapter describes characterization of both the haemolysin and the DNA that encodes this protein in order to gain insight into the production and export of HlyA. The corresponding region from a non-haemolytic Classical strain is also analysed.

3.2 Results

3.2.1 Classical strain 569B carries a *hlyA* mutation

Transformation of plasmid DNA in *V.cholerae* is not yet feasible due to the action of the deoxyribonucleases (Focareta and Manning, 1987). Thus, the only efficient method of introducing cloned DNA into *V.cholerae* is conjugal mobilization. The mobilization vectors described by Simon *et al.* (1983) possessed suitable restriction sites to enable recloning of the *hly* locus from plasmid pPM431 (Manning *et al.*, 1984). The 6.4 kb *Pst*I fragment encodes the structural gene, *hlyA*, for the haemolysin and two potential accessory genes, *hlyB* and *hlyC* (Manning *et al.*, 1984). This fragment was cloned into the *Pst*I site of vector pSUP201-1 to generate plasmid pPM2001 (Figure 3.1). In order to analyse the roles of the *hlyA* and *hlyB* genes independently, both genes, present on separate *Pst*I - *Eco*RI fragments were cloned into plasmid pSUP205 to generate plasmids pPM2004 (*hlyB*) and pPM2005 (*hlyA*) (Figure 3.2).

Plasmids pPM2001 and pPM2005 were introduced into the non-haemolytic Classical strain 569B. In both cases, the haemolysin produced by these strains was present in the extracellular medium (Figure 3.3). Strain 569B mimicked the El Tor strain O17 upon introduction of either of the plasmids harbouring the cloned *hlyA* gene. This implies that 569B possesses a structural gene defect and not an inability to secrete/excrete the protein since pPM2005 lacks the *hlyB* gene. This is in excellent agreement with results obtained by Goldberg and Murphy (1985) who

Figure 3.1 Construction of the mobilizable plasmid pPM2001.

Plasmids pPM431 and pSUP201-1 were digested with *Pst*I. The 6.4 kb *Pst*I fragment from pPM431 containing the *hly* locus was cloned into the *Pst*I site of pSUP201-1. The colonies were selected on Cm blood agar plates and one such clone was designated pPM2001.

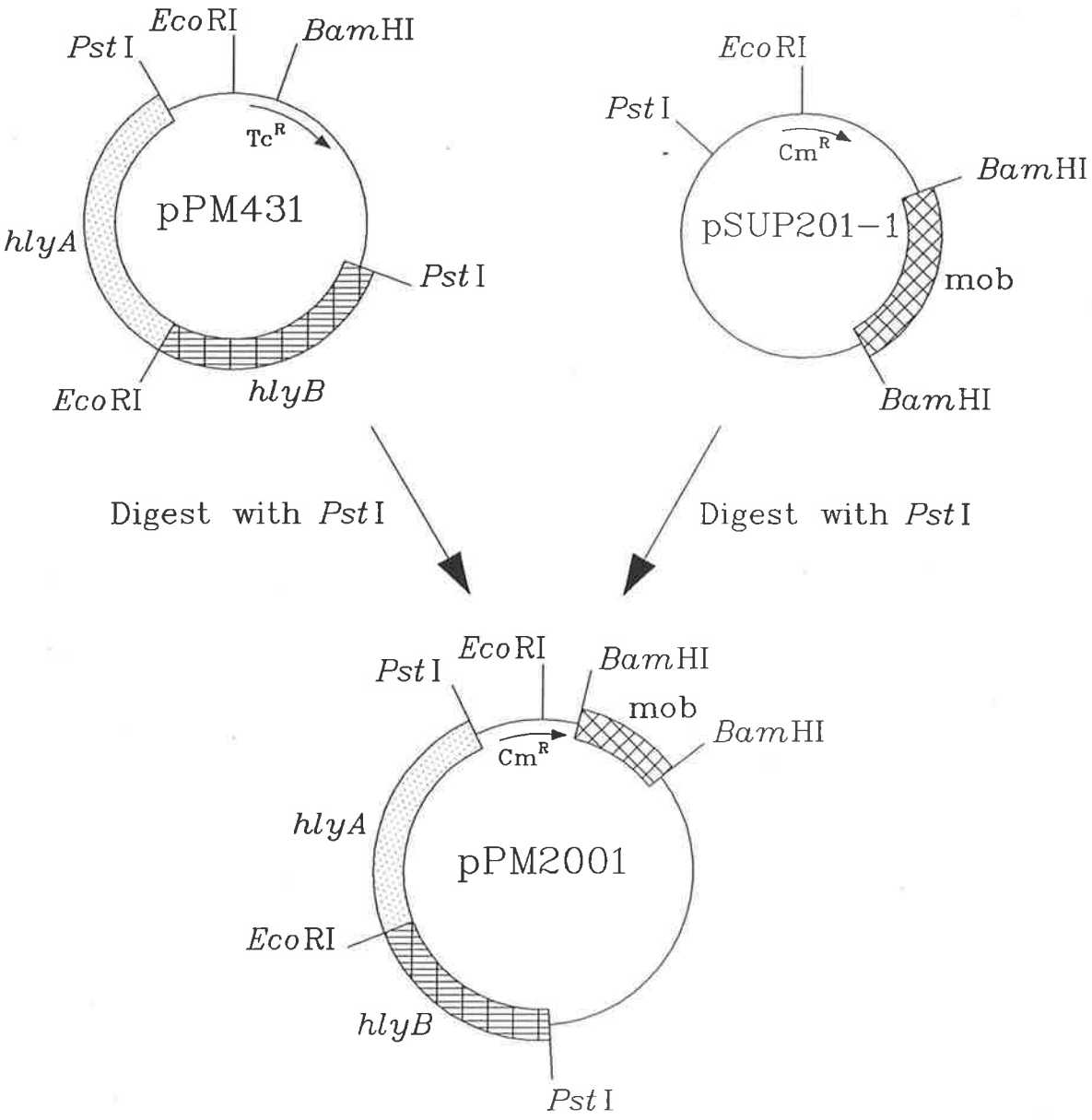

EcoRI
PstI
BamHI
TcR
pPM431
hlyA
PstI
EcoRI
hlyB
EcoRI
PstI
CmR
BamHI
pSUP201-1
mob
BamHI
Digest with PstI
Digest with PstI
PstI
EcoRI
BamHI
mob
BamHI
CmR
hlyA
pPM2001
EcoRI
hlyB
PstI

Figure 3.2 Construction of plasmids pPM2004 and pPM2005.

Plasmids pPM2001 and pSUP205 were digested with *Pst*I and *Eco*RI. Both the 3.5 kb *Pst*I - *Eco*RI fragment containing *hlyA* and the 2.9 kb *Eco*RI - *Pst*I fragment containing *hlyB* were cloned into pSUP205 and the representative plasmids were designated pPM2004 and pPM2005 respectively.

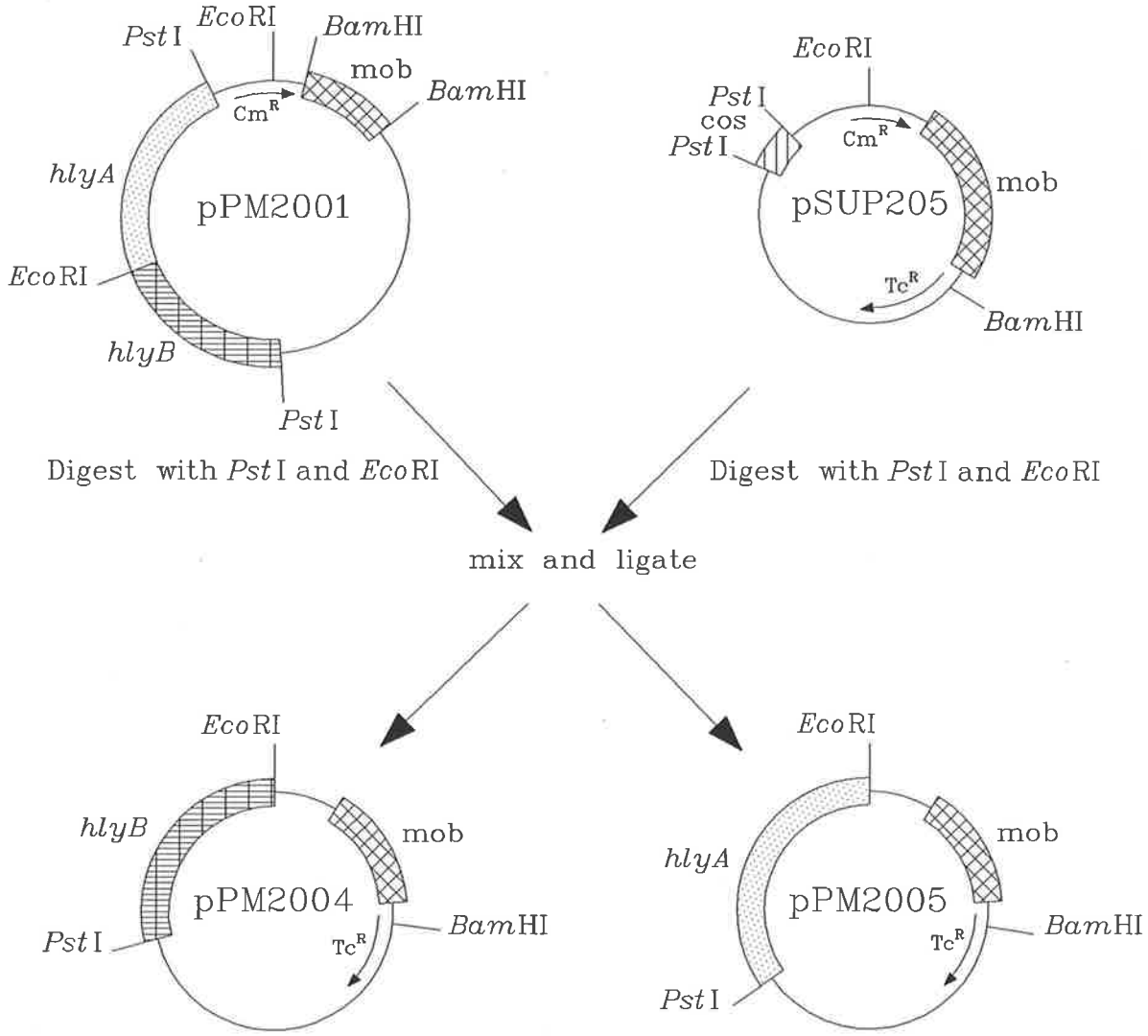
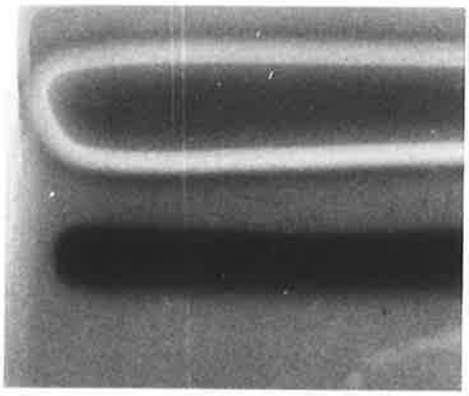

PstI
EcoRI
BamHI
mob
BamHI
Cm^R
hlyA
pPM2001
EcoRI
hlyB
PstI
Digest with PstI and EcoRI
EcoRI
PstI
cos
PstI
Cm^R
mob
pSUP205
Tc^R
BamHI
Digest with PstI and EcoRI
mix and ligate
EcoRI
hlyB
mob
pPM2004
PstI
Tc^R
BamHI
EcoRI
hlyA
mob
pPM2005
Tc^R
BamHI
PstI

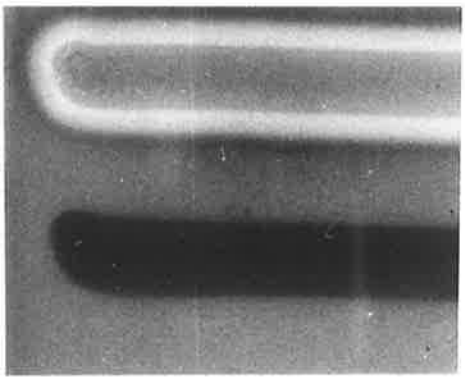
Figure 3.3 Haemolytic activity of *V.cholerae* strains.

The haemolytic activity of the following strains was detected by growth on nutrient agar containing 5% sheep erythrocytes. The strains are : O17 (El Tor, Hly^+); 569B (Classical, Hly^-); 569B + pPM2001; 569B + pSUP201-1; 569B + pPM2005. With the introduction of the structural gene alone (pPM2005), the non-haemolytic Classical strain 569B mimics the parental El Tor strain O17.



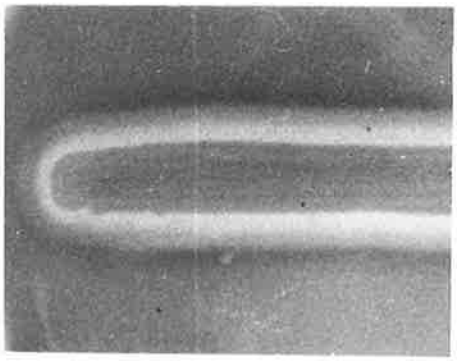
017

569B



569B + pPM2001

569B + pSUP201-1



569B + pPM2005

demonstrated slight differences in the restriction enzyme pattern between the *hlyA* genes from the two biotypes of *V.cholerae*.

Colonies of the Classical strain 569B harbouring pPM2001 were screened on blood agar for a non-reciprocal recombination event in which the defective chromosomal gene was recombined into the plasmid, but the *hlyA* gene from the plasmid did not recombine back into the 569B chromosome, resulting in a non-haemolytic phenotype (Figure 3.4). Plasmid pPM2002 which was derived from such an event (Figure 3.4) was isolated and its restriction pattern compared to pPM2001. No noticeable difference was detected between plasmid pPM2001 and pPM2002 when using restriction enzymes that have a 6 bp recognition sequence (Figure 3.5; panel A). However, plasmid pPM2002 has an identical *Sau*3A and *Taq*I restriction pattern to the cloned defective *hlyA* gene of 569B (Goldberg and Murphy, 1985) and consequently contains the structural defect present in 569B (Figure 3.5; panel B).

Plasmids pPM2001, pPM2002 and the vector pSUP201-1 were transformed into the *E.coli* K-12 minicell producing strain DS410, and the plasmid encoded proteins analysed. Plasmid pPM2002 differs from the parent pPM2001 in that it does not lead to the production of the 80 kDa HlyA protein (Figure 3.6). However, a truncated protein product of 27 kDa, designated HlyA* can be detected (Figure 3.6). This is further supported by results obtained when the plasmids were introduced into 569B and whole cell samples subjected to Western blot analysis (Figure 3.7). However, although both the 80 kDa and 65 kDa forms of HlyA were detected with the antiserum to the 65 kDa form of HlyA in 569B[pPM2001], HlyA* was not seen in 569B[pPM2002].

It can therefore be concluded that the Classical strain 569B does not possess a defect in excretion, but a structural gene defect that results in a non-haemolytic phenotype and the production of a 27 kDa truncated protein. These data also suggest that the region cleaved from the 80 kDa form of HlyA to produce the 65 kDa form is at the N-terminus.

Figure 3.4 Non-reciprocal recombination of the *hly* locus in 569B harbouring plasmid pPM2001.

Colonies of Classical strain 569B harbouring plasmid pPM2001 were patched onto Cm blood agar plates. The majority of the colonies now displayed a haemolytic phenotype. In one of these colonies a non-reciprocal recombination event has resulted in the defective chromosomal structural gene replacing the *hlyA*$^+$ gene on the plasmid, but the plasmid *hlyA*$^+$ gene has not recombined into the 569B chromosome. This produces a non-haemolytic colony as indicated by the arrow.

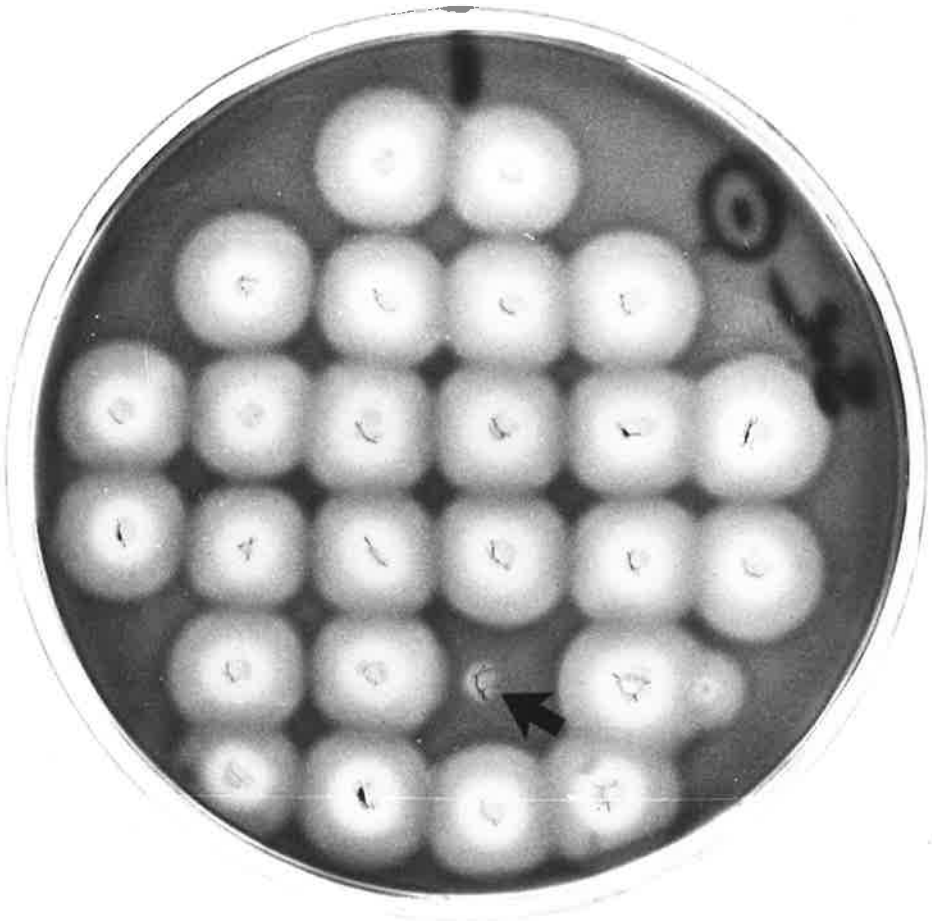


Figure 3.5 Analysis of the *hlyA* genes from strains of the Classical and El Tor biotypes by restriction endonuclease digestion.

The 3.5 kb *Pst*I - *Eco*RI fragment encoding *hlyA* from plasmids pPM2001 and pPM2002 were cloned into vector pBR322 to generate plasmids pPM2011 and pPM2012 respectively.

Panel A illustrates plasmids pPM2011 (tracks A) and pPM2012 (tracks B) after digestion with a variety of restriction enzymes that have a 6 bp recognition sequence but no difference could be detected. SPP1 phage DNA digested with *Eco*RI was used as a standard and the size of the fragments generated in kb are shown on the right.

Panel B illustrates pPM2011 and pPM2012 digested with *Sau*3A. The only fragments which differ are indicated by arrows and suggest that a small deletion has occurred in the Classical strain. Plasmid pBR322 digested with *Hae*III was used as a standard and the fragment sizes are shown on the right.

A
SPP1 (EcoRI)
8.37
7.20
6.05
4.90
3.55
2.63
1.73
1.61
1.29
1.19
0.99
0.86
0.63
A B A B A B A B A B A B A B A B
] PstI
] PstI + EcoRI
] MluI
] PstI + MluI
] EcoRI + MluI
] HindIII
] HpaI
] HindII

B

pPM2011(*Sau*3A)
pPM2012(*Sau*3A)
pBR322(*Hae* III)
104
184
267
434
587

Figure 3.6 Analysis of plasmid encoded proteins.

Plasmid encoded proteins were analysed using the *E.coli* minicell producing strain DS410. Minicells harbouring the plasmids were purified on sucrose gradients, labelled with [^{35}S]-methionine and solubilized in SDS sample buffer. The plasmid encoded proteins were visualized by autoradiography after electrophoretic separation in SDS on an 11-20% polyacrylamide gradient gel. The HlyA, HlyB and chloramphenicol acetyltransferase (Cat) proteins are indicated. The HlyC protein described by Manning *et al.* (1984) present in DS410[pPM431] is also indicated. The truncated *hlyA* product, HlyA*, in pPM2002 is indicated by an arrow.

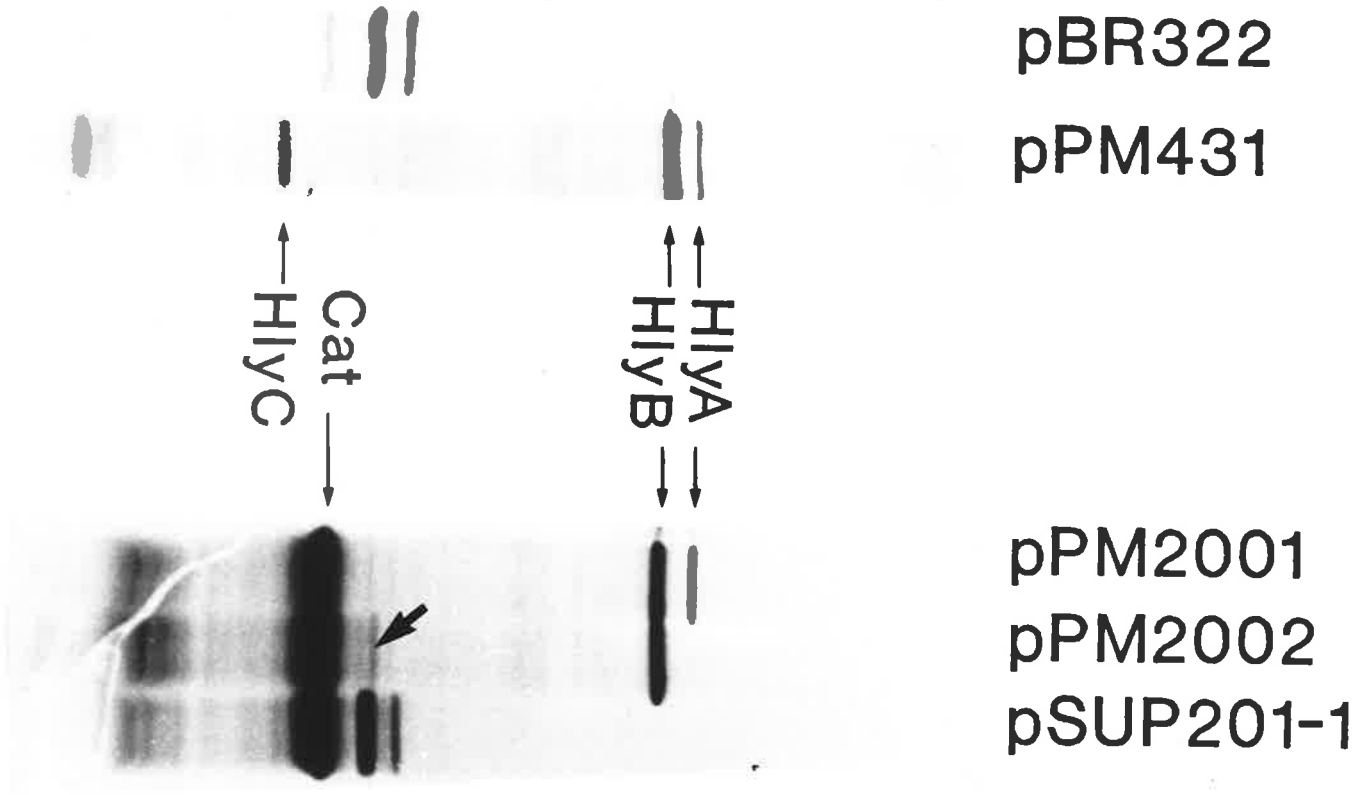

pBR322
pPM431
HlyC
Cat
HlyB
HlyA
pPM2001
pPM2002
pSUP201-1

Figure 3.7 Western blot analysis of Classical strain 569B carrying plasmids.

Whole cell preparations of 569B carrying the plasmids pPM2001, pPM2002 and pSUP201-1 in 1x sample buffer were separated on a 15% polyacrylamide gel. The proteins were then transferred to nitrocellulose. The antiserum used was raised against the purified 65 kDa form of HlyA. It can be seen that 569B[pPM2001] produces both the 80 kDa and 65 kDa forms of HlyA. In contrast, 569B[pPM2002] and 569B[pSUP201-1] do not produce either form of HlyA.

80 kDa
65 kDa
569B[pPM2001]
569B[pPM2002]
569B[pSUP201-1]

3.2.2 HlyA is exported as an 80 kDa protein

As mentioned previously, the extracellular form of HlyA in *V.cholerae* has been purified and shown to be approximately 65 kDa in size (Yamamoto *et al.*, 1986). However, when the cloned *hlyA* gene is expressed in *E.coli* K-12, an 80 kDa form remains localized in the periplasmic space (Mecurio and Manning, 1985). This can be visualized on blood agar by comparing a wild type *E.coli* and a *tolA* mutant which has a leaky outer membrane and releases periplasmic proteins (Bernstein *et al.*, 1972; Anderson *et al.*, 1979). Upon introduction of both pPM2001 and pPM2005 into the wild type and *tolA* mutant strains of *E.coli*, a marked difference in the zones of haemolysis could be detected (Figure 3.8). Using antiserum raised against the purified 65 kDa extracellular form of HlyA in Western blot analysis only the 65 kDa form of HlyA could be detected in the *V.cholerae* culture supernatant, whereas the periplasmic fraction of *E.coli* DH1[pPM431] contains both forms of HlyA (Figure 3.9). Cell fractions of *E.coli* K-12 harbouring pBR322 and pPM431 and *V.cholerae* strains 569B and O17 were examined on blood agar plates for haemolytic activity (Figure 3.10). After taking into account the relative concentrations of the fractions and the distribution of the 65 kDa and 80 kDa forms of HlyA, it is not possible to say whether both forms are haemolytically active or only the 65 kDa form after cleavage has occurred. This is compounded by the instability of the 80 kDa protein which rapidly breaks down into the 65 kDa form and also smaller proteolytic products (see section 3.2.3).

Culture supernatants of the *V.cholerae* strain O17 in exponential phase growth were precipitated with trichloroacetic acid (TCA) and analysed by SDS-PAGE and Western blotting. The culture supernatant of *V.cholerae* O17 contained both the 80 kDa and 65 kDa form of HlyA (Figure 3.11). Analysis of the periplasm of *E.coli*[pPM431] clearly demonstrated the degradation of HlyA into a number of smaller products which could still react with the anti-65 kDa antiserum.

Cell fractionation of *E.coli* SM10[pPM2005] and *V.cholerae* O17[pPM2005] localized the 80 kDa haemolysin in the periplasm of *E.coli* and extracellularly in *V.cholerae* (Figure 3.12). A marked increase in amounts of HlyA

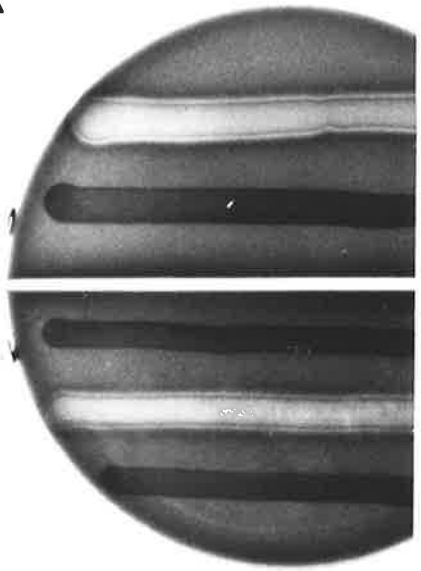
Figure 3.8 Effect of a *tolA* mutation in *E.coli* on the expression of haemolysin.

Haemolysis on blood agar containing 5% sheep erythrocytes of *E.coli* strain DH1 (panel A) and its *tolA* mutant, E851 (panel B) harbouring the plasmids pPM2001, pPM2004, pPM2005, pSUP201-1 and pSUP205.

Panel A: In DH1, the HlyA protein remains trapped within the periplasmic space and is only released upon cell lysis and consequently only small zones of haemolysis are seen with plasmids pPM2001 and pPM2005. As expected, the *hlyB* clone alone (pPM2004) and the two vectors produced no haemolysis.

Panel B: In E851, the *tolA* mutation results in a leaky outer membrane, thus allowing release of periplasmic proteins. The zones of haemolysis caused by E851 harbouring plasmids pPM2001 and pPM2005 are considerably larger.

A



DH1[pPM2001]

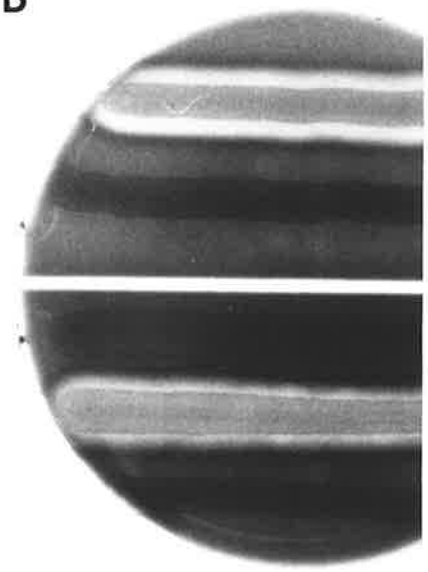
DH1[pSUP201-1]

DH1[pPM2004]

DH1[pPM2005]

DH1[pSUP205]

B



E851[pPM2001]

E851[pSUP201-1]

E851[pPM2004]

E851[pPM2005]

E851[pSUP205]

Figure 3.9 Western blot analysis of *V.cholerae* O17 culture supernatant and *E.coli* DH1[pPM431] periplasm.

The periplasmic fraction of *E.coli* DH1[pPM431] and the culture supernatant of *V.cholerae* O17 were analysed by SDS-PAGE followed by Western blotting with antiserum to the purified 65 kDa form of HlyA. The 80 kDa form of HlyA in the *E.coli* periplasm is indicated with an arrow whereas the smaller 65 kDa form present in the *V.cholerae* supernatant is shown by an arrowhead. The presence of proteolytically digested epitopes of HlyA in the periplasm of DH1[pPM431] that are still immunoreactive can also be seen.


DH1[pPM431] periplasm
O17 supernatant

Figure 3.10 Haemolytic activity of cell fractions.

Periplasmic, membrane and cytoplasmic fractions as well as culture supernatants of the strains DH1[pBR322], DH1[pPM431], O17 and 569B were added to preformed wells in nutrient agar containing 5% sheep erythrocytes and incubated overnight at 37°C. Both the periplasmic fraction of DH1[pPM431] and the culture supernatant of O17 displayed marked haemolytic activity. Traces of haemolytic activity were detected in the other fractions. No activity was seen with any fractions of the strains DH1[pBR322] and 569B.

DH1[pBR322]
DH1[pPM431]
O17
569B
supernatant
periplasm
membrane
cytoplasm

Figure 3.11 Analysis of cell fractions of *E.coli* and *V.cholerae*.

Periplasmic fractions of *E.coli* K-12 DH1 (lane 2) and DH1[pPM431] (lane 1) and the culture supernatant of *V.cholerae* strain O17 after TCA precipitation (lane 3) were analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (panel A) or Western blotting with antiserum to the purified 65 kDa form of HlyA (panel B). The 80 kDa form of HlyA in the *E.coli* periplasm is indicated by the arrows, and the marked proteolytic degradation within the periplasmic fraction can also be seen. The *V.cholerae* supernatant contains both the 80 kDa and 65 kDa form of HlyA.

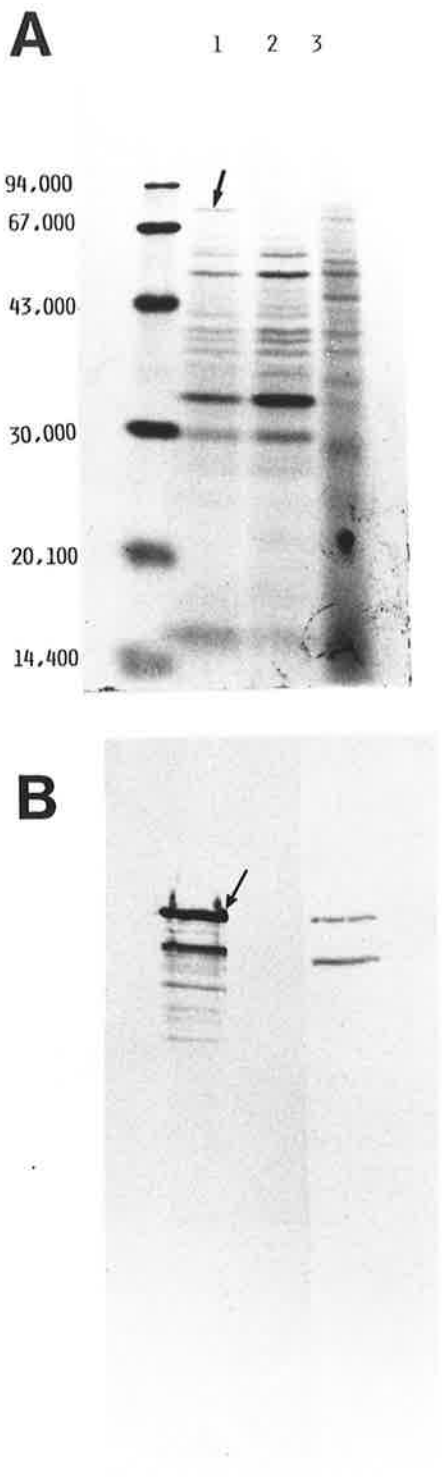

A
1 2 3
94,000
67,000
43,000
30,000
20,100
14,400
B

Figure 3.12 Localization of HlyA in *E.coli* and *V.cholerae*.

Analysis of *E.coli* SM10[pPM2005] and *V.cholerae* O17[pPM2005] cell fractions. Supernatant (s), periplasmic (p), membrane (m) and cytoplasmic (c) fractions were analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. The culture supernatants were precipitated with TCA and resuspended in sample buffer prior to loading. The 80 kDa form of HlyA is indicated by an arrow and is present in the periplasm of *E.coli* and the supernatant of *V.cholerae* but is not visualized in any other fractions.

spmc
SM10[pPM2005]
spmc
O17 [pPM2005]
HlyA

protein is seen when plasmid pPM2005 is present in strain O17 indicative of a gene dosage effect due to the gene being present on a high copy number plasmid. This can be more easily seen in Figure 3.13 which shows the relative amounts of HlyA protein in the supernatants of O17 and O17[pPM2005] and the periplasm of SM10[pPM2005].

3.2.3 Purification of the HlyA protein

The procedure reported by Yamamoto *et al.* (1986) for the purification of the HlyA protein involves ammonium sulphate precipitation followed by dialysis. Following this procedure, Yamamoto and co-workers purified the 65 kDa form but not the 80 kDa HlyA protein. The differences in size were originally attributed to excretion in *V.cholerae* but analysis of the effects following ammonium sulphate purification and dialysis help explain this result.

Periplasmic fractions of V218 (*E.coli* K-12[pPM431]) were saturated to various levels with ammonium sulphate, the precipitate collected and the ammonium sulphate removed by extensive dialysis. The samples were then analysed by SDS-PAGE. Most of the HlyA protein, both 80 kDa and 65 kDa forms, was precipitated by 40% ammonium sulphate saturation with very little remaining in the supernatant (Figure 3.14).

As mentioned previously, if culture supernatants of exponentially growing *V.cholerae* O17[pPM2005] are precipitated with TCA, then the 65 kDa form is not visualized by staining with Coomassie Brilliant Blue (Figure 3.15) whereas the 80 kDa form is seen instead. However, if the HlyA protein is concentrated by ammonium sulphate rather than TCA, marked cleavage into the 65 kDa form is seen (Figure 3.15). This cleavage appears to be very specific and a peptide (a) probably corresponding to the smaller of the products (Figure 3.15) with a size of 15 kDa is also evident. Yamamoto *et al.* (1986) used 60% ammonium sulphate saturation to concentrate the 65 kDa protein from *V.cholerae* culture supernatants. Thus, it is clear why these workers purified a 65 kDa form since virtually all of the 80 kDa HlyA protein had been cleaved to the 65 kDa form (Figure 3.15). The amount of ammonium sulphate needed to

Figure 3.13 Gene dosage effect of plasmid pPM2005.

Periplasmic fractions of *E.coli* strains SM10[pSUP205] and SM10[pPM2005] and TCA precipitated culture supernatants of *V.cholerae* strains O17, O17[pPM2005] and 569B were analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. The 80 kDa form of the haemolysin is indicated by an arrow. The presence of plasmid pPM2005 in strain O17 markedly increases the amount of HlyA seen in the supernatant, even when compared to the periplasmic fraction of *E.coli* strain SM10 carrying the same plasmid.

SM10[pSUP205] periplasm
SM10[pPM2005] periplasm
O17[pPM2005] supernatant
O17 supernatant
569B supernatant
HlyA

Figure 3.14 Ammonium sulphate precipitation of HlyA.

Periplasmic fractions of V218 (*E.coli* K-12[pPM431]) were saturated to 30%, 40%, 50%, 60% and 70% with ammonium sulphate. The precipitate was resuspended in sample buffer whereas the supernatant was dialyzed against water and then mixed with equal volumes of 2x sample buffer. Samples were analysed by SDS-PAGE followed by staining with Coomassie Blue. Most of both forms of the HlyA protein was precipitated by 40% ammonium sulphate and are indicated by arrows.

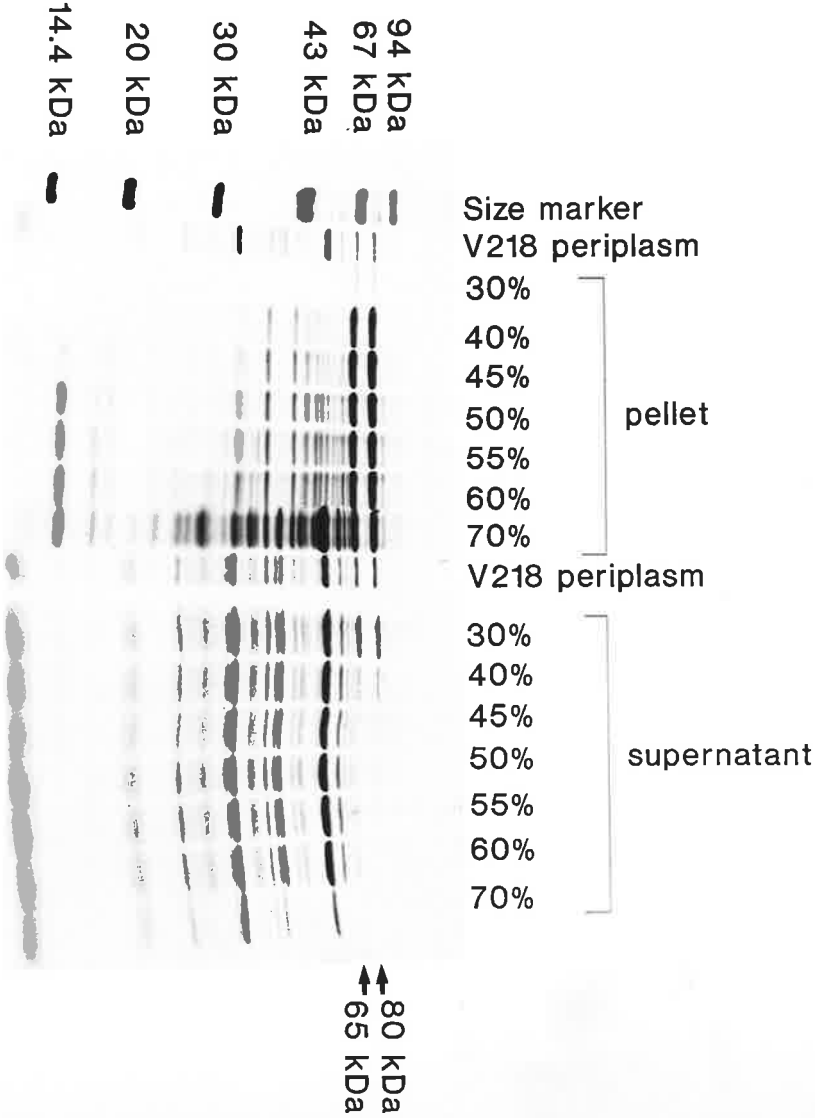

14.4 kDa
20 kDa
30 kDa
43 kDa
94 kDa
67 kDa
Size marker
V218 periplasm
30%
40%
45%
50%
55%
60%
70%
pellet
V218 periplasm
30%
40%
45%
50%
55%
60%
70%
supernatant
80 kDa
65 kDa

Figure 3.15 Analysis of *V.cholerae* culture supernatants.

Culture supernatants of *V.cholerae* strain O17[pPM2005] were analysed by SDS-PAGE for HlyA following precipitation with TCA or with 40%, 50% or 60% ammonium sulphate followed by dialysis. The cleavage products of HlyA ('a' and 'b') are indicated by arrows.

O17[pPM2005] - TCA
O17[pPM2005] - 40% Amm.SO4
O17[pPM2005] - 50% Amm.SO4
O17[pPM2005] - 60% Amm.SO4
O17[pPM2005] - TCA
HlyA
b
a

precipitate HlyA differs in *E.coli* and *V.cholerae* probably due to the difference in ionic environments between the culture supernatant and the periplasm.

Yamamoto and co-workers (1986) reported the presence of several immunoreactive bands smaller than 60 kDa and proposed that they were proteolytically digested epitopes of the haemolysin. Attempted purification of the 80 kDa HlyA protein from the periplasmic fraction of *E.coli* K-12[pPM431] by ion exchange resulted in significant degradation into many proteolytic products that were immunoreactive with the anti-65 kDa antiserum (Figure 3.16). The cleavage of the 80 kDa HlyA protein is not inhibited by the protease inhibitor phenyl-methyl-sulphonyl fluoride (PMSF) or EGTA.

3.2.4 Protease activity of HlyA

The marked degradation seen in HlyA during purification suggested either that a protease was being co-purified, or that HlyA possessed endogenous protease activity. In addition, the more extensive the purification of HlyA, the more degradation that was observed. Examination of *E.coli* DH1[pPM431] and DH1[pBR322] and *V.cholerae* O17 and its *hlyA*::Km^R mutant (Section 5.2.1) on casein agar revealed no difference in protease activity attributable to HlyA. Thus, if HlyA does have proteolytic activity it is clearly specific, also suggested by the sizes of the cleavage products.

3.2.5 Iron regulation of HlyA

The ability to establish and maintain infections often depends on the ability of the bacteria to acquire essential iron. It has been well documented that many bacteria including *E.coli*, *N.gonorrhoeae* and *V.cholerae* possess high-affinity iron transport systems. Growth *in vitro* under low-iron conditions leads to the expression of iron-regulated proteins such as iron-specific chelators or sideophores and membrane associated receptors (McIntosh and Earhart, 1976; Norquist *et al.*, 1978; Neilands, 1982; Sigel and Payne, 1982; Sciortino and Finkelstein, 1983; Braun, 1985). In *E.coli* this increase is mediated by the product of the *fur* locus (Hantke, 1982). The haem-

Figure 3.16 Proteolytic degradation of HlyA.

Ion exchange fractions generated during purification of the 80 kDa HlyA protein from the periplasm of *E.coli* K-12[pPM431] were analysed by Coomassie Blue staining (panel A) or Western blotting (panel B) with anti-65 kDa antiserum. The 80 kDa form is indicated by an arrow, but the large number of proteolytic fragments that are still immunoreactive are evident.

B
40% pellet
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
A
Size marker
40% pellet
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
94 kDa
67 kDa
43 kDa
30 kDa
20 kDa

bound iron present in erythrocytes represents an excellent, additional iron source for microbial pathogens. Thus, the haemolysin may play an important role in virulence by increasing the availability of iron in the host where iron is restricted.

To investigate whether the El Tor haemolysin is regulated by iron, the haemolytic activity of culture supernatants grown in iron-starved conditions were analysed. Overnight cultures grown in LB were subcultured into LB containing 2'-2'-dipyridyl and grown to an OD_{650} of 0.6, which corresponds to a viable count of approximately 2 x 10^8 organisms. The bacterial cells were removed by centrifugation and supernatants incubated with 5% sheep erythrocytes. The haemolytic activities are shown in Figure 3.17. It is clear that restricting the iron availability in the medium markedly increases the haemolytic activity of the bacterial strain.

3.2.6 Homology of *hlyA* with other Vibrionaceae

Most *V.cholerae* strains of the non-O1 serotype produce a large amount of a haemolysin. It has been demonstrated by Yamamoto *et al.* (1984, 1986) that this non-O1 haemolysin resembles that of El Tor strains. These workers showed both proteins to be approximately 60 kDa in size. Furthermore, no biological, physiochemical or immunological differences could be identified between the two purified haemolysins (Yamamoto *et al.*, 1986). In addition, when the DNA that encodes the El Tor haemolysin was used as a radiolabelled probe, strong homology was seen within the genomic DNA of haemolytic non-O1 strains of *V.cholerae* (Brown and Manning, 1985). In order to ascertain whether DNA homology existed throughout the Vibrionaceae family, the *hlyA* gene present on the 3.5 kb *Pst*I - *Eco*RI fragment was used to probe genomic DNA from a variety of species.

Southern hybridization analysis revealed that the *V.cholerae* O1 strains O17 (El Tor, Hly^+), 569B (Classical, Hly^-) and MAK757 (El Tor, Hly^-) all contained DNA fragments of the same size which displayed strong homology with the *hlyA* probe (Figure 3.18). However, in the non-O1 *V.cholerae* strain #65, the probe hybridized to a larger DNA fragment of 5.8 kb (Figure 3.18). *V.mimicus* DNA possesses a fragment of the same size as the O1 strains, but the intensity of binding of the probe is less

Figure 3.17 The effect of iron limitation on haemolytic activity of *V.cholerae*.

Culture supernatants of *V.cholerae* strain O17 were grown to mid exponential phase in either LB or LB + 0.2 mM 2'-2'-dipyridyl and mixed with 5% sheep erythrocytes at 37°C. In the presence of the iron chelator, the rate and amount of haemolysis is dramatically increased.

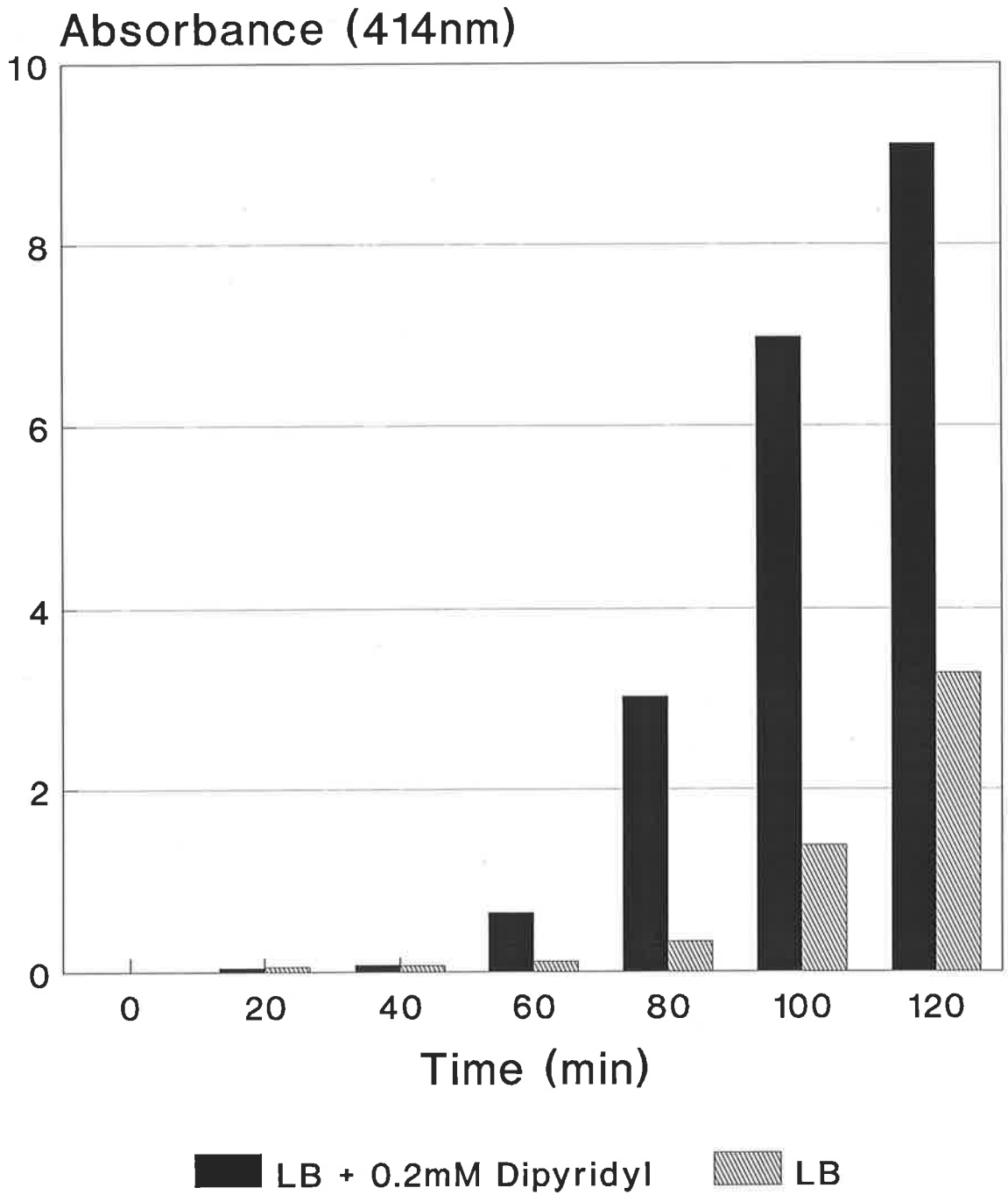
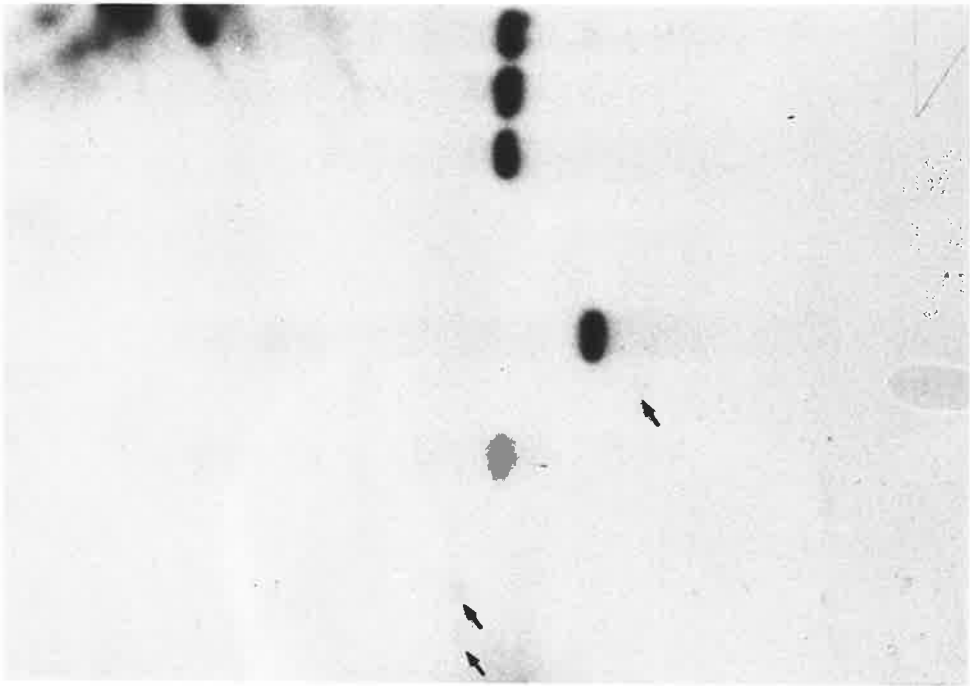

Absorbance (414nm)
10
8
6
4
2
0
0
20
40
60
80
100
120
Time (min)
LB + 0.2mM Dipyridyl
LB

Figure 3.18 Homology of *hlyA* with other Vibrionaceae

The 3.5 kb *Pst*I - *Eco*RI fragment that encodes HlyA contained within vector pGB2 represents plasmid pPM2008. This plasmid was radioactively labelled and used to probe genomic DNA from a variety of Vibrionaceae strains. Strong homology is seen with the *V.cholerae* O1 strains of both biotypes and with *V.cholerae* non-O1 #65. Some homology is detected with *V.mimicus*. Furthermore, slight homology can be seen with *V.fluvialis* and two *Aeromonas* isolates A321 and A191 (shown by arrows) at the stringency level used (Section 2.10.9). No significant homology can be detected with the other Vibrionaceae.



V.cholerae O17

V.cholerae 569B

V.cholerae MAK757

V.parahaemolyticus 1256/85

Pasturella multicida

V.cholerae non-O1 #65

V.fluvialis

V.mimicus

Aeromonas hydrophila AE-18

Aeromonas caviae A321

Aeromonas sobria A191

*Pst*I + *Eco*RI

suggesting less homology. *V.fluvialis* DNA also possesses a fragment of size 6.5 kb that displays very weak hybridization to the probe under the stringency level used (Figure 3.18). Slight homology was also detected with a 3 kb fragment in Aeromonas strains *A.sobria* A191 and *A.caviae* A321. No homology could be detected with *A.hydrophila* AE-18, *V.parahaemolyticus* 1256 or *Pasturella multicida*.

3.3 Summary and Conclusions

By subcloning the *hlyA* and *hlyB* genes separately into mobilizable vectors and reintroducing them back into the non-haemolytic Classical strain 569B, it was possible to demonstrate that 569B does not contain a defect in the excretion of HlyA but rather in the structural gene itself. This confirmed the findings of Goldberg and Murphy (1985) which implied that 569B possessed a small deletion in *hlyA*. Plasmid pPM2002 was shown to represent the structural defect in 569B and its restriction pattern was identical to that seen with an independently isolated clone (Goldberg and Murphy, 1985). Furthermore, pPM2002 was shown by minicell and Western blot analysis not to produce the mature 80 kDa HlyA protein but revealed the presence of a truncated product of 27 kDa, designated HlyA*.

Purified haemolysin from *V.cholerae* was 65 kDa (Yamamoto *et al.*, 1986) whereas the form trapped in the *E.coli* periplasm was 80 kDa. However, by concentrating *V.cholerae* culture supernatants by TCA precipitation rather than with ammonium sulphate, it is possible to visualize the 80 kDa form in the culture supernatant. Western blot analysis enabled both the 80 kDa and the 65 kDa forms to be seen. Therefore, it would appear that in *V.cholerae*, the protein is exported out of the cell as an 80 kDa protein and is cleaved extracellularly initially to give two fragments 'a' and 'b' (Figure 3.15). Together with previous analyses (Manning *et al.*, 1984, Yamamoto *et al.*, 1986) it is predicted that the 'a' fragment is derived from the amino terminus. Truncated proteins which are produced in normal amounts but have lost less than 10 kDa from the carboxy terminus as a result of Tn*1725* insertion into the gene are haemolytically inactive. Additionally, the haemolysin purified by Yamamoto *et al.* (1986) that is 'b' has lost approximately 15 kDa but is still haemolytically active.

Thus it can be concluded that the active site of the protein necessary for haemolysis is found in the C-terminal portion.

The marked proteolytic degradation seen with HlyA may occur for two reasons. Firstly, the HlyA protein may possess some endogenous protease activity and either cleave other HlyA molecules or be autoproteolytic. Secondly, it may be that during the dialysis following ammonium sulphate precipitation, a protease that co-precipitates with the haemolysin can efficiently cleave the HlyA molecule. This seems unlikely, as *E.coli* K-12[pPM431] periplasmic fractions show marked degradation where ammonium sulphate was not used. However, it is obvious that the 80 kDa HlyA protein is extremely unstable.

Haemolytic activity could be greatly enhanced by starving the bacterium of iron by the introduction into the growth medium of the iron-chelator 2'-2'-dipyridyl. This increased lytic activity on erythrocytes could release haem-bound iron and thus the haemolysin may play an important role in the virulence of *V.cholerae*. The DNA encoding HlyA is highly conserved throughout both O1 and non-O1 serotypes of *V.cholerae*. *V.mimicus*, *V.fluvialis* and two Aeromonas strains showed slight homology with *hlyA*.

CHAPTER 4

GENETIC ORGANIZATION OF THE *hlyA* GENE FROM CLASSICAL AND EL TOR STRAINS OF *Vibrio cholerae*

4.1 Introduction

The nucleotide sequence of very few *V.cholerae* genes have been determined. These include the genes that encode the following proteins: cholera enterotoxin (CT) (Lockman and Kaper, 1983; Mekalanos *et al.*, 1983; Lockman *et al.*, 1984); an extracellular DNase (Focareta and Manning, 1987); a major outer membrane protein, OmpV (Pohlner *et al.*, 1986); ToxR and ToxS, responsible for the co-ordinate regulation of a number of virulence determinants (Miller and Mekalanos, 1984; Miller *et al.*, 1987; Miller *et al.*, 1989); TcpA, the major pilin for the Tcp pilus (Faast *et al.*, 1989) and a group of genes involved in the production of the mannose-fucose resistant haemagglutinin (Van Dongen *et al.*, 1987; Franzon *et al.*, 1989). The analysis of genes at their primary level, the nucleotide sequence provides an insight into the expression and regulation of these genes and simplifies protein sequence determination.

V.cholerae O1 strains of both Classical and El Tor biotypes contain DNA that is essentially homologous to the cloned *hly* locus (Goldberg and Murphy, 1984; Manning *et al.*, 1984; Brown and Manning, 1985). Analysis of the DNA within the vicinity of the structural gene, *hlyA*, using restriction endonucleases with a 4 bp recognition sequence, allowed Goldberg and Murphy (1985) to detect a minor change between the biotypes.

In this chapter, the nucleotide sequence and genetic organization within the 3.5 kb *Pst*I - *Eco*RI DNA fragment containing *hlyA* from both a haemolytic El Tor and a non-haemolytic Classical strain is examined. The gene and the potential regulatory regions that surround *hlyA* are analyzed.

4.2 Results

4.2.1 Generation of overlapping fragments for sequencing

Manning *et al.* (1984) localized the structural gene for the haemolysin, *hlyA*, by transposon and deletion analysis of plasmid pPM431 to a 3.5 kb *Pst*I - *Eco*RI fragment. This fragment was cloned into the multiple cloning site polylinker of both M13mp18 and M13mp19 (Vieira and Messing, 1982). Sequential overlapping deletions were made within this fragment by employing the exonuclease activity of T4 DNA polymerase and varying the incubation times with this enzyme prior to heat inactivation (Section 2.12.4). Deletions were made in both M13mp18 and M13mp19 so that the gene could be sequenced by the dideoxy sequencing method in both directions using the universal primer. Unfortunately, the deletions did not always overlap and so synthetic oligodeoxynucleotides were employed to sequence across these regions. A map of the sequencing strategy is shown in Figure 4.1.

4.2.2 Nucleotide sequence determination and analysis of *hlyA* from El Tor strain O17

The entire nucleotide sequence of the 3533 bp *Pst*I - *Eco*RI fragment was determined from both strands by the chain termination method (Figure 4.1) and is shown in Figure 4.2.

4.2.2.1 Nucleotide sequence of *hlyA*

Analysis of the determined sequence revealed the presence of an open reading frame (ORF) beginning at position 1141 after the *Pst*I site, and terminating at nt 3361, 170 bases before the *Eco*RI site. This location of the ORF is in excellent agreement with that proposed by Manning *et al.* (1984) for the *hlyA* gene. This ORF

Figure 4.1 Strategy used for dideoxy sequencing of *hlyA*.

Overlapping deletions were made in the 3.5 kb *Pst*I - *Eco*RI fragment cloned into both M13mp18 and M13mp19. The fragment was digested from opposite ends in both vectors with T4 DNA polymerase, and universal primer used to generate the nucleotide sequence. The M13mp18 clones are shown in blue while the M13mp19 clones are diaplayed in red. The 3.5 kb fragment was also sequenced with the -35 primer to accurately determine the extreme ends at the junction of the polylinker. The regions indicated with an asterisk were generated using a specifically synthesized oligodeoxynucleotide primer.

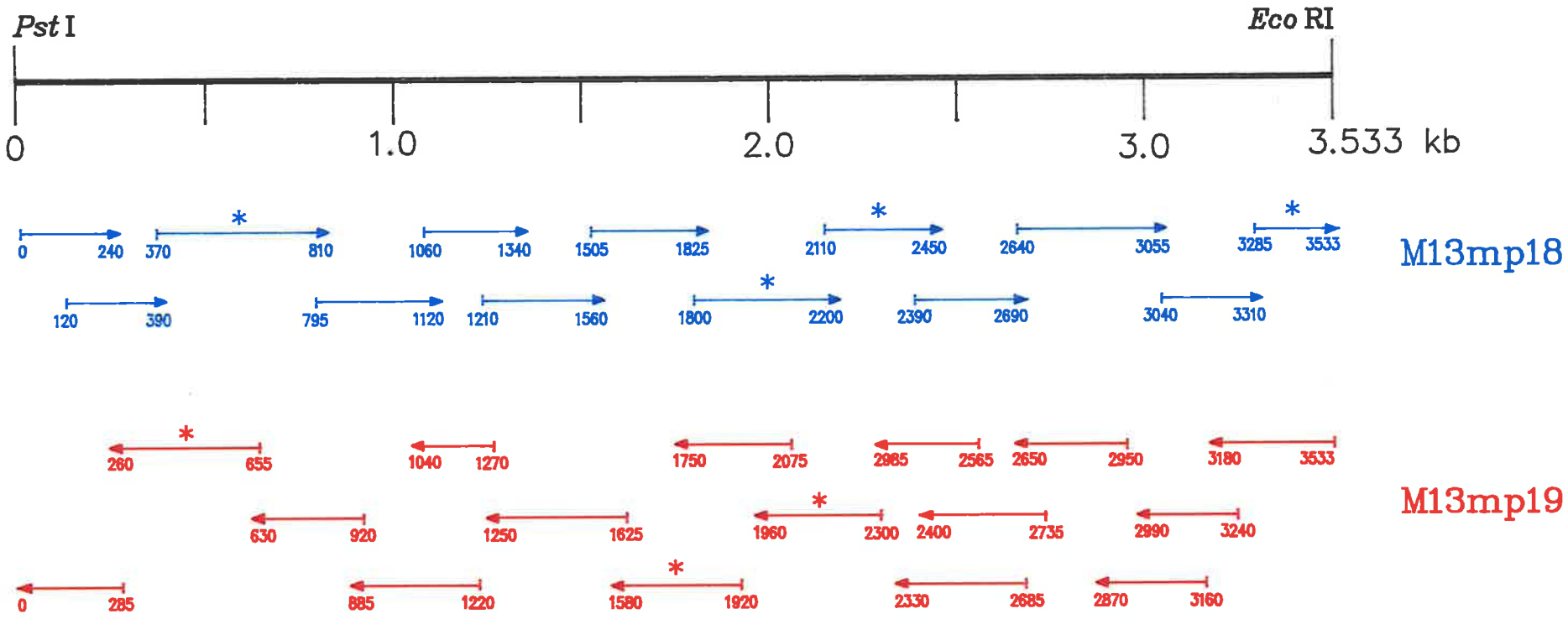

Pst I
Eco RI
0
1.0
2.0
3.0
3.533 kb
0
240
370
810
1060
1340
1505
1825
2110
2450
2640
3055
3285
3533
M13mp18
120
390
795
1120
1210
1560
1800
2200
2390
2690
3040
3310
260
655
1040
1270
1750
2075
2985
2565
2650
2950
3180
3533
M13mp19
630
920
1250
1625
1960
2300
2400
2735
2990
3240
0
285
885
1220
1580
1920
2330
2685
2870
3160

Figure 4.2 Nucleotide sequence of the 3.5 kb *Pst*I - *Eco*RI fragment of cloned *V.cholerae* DNA.

The *E.coli* promoter sequences for the "Pribnow box" and the "-35 region" are shown under two potential *hlyA* promoters. The transcriptional terminator (nt 3401 to 3422) is indicated by head to head arrows. The nucleotides are numbered on the right whereas the amino acids within the open reading frame that codes for HlyA are numbered below the sequence beginning at the initiation codon (ATG-Met). The signal peptidase cleavage site of the signal peptide is indicated by an upward arrow head at Ala_{25}.

```
CTGCAGGACG ATACCAACAA CGCACATAGG TATAGGTTTG TTTACCTTGT ACTTGTTGAA TTTGCGCGCG GCTAACGACT TCTGCCTCAG GGGAAGCCCA   100
TGGTTCAGTG GCGGCATTAA CCGATAAGCT AGCTAAGCCA GCGATTAGAA TAGAGAGTCT TTTTTTCATC GTTTACTTCT TATCATTGAG TAATCAGACG   200
TGAGGATCTG GTCAGATCTG TGGGTATTGT GAATGAGGTA TTCATCTGGT CAATGAAAAC ACCCAGCGGC TTCCCTAGGT TGGAAGTAAG ATCAAGTCTA   300
GTTGATGAAA GCATCACTAT CTTCTTCTTA CTTGAAGCGG CTACTGTGTC GGTTAGGCGG GTTCATTTTG ATTGCATAGT CAATCTAGTC TTATACGGGT   400
TCACTGACTC TTAAATTATG CTTTCTTATG TGTAAGCGTA TTGAAATCTT TAGAGTTAAA ATGGAGAAGA CTTAACTTTT TTTAAATTAT TCAATTAATC   500
TTTAAATCAA CTTTATAAAT TAATTCAGAC TAAATTAGTT CAAATTAAAT TAGGCTCATT AAATAATATG AATATCAGTA ATTGTTATTT TAGTAAGAAT   600
TATTTTACAG CAAATAAAAA GTCTTTAGAG GCTAAAATCT GTGATCCGCT GTGAATTTTC AATTTTACCG TATTTTACAT TTAGAAACAT AAGTGATATT   700
TCAGTAAGTA TGTGGTGGCA GAAAATATAT ACCAAAACTC CTTGGAGTTG CAGGTAGGCG GCAAGAGAGC GAATCCTCAT GAGCATGGAT AAACTGTGTG   800
ATTAGGATGA ACGAACGTGC ACAACACCGC TGCCGCTTCG AGTAAGAAGG GGATATGCAT TTCTGCTAAA AGGATACGCG GTAAGCCGTA GCAGTAAAGC   900
CACACGCAAA CTCAAGGATG ACGAGGGTAA CCCATGAGAC ACATGCAAAA TGGGTATGTT CTAATTACTT GAAAATATAA GAATATTACT CAACTCAGAA  1000
TTATAGAAGA GATTTATTAG CAACTATTAA TTTGAGTGTT TGATATATTT CTTGTTTTTT CAGTAGTTTG AGTATAAGTC ACTTTGTTTG GAAAATCTCTC  1100
                                                                    TTGAC A     TTG ACA

TTGTAATAAC ACTAAAAATA ACAGAGTCAG TGAGGTTTAT ATG CCA AAA CTC AAT CGT TGC GCA ATC GCG ATA TTC ACA ATA TTA AGC      1188
 TATAAT      TATAAT           AA GGAGGT          Met Pro Lys Leu Asn Arg Cys Ala Ile Ala Ile Phe Thr Ile Leu Ser
                                                                  10

GCA ATA TCC AGT CCA ACC CTG TTG GCA AAT ATC AAT GAA CCA AGT GGT GAA GCG GCG GAT ATT ATT AGT CAA GTC GCT GAT      1269
Ala Ile Ser Ser Pro Thr Leu Leu Ala Asn Ile Asn Glu Pro Ser Gly Glu Ala Ala Asp Ile Ile Ser Gln Val Ala Asp
            20                          30                                  40

AGT CAT GCA ATA AAA TAT TAC AAT GCT GCT GAT TGG CAA GCC GAA GAC AAC GCA TTA CCG AGC TTA GCT GAG CTG CGA CGT      1350
Ser His Ala Ile Lys Tyr Tyr Asn Ala Ala Asp Trp Gln Ala Glu Asp Asn Ala Leu Pro Ser Leu Ala Glu Leu Arg Arg
                    50                                  60                                  70

TTG GTG ATT AAC CAG CAA AAA CGC GTT TTG GTT GAT TTC AGT CAG ATC AGT GAT GCT GAA GGT CAA GCA GAG ATG CAA GCC      1431
Leu Val Ile Asn Gln Gln Lys Arg Val Leu Val Asp Phe Ser Gln Ile Ser Asp Ala Glu Gly Gln Ala Glu Met Gln Ala
                            80                                  90

CAA TTC AGA AAG GCT TAT GGG GTG GGT TTT GCT AAT CAA TTT ATT GTC ATC ACT GAA CAT AAA GGG GAA CTG CTG TTT ACA      1512
Gln Phe Arg Lys Ala Tyr Gly Val Gly Phe Ala Asn Gln Phe Ile Val Ile Thr Glu His Lys Gly Glu Leu Leu Phe Thr
        100                                 110                                 120

CCT TTT GAT CAG GCA GAA GAG GTT GAC CCT CAA TTA CTC GAA GCG CCG CGT ACC GCT CGC TTA TTA GCG CGC TCT GGT TTT      1593
Pro Phe Asp Gln Ala Glu Glu Val Asp Pro Gln Leu Leu Glu Ala Pro Arg Thr Ala Arg Leu Leu Ala Arg Ser Gly Phe
                130                                 140                                 150

GCA AGT CCG GCA CCG GCA AAC AGC GAA ACA AAT ACC TTG CCG CAT GTG CGT TTT TAC ATC AGT GTC AAC CGT GCG ATC ACG      1674
Ala Ser Pro Ala Pro Ala Asn Ser Glu Thr Asn Thr Leu Pro His Val Arg Phe Tyr Ile Ser Val Asn Arg Ala Ile Thr
                        160                                 170

GAT GAA GAG TGT ACC TTT AAC AAC TCT TGG TTG TGG AAA AAC GAA AAG GGC AGT CGT CCG TTC TGT AAA GAT GCC AAT ATC      1755
Asp Glu Glu Cys Thr Phe Asn Asn Ser Trp Leu Trp Lys Asn Glu Lys Gly Ser Arg Pro Phe Cys Lys Asp Ala Asn Ile
    180                                 190                                 200

TCA TTG ATT TAT CGA GTT AAC CTA GAG CGT TCA TTG CAA TAC GGC ATT GTG GGT TCC GCG ACA CCG GAT GCC AAA ATT GTG      1836
Ser Leu Ile Tyr Arg Val Asn Leu Glu Arg Ser Leu Gln Tyr Gly Ile Val Gly Ser Ala Thr Pro Asp Ala Lys Ile Val
                210                                 220                                 230

CGT ATC AGC CTA GAT GAT GAC AGC ACG GGA GCC GGC ATT CAT CTG AAT GAT CAA CTC GGT TAT CGT CAG TTT GGA GCC AGT      1917
Arg Ile Ser Leu Asp Asp Asp Ser Thr Gly Ala Gly Ile His Leu Asn Asp Gln Leu Gly Tyr Arg Gln Phe Gly Ala Ser
                        240                                 250

TAT ACG ACG TTA GAT GCC TAT TTC CGT GAG TGG TCA ACC GAT GCG ATT GCC CAA GAT TAT CGC TTC GTG TTT AAC GCA TCG      1998
Tyr Thr Thr Leu Asp Ala Tyr Phe Arg Glu Trp Ser Thr Asp Ala Ile Ala Gln Asp Tyr Arg Phe Val Phe Asn Ala Ser
260                                 270                                 280

AAC AAT AAA GCG CAG ATC CTG AAA ACC TTT CCT GTC GAT AAC ATT AAC GAG AAA TTT GAG CGC AAA GAG GTT TCA GGT TTT      2079
Asn Asn Lys Ala Gln Ile Leu Lys Thr Phe Pro Val Asp Asn Ile Asn Glu Lys Phe Glu Arg Lys Glu Val Ser Gly Phe
            290                                 300                                 310

GAG CTT GGG GTG ACT GGT GGG GTG GAA GTC AGT GGA GAT GGC CCG AAA GCC AAA CTA GAG GCG AGA GCA AGT TAT ACC CAG      2160
Glu Leu Gly Val Thr Gly Gly Val Glu Val Ser Gly Asp Gly Pro Lys Ala Lys Leu Glu Ala Arg Ala Ser Tyr Thr Gln
                320                                 330                                 340

AGT CGC TGG TTA ACC TAC AAC ACA CAA GAC TAT CGT ATT GAG CGT AAT GCG AAG AAT GCC AAG CGG TTA GCT TTA CAT GAA      2241
Ser Arg Trp Leu Thr Tyr Asn Thr Gln Asp Tyr Arg Ile Glu Arg Asn Ala Lys Asn Ala Lys Arg Leu Ala Leu His Glu
                        350                                 360

TCG TCA ACA ATA CGC GAC AGC AGA ATC GCT ACT CAA TCG TTC GAC CGA TGC TTT GTG GGT GAA TAC TAC CCG GTA GAT GTA      2322
Ser Ser Thr Ile Arg Asp Ser Arg Ile Ala Thr Gln Ser Phe Asp Arg Cys Phe Val Gly Glu Tyr Tyr Pro Val Asp Val
        370                                 380                                 390

AAC CGT ATT AGC GCG CTG ACG TAC GCG AGT TTT GTG CCG AAA ATG GAT GTG ATT TAT AAA GCC TCA GCC ACA GAG ACA GGC      2403
Asn Arg Ile Ser Ala Leu Thr Tyr Ala Ser Phe Val Pro Lys Met Asp Val Ile Tyr Lys Ala Ser Ala Thr Glu Thr Gly
                400                                 410                                 420

AGT ACG GAT TTT ATC ATC GAC TCT TCG GTC AAT ATC CGC CCA ATC TAT AAC GGT GCT TAT AAG CAC TAC TAT GTG GTC GGT      2484
Ser Thr Asp Phe Ile Ile Asp Ser Ser Val Asn Ile Arg Pro Ile Tyr Asn Gly Ala Tyr Lys His Tyr Tyr Val Val Gly
                        430                                 440

GCT CAT CAG TCC TAC CAT GGC TTT GAA GAT ACC CCA CGT CGT CGA ATC ACG AAA TCG GCA AGC TTT ACG GTC GAT TGG GAT      2565
Ala His Gln Ser Tyr His Gly Phe Glu Asp Thr Pro Arg Arg Arg Ile Thr Lys Ser Ala Ser Phe Thr Val Asp Trp Asp
    450                                 460                                 470

CAC CCA GTA TTC ACG GGT GGC CGC CCG GTC AAC CTA CAA CTT GCC AGC TTT AAC AAC CGC TGT ATT CAA GTC GAT GCT CAA      2646
His Pro Val Phe Thr Gly Gly Arg Pro Val Asn Leu Gln Leu Ala Ser Phe Asn Asn Arg Cys Ile Gln Val Asp Ala Gln
                480                                 490                                 500

GGT CGC TTG GCG GCC AAT ACG TGC GAT AGC CAG CAA TCA GCG CAA TCG TTC ATC TAT GAT CAG CTT GGT CGT TAT GTG AGT      2727
Gly Arg Leu Ala Ala Asn Thr Cys Asp Ser Gln Gln Ser Ala Gln Ser Phe Ile Tyr Asp Gln Leu Gly Arg Tyr Val Ser
                        510                                 520

GCG AGT AAC ACC AAG CTC TGT CTT GAT GGT GAG GCA TTA GAC GCA TTG CAA CCC TGT AAC CAA AAC CTG ACT CAG CGT TGG      2808
Ala Ser Asn Thr Lys Leu Cys Leu Asp Gly Glu Ala Leu Asp Ala Leu Gln Pro Cys Asn Gln Asn Leu Thr Gln Arg Trp
530                                 540                                 550

GAG TGG CGT AAA GGC ACA GAT GAA TTG ACC AAT GTC TAC AGC GGC GAT GCC CTT GGA CAT GAC AAA CAA ACC GGT GAG CTT      2889
Glu Trp Arg Lys Gly Thr Asp Glu Leu Thr Asn Val Tyr Ser Gly Asp Ala Leu Gly His Asp Lys Gln Thr Gly Glu Leu
            560                                 570                                 580

GGT TTG TAT GCG AGC AGC AAC GAT GCG GTA AGT TTA CGT ACC ATC ACC GCT TAT ACC GAT GTG TTT AAT GCG CAA GAA AGT      2970
Gly Leu Tyr Ala Ser Ser Asn Asp Ala Val Ser Leu Arg Thr Ile Thr Ala Tyr Thr Asp Val Phe Asn Ala Gln Glu Ser
                590                                 600                                 610

TCG CCG ATT CTG GGT TAC ACA CAA GGG AAA ATG AAT CAG CAG CGT GTG GGA CAA GAT CAT CGT TTG TAT GTG CGA GCG GGT      3051
Ser Pro Ile Leu Gly Tyr Thr Gln Gly Lys Met Asn Gln Gln Arg Val Gly Gln Asp His Arg Leu Tyr Val Arg Ala Gly
                        620                                 630

GCT GCC ATT GAT GCA TTA GGG TCC GCC TCC GAT TTA TTG GTT GGT GGC AAT GGT GGT AGC TTG AGT TCG GTG GAT CTG TCC      3132
Ala Ala Ile Asp Ala Leu Gly Ser Ala Ser Asp Leu Leu Val Gly Gly Asn Gly Gly Ser Leu Ser Ser Val Asp Leu Ser
    640                                 650                                 660

GGC GTG AAA TCC ATC ACG GCA ACC TCT GGT GAT TTC CAA TAT GGC GGT CAC GAG TTG GTG GCG CTG ACA TTC ACC TAC CAA      3213
Gly Val Lys Ser Ile Thr Ala Thr Ser Gly Asp Phe Gln Tyr Gly Gly His Glu Leu Val Ala Leu Thr Phe Thr Tyr Gln
                670                                 680                                 690

GAT GGA CGT CAG CAA ACG GTA GGC TCG AAA GCG TAT GTC ACC AAT GCT CAT GAA GAC CGT TTC GAT TTA CCG GCT GCC GCT      3294
Asp Gly Arg Gln Gln Thr Val Gly Ser Lys Ala Tyr Val Thr Asn Ala His Glu Asp Arg Phe Asp Leu Pro Ala Ala Ala
                        700                                 710

AAG ATC ACT CAA CTG AAA ATT TGG TCT GAC GAT TGG TTG GTG AAA GGG GTT CAA TTT GAT TTG AAC TAA AAAG AGACCGTCAA      3377
Lys Ile Thr Gln Leu Lys Ile Trp Ser Asp Asp Trp Leu Val Lys Gly Val Gln Phe Asp Leu Asn ***
    720                                 730                                 740

CATAGCCATT AAACAATGGA AGCATGCCCT GAAATTTCAG GGCATTTTAT GTATTTAATG TTCTTTATTT AGTCATTTAT CGACTTAACA ATTCAGAAAC      3477
AGAATAATTT TGCCATACTA CTTCAAAGGC TGAACTTATT TAGGTGTACA GAATTC                                                      3533
```

codes for 740 amino acids which, when translated would give rise to a protein of 81,981 Da. Furthermore, as might be predicted for for an extracellular protein, a typical potential leader peptide is present at the N-terminal end. The first 25 amino acids conforms well to the general rules defined by von Heijne (1984, 1985) for signal sequences, and will be discussed later (Section 4.2.3.1).

Analysis of a large number of *E.coli* promoter sequences has defined two consensus hexamer regions (Pribnow, 1975a, 1975b; Schaller *et al.*, 1975; Takanami *et al.*, 1976; Seeburg *et al.*, 1977; Hawley and McClure, 1983). The "Pribnow box" is located 10 bases upstream from the initiation site. The "Pribnow box" consensus sequence is T_{80} A_{95} T_{45} A_{60} A_{50} T_{96} where the subscript represents the percent occurrence of the base most frequently found at that position (Siebenlist *et al.*, 1980; Hawley and McClure, 1983). The other site is located 35 base pairs upstream of the transcription initiation site and is termed the "-35 region" or "recognition sequence" (Maniatis *et al.*, 1975; Pribnow, 1979; Hawley and McClure, 1983; Studnicka, 1987). The "-35 region" has been implicated in the initial recognition of the promoter site by the RNA polymerase. Following the same pattern of conservation as before, the "-35 region" consensus sequence is T_{82} T_{84} G_{78} A_{65} C_{54} A_{45} (Hawley and McClure, 1983). The importance of the individual bases within these hexamers in efficient promoter function has been well characterized by specific mutations which result in a marked decrease/increase in promoter activity (Rosenberg and Court, 1979; Hawley and McClure, 1983).

A potential promoter can be detected 5' to the *hlyA* gene by virtue of the homology displayed between this region and the known *E.coli* promoter consensus sequences. The suggested "-10 region" of this promoter is located between nt 1113 and 1118 (Figure 4.2) and reads **TAAAAA**. This sequence displays reasonable homology with the consensus sequence, however, the last base of this hexamer which is usually the most conserved does differ. A similar divergence from the consensus sequence is also seen with the *V.cholerae* MFRHA putative promoter (Franzon *et al.*, 1989). The corresponding "-35 region" located between nt 1088 and 1093 (Figure 4.2) is **TTGGAA**, where 4 of the 6 bases are identical to the consensus.

A second potential "-10 region" can be detected between nt 1102 and 1107 (Figure 4.2) and reads **TGTAAT**. This hexamer also displays good homology with the *E.coli* consensus sequence, however the second base differs which is usually highly conserved The corresponding "-35 region", GTCACT can be detected between nt 1078 and 1083 (Figure 4.2), although only 3 of the 6 bases match the consensus sequence.

The spacing between the -10 and -35 regions has been implicated to play an important role in promoter strength. The limits of the spacing are 15 to 21 nt, with promoter strength being maximal at 17$\pm$1 nt (Rosenberg and Court, 1979; Hawley and McClure, 1983). Thus, the 19 nt space between the -10 and -35 regions of the first potential promoter is not optimal. However, the spacing between the alternative -10 and -35 regions is 18 nt which is in the optimal range for *E.coli* consensus promoters (Rosenberg and Court, 1979; Hawley and McClure, 1983)

Gentz and Bujard (1985) have identified a hexamer of A's centered around position -43 which may play an additional role in promoter activity. The lack of a "-43 region" coupled with the 19 nt spacing between the -10 and -35 regions suggests that the promoter is not highly expressed (Gentz and Bujard, 1985). However, high levels of HlyA are produced by *V.cholerae* which may indicate that *hlyA* is not constitutively expressed but subject to a regulatory system. Indeed, von Mechow *et al.* (1985) have mapped a potential regulatory gene, *hlyR*, which is tightly linked to the well-characterized *toxR* locus. The possibility of *hlyA* being regulated will be discussed later (Section 4.2.2.4).

A ribosome binding site is required for the efficient initiation of protein translation. The ribosome binding site consists of the required initiation codon (AUG) and also a sequence situated just upstream of this codon that displays homology with the free 3' end of the 16S RNA, known as a Shine-Dalgarno sequence (Shine and Dalgarno, 1974). The Shine-Dalgarno sequence is AAGGAGGU and mutations leading to a further divergence from this region drastically reduce the level of translation initiation (Gold *et al.*, 1981; Kozak, 1983). The Shine-Dalgarno sequence located 4 nt before the AUG start codon of *hlyA* is **AGUGAGGU** which displays excellent homology to the consensus. This spacing is not optimal with the average

being 7 nt and spacings of less than 5 and greater than 9 nt are rare. Reducing this spacing results in less efficient translation (Gold *et al.*, 1981; Kozak, 1983).

There is a potential stem loop structure that incorporates the Shine-Dalgarno sequence but not the initiation codon which could be formed in this region to affect the expression of the gene (Figure 4.3). However, analysis of the stability of this structure suggests that it is not energetically favourable (ΔG = -1.2 kCal/mol) and would require some other factor to enable it to form.

4.2.2.2 Codon Usage

A summary of the codon usage within the coding region of the *hlyA* mRNA is shown in Table 4.1. Table 4.2 shows the *hlyA* codon usage as compared to the average usage in other sequenced *V.cholerae* genes. These genes include *toxR* (Miller *et al.*, 1987), *ctxA,B* (Mekalanos *et al.*, 1983), *pac* (Guidolin and Manning, 1988), *ompV* (Pohlner *et al.*, 1986), Dnase (Focareta and Manning, 1987), *tcpA* (Faast *et al.*, 1989) and a locus consisting of four genes including the MFRHA (Van Dongen *et al.*, 1987; Franzon *et al.*, 1989; Franzon, V.L., Clark, C.A., Barker, A., Williams, S.G. and Manning, P.A., manuscript in preparation). It can be seen that the preferred codon usage within *hlyA* conforms well with other sequenced *V.cholerae* genes, although the codon usage presented may be slightly biased since they represent a select group of proteins, namely outer membrane and secreted proteins.

4.2.2.3 Transcriptional Terminators

The termination of transcription occurs at an inverted repeat structure with the potential ability to form a stable base-paired stem loop structure which may or may not require the presence of the termination factor Rho (Rosenberg and Court, 1979). Analysis of the sequence after the UAA stop codon showed the presence of a region of dyad symmetry followed by a poly-U tail 37 nt downstream from the stop codon (Figure 4.4). This potential transcriptional terminator has a free energy value of -19.4 kCal/mol according to Tinoco *et al.* (1973) and is typical of a Rho-independent terminator.

Figure 4.3 Initiation stem loop.

This is a potential hairpin loop that may form at the beginning of *hlyA*. The free energy of this loop is poor, at -1.2 kCal/mol and would require some other factor in order for it to form. The Shine-Dalgarno sequence is shown in red and is paired in the stem-loop while the initiation codon (shown in blue) is present in the loop-out.

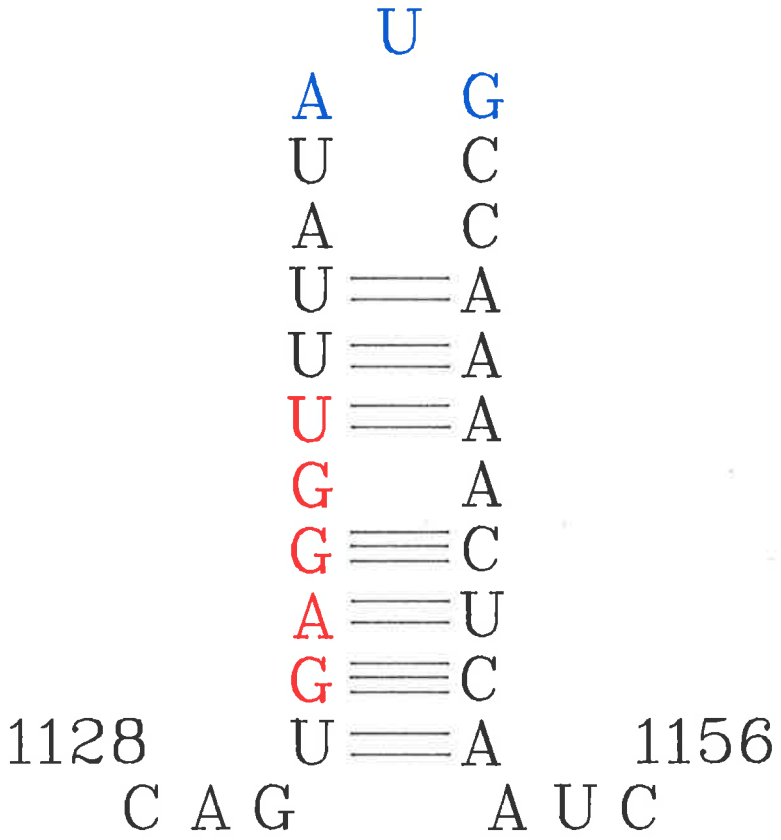

U
A G
U C
A C
U A
U A
U A
G A
G C
A U
G C
1128 U A 1156
C A G A U C

Table 4.1 Codon usage within *hlyA*

Codon	Number	Codon	Number
UUU-Phe	20	UAU-Tyr	20
UUC-Phe	12	UAC-Tyr	12
UUA-Leu	15	UAA-ochre	1
UUG-Leu	17	UAG-amber	0
CUU-Leu	6	CAU-His	10
CUC-Leu	4	CAC-His	3
CUA-Leu	4	CAA-Gln	29
CUG-Leu	12	CAG-Gln	13
AUU-Ile	17	AAU-Asn	18
AUC-Ile	19	AAC-Asn	23
AUA-Ile	5	AAA-Lys	23
AUG-Met	4	AAG-Lys	7
GUU-Val	7	GAU-Asp	40
GUC-Val	12	GAC-Asp	11
GUA-Val	5	GAA-Glu	18
GUG-Val	20	GAG-Glu	17
UCU-Ser	5	UGU-Cys	5
UCC-Ser	7	UGC-Cys	3
UCA-Ser	7	UGA-opal	0
UCG-Ser	9	UGG-Trp	10
CCU-Pro	3	CGU-Arg	23
CCC-Pro	1	CGC-Arg	11
CCA-Pro	6	CGA-Arg	5
CCG-Pro	13	CGG-Arg	1
ACU-Thr	5	AGU-Ser	20
ACC-Thr	18	AGC-Ser	14
ACA-Thr	11	AGA-Arg	3
ACG-Thr	12	AGG-Arg	0
GCU-Ala	18	GGU-Gly	24
GCC-Ala	18	GGC-Gly	13
GCA-Ala	17	GGA-Gly	6
GCG-Ala	22	GGG-Gly	7

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

Codon	% *hlyA*	% *V.cholerae*	Codon	% *hlyA*	% *V.cholerae*
UUU-Phe	62.5	67.1	GCU-Ala	24.0	27.8
UUC-Phe	37.5	32.9	GCC-Ala	24.0	18.8
			GCA-Ala	22.7	25.6
UUA-Leu	25.9	25.3	GCG-Ala	29.3	27.8
UUG-Leu	29.3	19.5			
CUU-Leu	10.3	13.0	UAU-Tyr	62.5	56.9
CUC-Leu	6.9	9.6	UAC-Tyr	37.5	43.1
CUA-Leu	6.9	11.7			
CUG-Leu	20.7	20.9	CAU-His	76.9	70.5
			CAC-His	23.1	29.5
AUU-Ile	41.5	48.3			
AUC-Ile	46.3	35.8	CAA-Gln	69.0	69.1
AUA-Ile	12.2	15.9	CAG-Gln	31.0	30.9
AUG-Met	100.0	97.3	AAU-Asn	43.9	52.0
GUG-Met	0.0	2.7	AAC-Asn	56.1	47.0
GUU-Val	15.9	30.8	AAA-Lys	76.7	74.7
GUC-Val	27.3	17.2	AAG-Lys	23.3	25.3
GUA-Val	11.3	17.6			
GUG-Val	45.5	34.4	GAU-Asp	78.4	76.8
			GAC-Asp	21.6	23.2
UCU-Ser	8.1	13.2			
UCC-Ser	11.3	8.7	GAA-Glu	51.4	64.5
UCA-Ser	11.3	22.9	GAG-Glu	48.6	35.5
UCG-Ser	14.5	12.9			
AGU-Ser	32.2	26.0	UGU-Cys	62.5	67.5
AGC-Ser	22.6	16.3	UGC-Cys	37.5	32.5
CCU-Pro	13.1	23.8	UGG-Trp	100.0	100.0
CCC-Pro	4.3	11.4			
CCA-Pro	26.1	31.5	CGU-Arg	53.5	38.2
CCG-Pro	56.5	33.3	CGC-Arg	25.6	21.3
			CGA-Arg	11.6	15.2
ACU-Thr	10.9	26.1	CGG-Arg	2.3	5.1
ACC-Thr	39.1	28.5	AGA-Arg	7.0	15.7
ACA-Thr	23.9	22.2	AGG-Arg	0.0	4.5
ACG-Thr	26.1	23.2			
			UAA-ochre	100.0	28.6
GGU-Gly	48.0	47.0	UAG-amber	0.0	35.7
GGC-Gly	26.0	30.0	UGA-opal	0.0	35.7
GGA-Gly	12.0	12.7			
GGG-Gly	14.0	10.3			

Figure 4.4 Transcriptional terminator.

A typical Rho-independant transcriptional terminator exists after *hlyA* corresponding to bp 3401 to 3422 in Figure 4.2. It has a potential free energy of -19.4 kCal/mol (Tinoco *et al.*, 1973). The UAA termination codon is indicated by asterisks followed by a spacer region of 37 bases.


A U
A U
A U
G C
U A
C G
C G
C G
G C
U A
A U
3361
U A A A A A /31 bases/ A G C U U U A U
3427
* * *

4.2.2.4 Possible regulation of *hlyA*

Analysis of the 5' precoding region of *hlyA* shows that no significant ORF exists within an 1140 bp region indicating that the provisional localization of the potential *hlyC* gene mapped to this region was incorrect (Manning *et al.* 1984). Additionally, when the 3.5 kb *Pst*I - *Eco*RI fragment was cloned into pGB2 and introduced into the *E.coli* minicell producing strain DS410, no HlyC product could be seen (Figure 4.5). This suggests that the 22 kDa HlyC protein seen by Manning *et al.* (1984) was not encoded by this region and was either an artificially generated fusion with the β-lactamase of the vector or came from the remaining *Eco*RI - *Pst*I fragment of the *hly* locus. The 1140 bp 5' to the ATG codon are relatively AT rich (64%). This is unusual for *V.cholerae* which has an average GC content of 47-49% (Bauman and Schubert, 1984). There are also two regions (nt 420 - 620 and 940 - 1140) which have markedly lower GC content of 20% and 27%, respectively. However, the *hlyA* gene is typical with a GC content of 47.6%.

The ToxR protein which positively regulates the cholera enterotoxin genes (*ctxA,B*) has been shown to bind to multiple copies of the heptamer TTTTGAT that lie before the *ctxAB* operon (Miller and Mekalanos, 1984; Miller *et al.*, 1987). This heptamer is not present before the *hlyA* gene but there are three copies of the heptamer TATTTTA (nt 586-592, 601-607 and 671-677; Figure 4.2). The role of these sequences is unknown but it is tempting to speculate that they may serve a similar function to the heptamer that lies before the *ctxAB* operon although they are not contiguous. A further sequence, TAAATTA, is repeated 5 times (nt 412-417, 483-488, 516-521, 531-536 and 546-551; Figure 4.2). Within the 5' precoding region there are a large number of "AT-rich" regions that can be used to build up a consensus sequence which displays dyad symmetry (Figure 4.6). This consensus may be an artefact due to the unusually high AT content, however, this content itself is atypical. Thus, these repeat sequences may play a role in the regulation of *hlyA*. In this regard it is interesting to note a similarity with the known Fur binding sites of iron-regulated proteins of *E.coli*. Poole and Braun (1988) demonstrated that reducing the level of iron dramatically increases the haemolytic activity of *S.marcescens*. These workers reported

Figure 4.5 Expression of plasmid encoded proteins.

Plasmid encoded proteins were analysed using the *E.coli* minicell producing strain DS410. Minicells harbouring the various plasmids were purified on sucrose gradients, labelled with [^{35}S]-methionine and solubilized in SDS sample buffer. The plasmid encoded proteins were visualized by autoradiography after electrophoretic separation on an 11-20% polyacrylamide gradient gel. The HlyA protein (pPM2008) and the HlyA* protein (pPM2010) are indicated by arrows. A potential precursor form of HlyA* is indicated with an arrowhead.

pPM2008
pPM2010
pGB2
HlyA*
HlyA

Figure 4.6 Analysis of the 5'-precoding region of *hlyA*.

An extremely AT-rich region is found prior to the start of the *hlyA* gene. Analysis of this region has revealed the presence of a number of highly conserved repeat sequences. Using these conserved regions, a consensus sequence was generated. This sequence corresponds to a region of dyad symmetry indicated by head to head arrows.


365 369
411 419
456 460
477 491
493 499
501 507
512 525
531 537
542 552
558 569
571 575
579 583
585 592
598 607
612 620
633 638
654 659
661 667
671 677
679 683
696 701
722 731
789 793
867 871
947 951
961 965
972 980
982 988
999 1005
1012 1019
1025 1033
1043 1050
1055 1060
1116 1121
1136 1142
Consensus

the presence of a nucleotide sequence upstream of the *shlAB* locus that displays homology with the "Fur-consensus" sequences of *E.coli* and is responsible for the iron regulation seen with the haemolysin. This would explain increase in haemolytic activity under iron-starvation (Section 3.2.5).

4.2.3 The HlyA protein

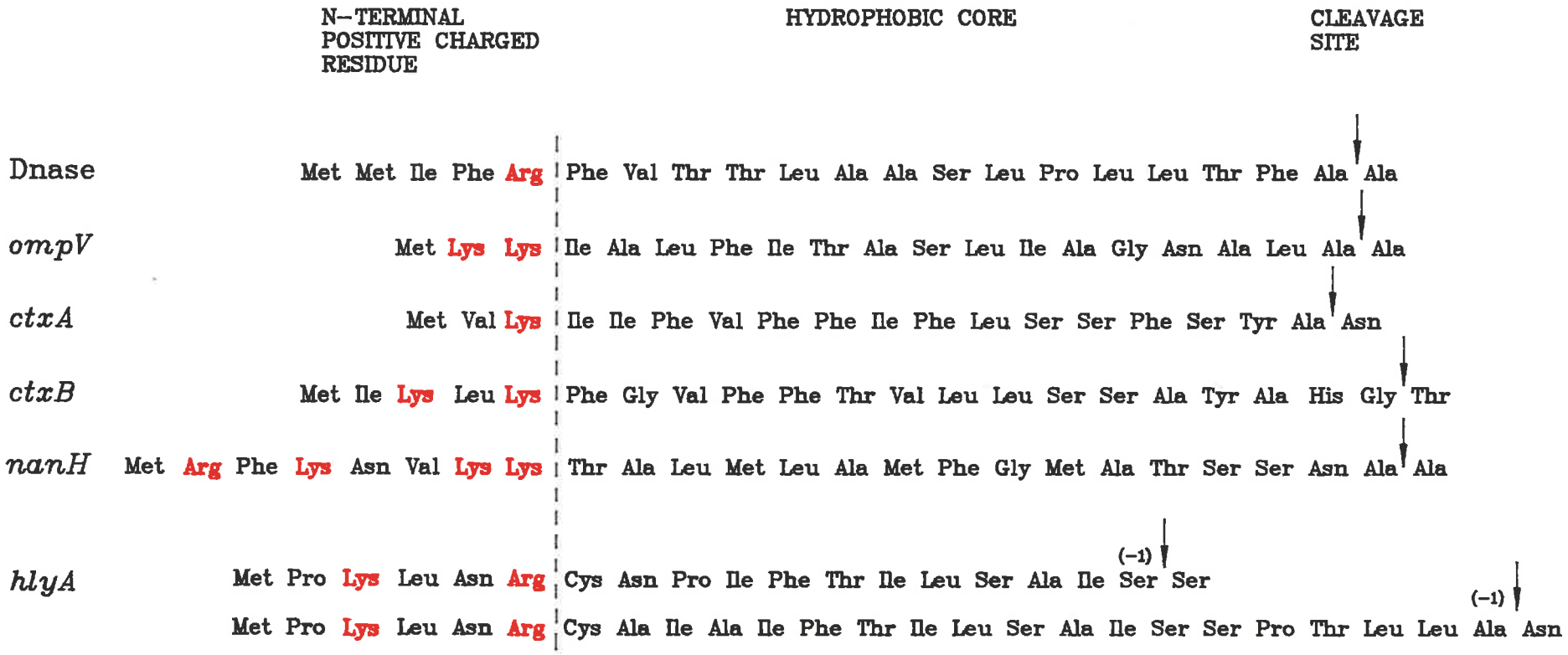
4.2.3.1 The signal sequence

The existence of a signal sequence in prokaryotes was first demonstrated with the major outer membrane lipoprotein of *E.coli* (Halegoua *et al.*, 1976; Inouye *et al.*, 1977). Many examples of signal sequences or leader peptides on proteins which are located in the periplasm, outer membrane or excreted into the extracellular medium have since been described. A comparison of different signal sequences from a variety of origins has demonstrated that they share a number of common features which may be related to their role in protein secretion (Inouye and Halegoua, 1980; von Heijne, 1983). These features include the presence of positively charged amino acids at the N-terminal end (n region) followed by a hydrophobic stretch of 9-20 amino acids which contain one or two helix-breaking residues such as Pro or Gly (h region). There is also a c region at the C-terminal end of the hydrophobic segment (h region) within which there is a suitable cleavage site for the leader peptidase. The residue at position -1 is usually Ala or Gly and to a lesser extent Ser. Usually one of these aa's is also found at position -3 leading to the -3, -1 rule as defined by von Heijne (1984, 1985).

The first 25 aa's conform well to the rules defined for signal sequences and within this region there are two potential cleavage sites (von Heijne, 1984, 1985). A comparison of the two putative HlyA signal sequences and those of other known signal peptides for *V.cholerae* proteins is shown in Figure 4.7. There is a 'n' region of 6 aa's containing 2 positively charged residues (Lys and Arg). These are thought to play a role in the initial interaction between the signal peptide and the negatively charged phosphate residues of the phospholipids inserted in the inner surface of the cytoplasmic membrane. This region is followed by a hydrophobic core ('h' region). The predicted

Figure 4.7 Signal sequences of some *V.cholerae* exported proteins

The signal peptides of some exported *V.cholerae* proteins have been determined. The positively charged amino acids at the N-terminal end are shown in red. All leader peptides contain a hydrophobic core and a suitable cleavage site consistent with the rules of von Heijne (1983). The cleavage sites of OmpV (Pohlner *et al.*, 1986), CtxA and CtxB (Mekalanos *et al.*, 1983), and nanH (Vimr *et al.*, 1988) were determined by N-terminal analysis of the mature protein, whereas those for the DNase (Focareta and Manning, 1987) and HlyA are only predicted based on the rules of von Heijne (1983, 1984, 1985).


N–TERMINAL
POSITIVE CHARGED
RESIDUE
HYDROPHOBIC CORE
CLEAVAGE
SITE
Dnase
Met Met Ile Phe Arg
Phe Val Thr Thr Leu Ala Ala Ser Leu Pro Leu Leu Thr Phe Ala Ala
ompV
Met Lys Lys
Ile Ala Leu Phe Ile Thr Ala Ser Leu Ile Ala Gly Asn Ala Leu Ala Ala
ctxA
Met Val Lys
Ile Ile Phe Val Phe Phe Ile Phe Leu Ser Ser Phe Ser Tyr Ala Asn
ctxB
Met Ile Lys Leu Lys
Phe Gly Val Phe Phe Thr Val Leu Leu Ser Ser Ala Tyr Ala His Gly Thr
nanH
Met Arg Phe Lys Asn Val Lys Lys
Thr Ala Leu Met Leu Ala Met Phe Gly Met Ala Thr Ser Ser Asn Ala Ala
hlyA
Met Pro Lys Leu Asn Arg
Cys Asn Pro Ile Phe Thr Ile Leu Ser Ala Ile Ser Ser
(–1)
Met Pro Lys Leu Asn Arg
Cys Ala Ile Ala Ile Phe Thr Ile Leu Ser Ala Ile Ser Ser Pro Thr Leu Leu Ala Asn
(–1)

signal peptide cleavage site after Ala_{25} does not conforms with the (-3, -1) rule (von Heijne, 1984, 1985). However, cleavage after Ser_{19} does fit this rule. The broad uninterrupted neutral/hydrophobic domain would permit insertion into the lipid bilayer and enable spanning of the membrane in both cases. But, a helix-breaking residue, Pro_{21}, is present in the hydrophobic core, and is usually 4-8 residues from the cleavage site (von Heijne, 1983). If cleavage occurred after Ser_{19}, no helix-breaking residue would be present, but precedents do exist for this with the DNase and OmpV signal peptides which do not have such a residue in this region. This cleavage point would place Pro_{21} at position +2, which is not an uncommon configuration since it has been postulated that these helix-breaking residues may act to place the cleavage site into a position that allows more efficient cleavage by the signal peptidase (Inouye *et al.*, 1984).

Due to the presence of two N-terminal extensions that conform reasonably well to the rules set for prokaryotic signal sequences (von Heijne, 1983), the exact cleavage site could only be predicted. To confirm the cleavage site, N-terminal analysis was performed on purified mature 80 kDa protein. The protein was purified by precipitating culture supernatants of *V.cholerae* O17 with trichloroacetic acid, electrophoresing this on a polyacrylamide gel and electroelution of the 80 kDa protein. Figure 4.8 shows the first 50 amino acid residues of the HlyA precursor. Amino acid sequence analysis defined the cleavage site after Ala_{25}. This places the Pro_{21} residue at the -5 position which is optimal for the helix-breaker.

4.2.3.2 Analysis of the HlyA protein

A plot of the hydropathic nature of the predicted HlyA protein was made according to Kyte and Doolittle (1982) (Figure 4.9). This plot suggests that overall the protein is hydrophilic with a mean hydropathic value of -0.4. This overall hydrophilicity is consistent with its extracellular location. However, there are a number of large hydrophobic domains. Firstly the core of the signal sequence, but a large domain

Figure 4.8 N-terminal analysis of HlyA.

The first fifty amino acid residues of HlyA are shown. The arrow heads indicates potential cleavage points as predicted according to the rules for signal peptides (von Heijne, 1983). The underlined residues are the amino acids unequivocally determined by N-terminal analysis of the exported protein. The questioned amino acids could not be precisely identified due to the high glycine content of the buffer used. The solid arrow head therefore represents the cleavage point of the HlyA signal peptide, after Ala_{25}.

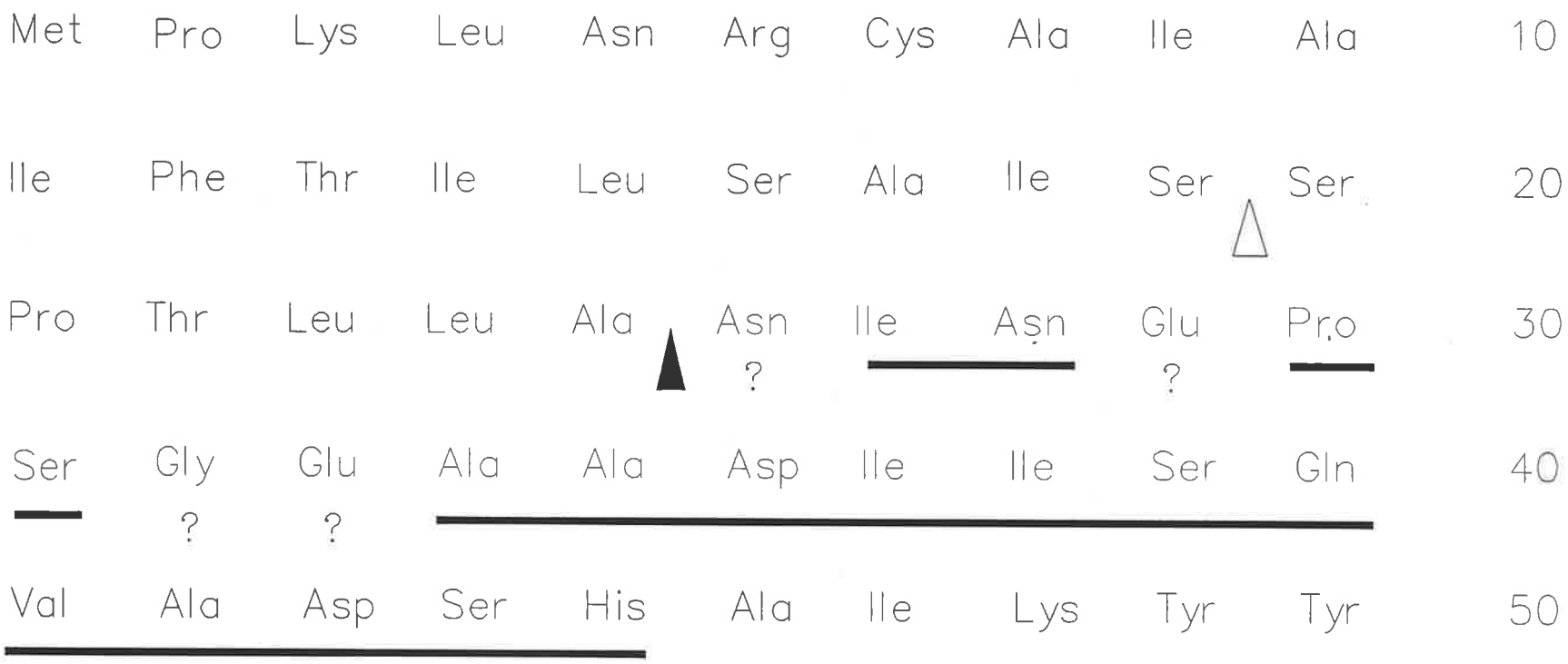
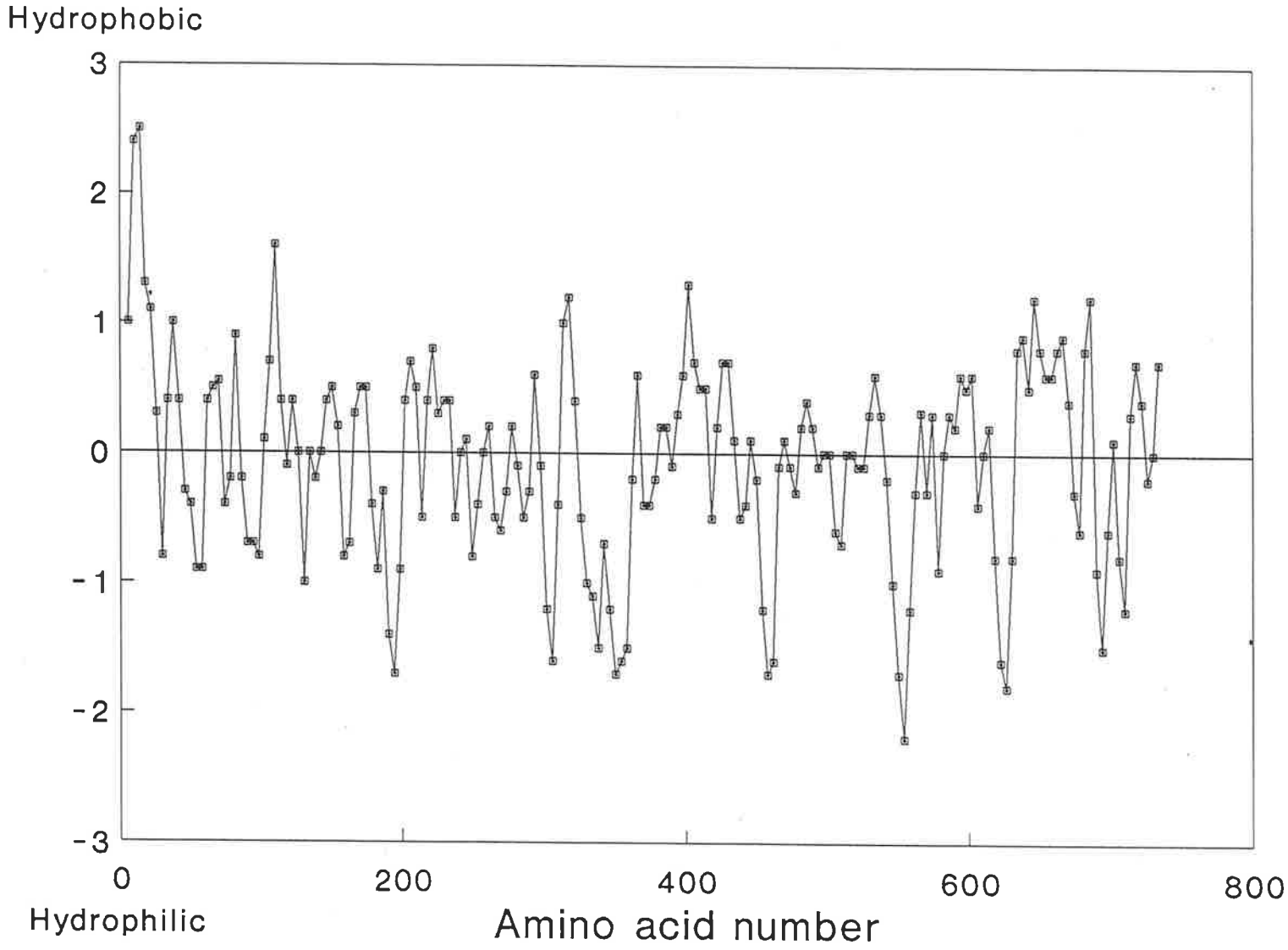

Met Pro Lys Leu Asn Arg Cys Ala Ile Ala 10
Ile Phe Thr Ile Leu Ser Ala Ile Ser Ser 20
Pro Thr Leu Leu Ala Asn ? Ile Asn Glu ? Pro 30
Ser Gly ? Glu ? Ala Ala Asp Ile Ile Ser Gln 40
Val Ala Asp Ser His Ala Ile Lys Tyr Tyr 50

Figure 4.9 Hydropathic nature of the pro-HlyA.

The amino acid sequence of the precursor form of the HlyA protein was analyzed according to Kyte and Doolittle (1982) using a window of nine amino acids.


Hydrophobic
3
2
1
0
-1
-2
-3
0
200
400
600
800
Hydrophilic
Amino acid number

between aa's 130 and 150 displays marked hydrophobicity. The other strong hydrophobic region is found between residues 675 and 685.

The rules of Chou and Fasman (1974a, 1974b, 1978) were applied to predict the secondary structure of HlyA from the predicted aa sequence (Figure 4.10). The arrangement of the cysteine residues is most interesting. Cys_7 is removed with the cleavage of the signal peptide, leaving an odd number of Cys residues. Cys_{182}, Cys_{200}, Cys_{384}, Cys_{496}, Cys_{510}, Cys_{536} and Cys_{548} are all closely linked to turn regions and the pairs Cys_{182}/Cys_{200}; Cys_{496}/Cys_{510}; Cys_{536}/Cys_{548} are very close to each other, making it feasible to suggest that they may be cross-linked to form disulphide bridges. Cys_{384} is located some distance from the other pairs and this residue may be involved in the tertiary structure of HlyA. Alternatively, Cys_{384} may form part of the active site of the haemolysin. Site directed mutagenesis using synthetic oligodeoxynucleotides of these Cys residues to other amino acids may indicate whether they are located in critical positions for effective protein function.

4.2.4 Nucleotide sequence of *hlyA* from Classical 569B

Nucleotide sequence analysis of the Classical *hlyA* gene in pPM2002 revealed only minor changes, which account for the slight difference in the restriction patterns with *Sau*3A and *Taq*I of the El Tor and Classical *hlyA* genes (Goldberg and Murphy, 1985). Analysis of the region of difference within the Classical *hlyA* gene shows an eleven base pair deletion which would result in a frameshift leading to the generation of a stop codon at its end (Figure 4.11). This premature stop codon would produce a truncated *hlyA* product ($HlyA^*$), with a size of 26,918 Da. This is in excellent agreement with the truncated protein product detected in *E.coli* minicells (Section 3.2.1). This deletion occurs within a region of inverted repeat homology (Figure 4.12) which may have looped out to generate the deletion in Classical 569B by homologous recombination, rendering it non-haemolytic. A hydropathy plot (Figure 4.9) predicts that the truncated polypeptide $HlyA^*$ generated by Classical strain 569B is still slightly hydrophilic with a mean hydropathic value of -0.2 and -0.4 after removal of the signal peptide. The $HlyA^*$ protein still possesses the signal sequence and would

Figure 4.10 Predicted secondary structure of HlyA.

The amino acid sequence of the HlyA protein was subjected to analysis using the algorithm of Chou and Fasman (1978). The various regions are represented as follows:

α-helix : 

β-sheet : 

β-turn : 

random coil :

The position of the Cysteine residues are indicated by arrows.

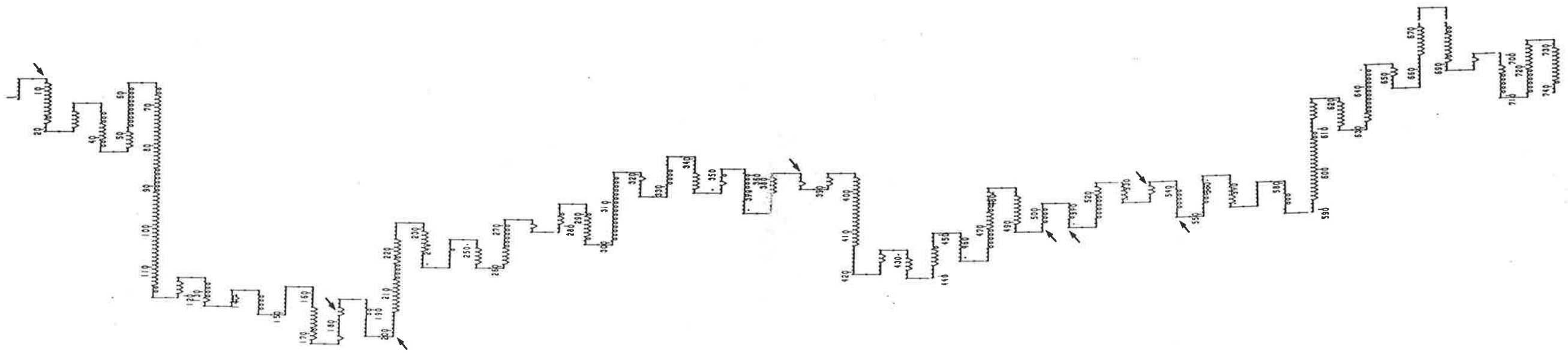


Figure 4.11 Comparison of regions of *hlyA* of *V.cholerae* strains 569B (Classical) and O17 (El Tor).

A synthetic oligodeoxynucleotide was employed as a primer 40 bases downstream of the site of variation in the *hlyA* gene observed in *V.cholerae* strain 569B (Classical). The region of difference is an 11 bp deletion which results in a frameshift generating a stop codon. The corresponding bases in strain O17 (El Tor) are boxed.

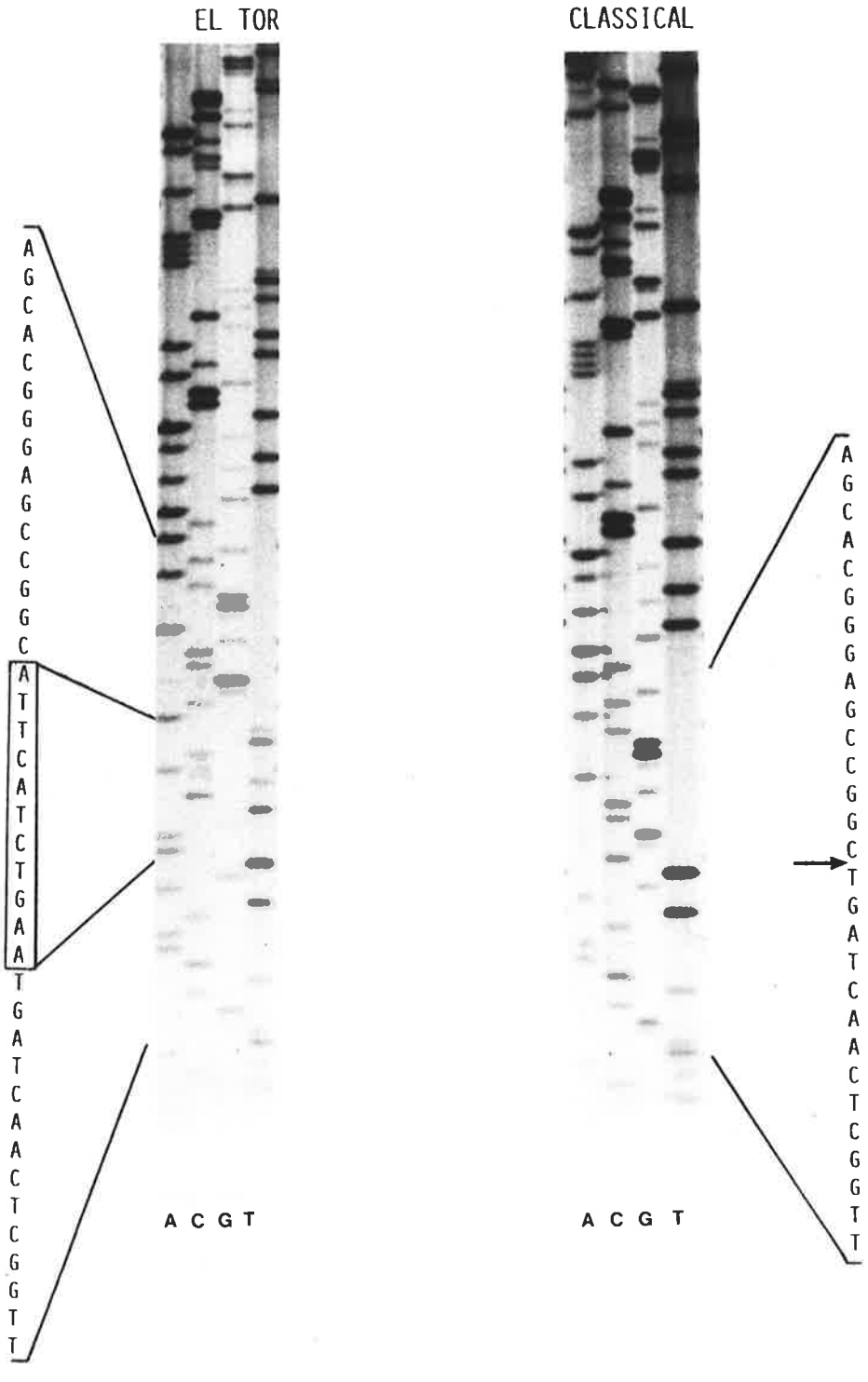
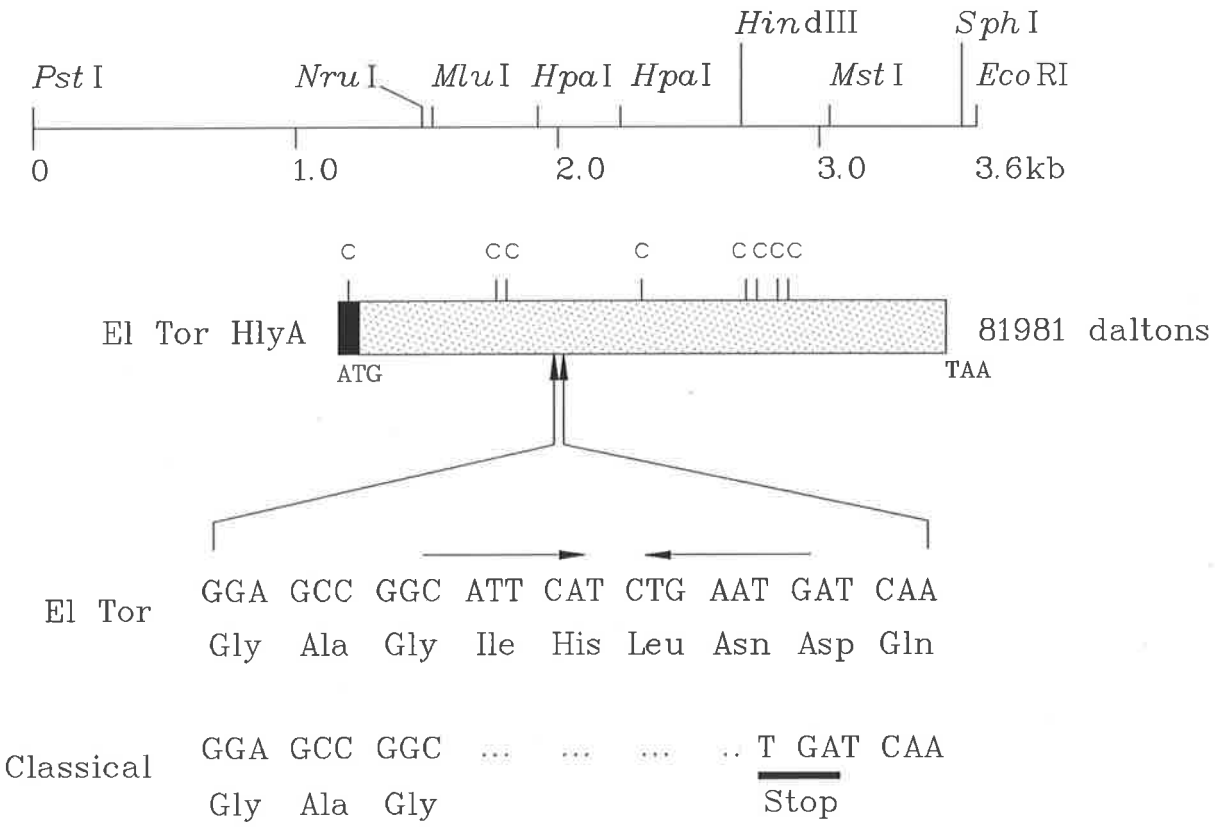

EL TOR
CLASSICAL
AGCACGGGAGCCGGC
ATTCATCTGAA
TGATCAACTCGGTT
A C G T
A C G T
AGCACGGGAGCCGGCTGATCAACTCGGTT

Figure 4.12 A diagrammatic representation indicating the relative position of the Classical deletion in *hlyA*.

Map of the region of DNA containing the *hlyA* gene of *V.cholerae* showing the differences between strains O17 (El Tor) and 569B (Classical). The stretch of 11 bp deleted in the Classical strain which results in a truncated protein product is shown. The inverted repeat homology within this region is indicated by the head to head arrows.


Hin dIII
Sph I
Pst I
Nru I
Mlu I
Hpa I
Hpa I
Mst I
Eco RI
0
1.0
2.0
3.0
3.6kb
C
CC
C
CCCC
El Tor HlyA
81981 daltons
ATG
TAA
El Tor
GGA GCC GGC ATT CAT CTG AAT GAT CAA
Gly Ala Gly Ile His Leu Asn Asp Gln
Classical
GGA GCC GGC ... ... T GAT CAA
Gly Ala Gly
Stop

therefore be secreted at least to the periplasmic space. However, whether this protein still contains the topogenic sequences or signals required for export into the extracellular medium is not known. After cleavage of the signal peptide, HlyA* would contain only two Cysteine residues, Cys_{182} and Cys_{200}.

4.2.5 Comparison of Vibrionaceae strains

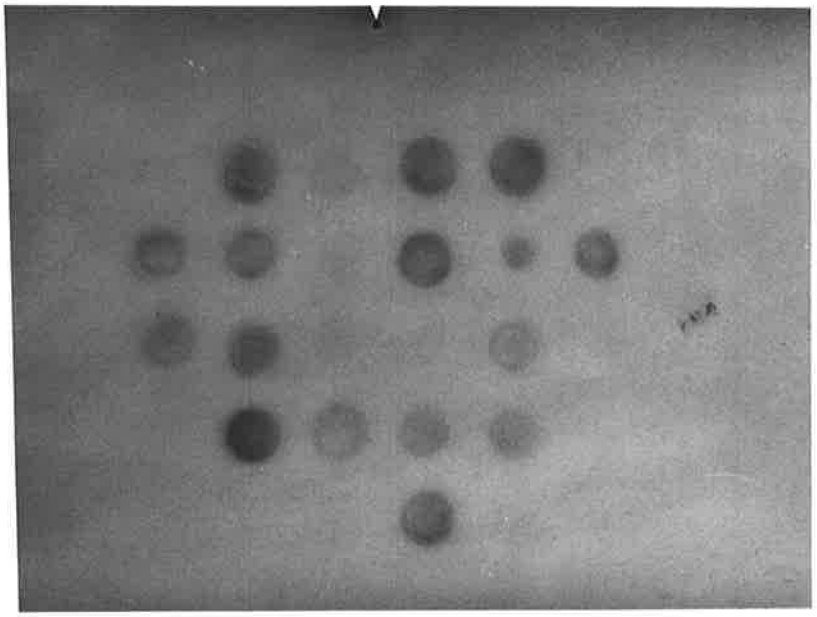
V.cholerae strains of the Classical biotype are invariably non-haemolytic, however, El Tor isolates differ in their haemolytic phenotype (De Moor, 1963). The Classical strain 569B has been shown to possess an 11 bp deletion which would result in its non-haemolytic phenotype. The question of whether all Classical isolates and indeed non-haemolytic El Tor strains possess a similar deletion was investigated.

A 19 base oligodeoxynucleotide was synthesized that spans this 11 bp deleted region with 4 nt on either side. This oligomer (5'-CGGCATTCATCTGAATGAT-3') was radiolabelled and used to probe whole genomic DNA from 132 Vibrionaceae strains including non-O1 *V.cholerae*, environmental *V.cholerae* O1 isolates as well as non-cholera and other *Vibrio* spp. (Figure 4.13). The results show that all El Tor isolates regardless of their haemolytic phenotype possess homology to this probe whereas the Classical isolates do not, indicating that the genetic basis for the non-haemolytic phenotype in Classical and non-haemolytic El Tor strains is clearly different. The *V.cholerae* O1 El Tor haemolysin has been shown to be immunologically related to the haemolysin produced by non-O1 *V.cholerae* isolates, and furthermore, the genes that encode these proteins display very strong DNA homology (Brown and Manning, 1985; Yamamoto *et al.*, 1986; Section 3.2.6). The probe binds to all the non-O1 isolates tested. Amongst other organisms, *V.mimicus* displays strong homology while *V.fluvialis* exhibits none (data not shown).

These data imply that all Classical strains tested have the same deletion mutation in *hlyA* and that this 19-mer oligodeoxynucleotide is an effective probe to distinguish between the two biotypes of *V.cholerae* O1, compared with the other commonly used methods which are less reliable and often difficult to interpret.

Figure 4.13 Biotype specificity of *V.cholerae* O1.

Autoradiograph of a nitrocellulose filter showing a sample of the 132 strains probed with the labelled oligodeoxynucleotide that spans the 11 bp deletion originally seen in 569B (Classical). The strains, together with their biotype and haemolytic status, are listed in Table 2.1



		569B-165	569B		
	T51	KB5	PH8	PH9	
MAK757	RV79	RV69	NSW14	B149	M791
26-3	3083	CA401	029	909	903
	V.MIMICUS	NON-01#52	NON-01#59	NON-01#67	
		569B	017		

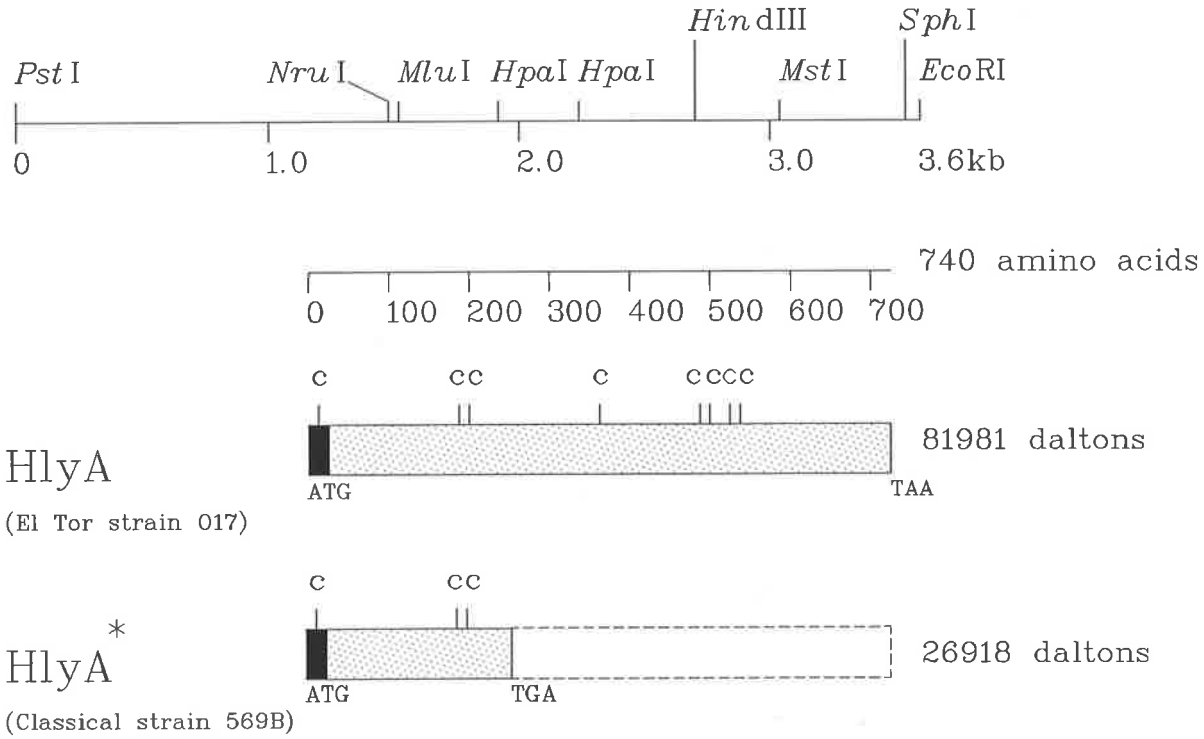
4.3 Summary and Conclusions

A summary of the results presented in this chapter is shown in Figure 4.14. The nucleotide sequence of the *hlyA* gene was determined from a haemolytic El Tor strain of *V.cholerae* (O17), and contained an ORF which, when translated, would give rise to a protein of 740 amino acid residues with a size of 82 kDa. The relatively poor promoter region together with the extremely AT-rich precoding region are also suggestive of additional regulatory functions. The HlyA protein has a typical 25 amino acid signal sequence (von Heijne, 1984, 1985) as determined by N-terminal analysis of the purified protein. Computer homology searches of both the nt and aa sequence using the GenBank and EMBL Gene/Protein Sequence Database, failed to find any other prokaryotic haemolysins with any significant homology.

Classical strain 569B contains a small deletion (Goldberg and Murphy, 1985) which has now been confirmed, by sequence analysis, to correspond to an 11 bp deletion resulting in a truncated *hlyA* product designated HlyA*. Analysis of the sequence around the deletion shows a region of inverted repeat homology comprising repeats of 6 bp with a 2 bp loop. Perhaps the formation of the potential stem loop structure, although not very energetically favourable (ΔG = -1.2 kCal/mol), has during replication at some time past resulted in this deletion. We have confirmed that all Classical *V.cholerae* strains tested harbour a similar deletion and that non-haemolytic El Tor strains display that phenotype for another, as yet, unknown reason. Hydropathic plots of HlyA and HlyA* suggest that they are extracellular. It is, however, interesting to speculate that perhaps the *hlyA* region has been conserved in non-haemolytic Classical strains because HlyA* is produced and has another, as yet unknown, function. This will be discussed in Chapter 5.

Figure 4.14 Summary of the organization of *hlyA* in *V.cholerae* strains O17 and 569B.

The relative position of the open reading frame that encodes the complete HlyA protein in El Tor strain O17 is shown with respect to the surrounding restriction endonuclease cleavage sites. The HlyA protein is produced as a precursor with a N-terminal signal peptide (solid region). The Classical strain 569B has an 11 bp deletion leading to premature termination of translation and a truncated protein, $HlyA^{*}$. The position of the cysteine residues (C) are also indicated and may play a role in the tertiary structure of the haemolysin.


HindIII
SphI
PstI
NruI
MluI
HpaI
HpaI
MstI
EcoRI
0
1.0
2.0
3.0
3.6kb
740 amino acids
0
100
200
300
400
500
600
700
c
cc
c
cccc
81981 daltons
HlyA
ATG
TAA
(El Tor strain 017)
c
cc
26918 daltons
HlyA*
ATG
TGA
(Classical strain 569B)

CHAPTER 5

CONSTRUCTION OF *hlyA* MUTATIONS IN *V.cholerae* AND THEIR EFFECT ON BIOLOGICAL ACTIVITY

5.1 Introduction

Induction of diarrhoea by *V.cholerae* is multifactorial with the prime virulence determinants being those which aid colonization and adherence of the gut epithelium and the enterotoxins responsible for the fluid accumulation. Much of the work with *V.cholerae* of the O1 serotype has concentrated on CT as the major diarrhoeagenic agent responsible for the manifestations of cholera in humans. Some non-O1 isolates produce an enterotoxin that is similar to CT (Zinnaka and Carpenter, 1972; Craig *et al.*, 1981; Yamamoto *et al.*, 1983a, 1983b), however, others lacking the *ctxA,B* genes, have been isolated from clinical cases of diarrhoea indicating that there are other factors which can generate a diarrhoeal response. These non-O1 strains have been shown to produce a haemolysin, and, although its role in pathogenesis remains unclear, it has been implicated as a potential diarrhoeagenic factor (McIntyre and Feeley, 1965). The haemolysin produced by non-O1 isolates has been shown by Yamamoto *et al.* (1986) to be biologically, physiochemically and immunologically indistinguishable from the haemolysin that is produced by El Tor strains of *V.cholerae* O1. This is supported by Southern hybridization analyses which imply that the genes are essentially identical (Brown and Manning, 1985).

A haemolytic factor from *V.cholerae* O1 characterized by Honda and Finkelstein (1979b) was cytotoxic, cardiotoxic and rapidly lethal for mice. McCardell *et al.* (1985) and Spira *et al.* (1986) have described a cytolysin that is produced by non-O1

and O1 strains of *V.cholerae*. This cytolysin causes fluid accumulation in the rabbit gut as well as being cytotoxic for Chinese hamster ovary (CHO) and Y-1 adrenal cells. It has been proposed to be identical to the haemolysin produced by El Tor strains of *V.cholerae* O1 (Madden *et al.*, 1984).

In order to evaluate the contribution that a particular determinant makes to the disease process, it is necessary to construct specific mutations in the genes for the respective molecules, followed by evaluating the virulence of these mutants either in the animal host or in a model infection system. Classically, this has been accomplished by chemical mutagenesis, however, it is not a directed procedure. For example, by mutagenesis with NTG, Honda and Finkelstein (1979a) isolated a non-toxigenic *V.cholerae* strain, Texas Star-SR that did not produce the A subunit of CT whilst leaving the B subunit intact. However, due to the nature of the mutagenesis procedure, other phenotypic alterations unrelated to toxin production are likely to be present, and these may effect virulence. Furthermore, it is not possible to precisely define and localize mutations obtained in this fashion and reversion to virulence can occur.

More recently, recombinant DNA techniques have enabled precise deletion mutations to be introduced into otherwise wild-type strains. This avoids the possibility of reversion, and also leaves the genes for other determinants untouched. Several *V.cholerae* O1 candidate vaccine strains have been constructed using recombinant techniques to remove either the A or B subunit or both of the cholera enterotoxin (Kaper *et al.*, 1984a, 1984b). When these strains were tested in volunteers, greater than 50% developed mild clinical diarrhoea (Levine *et al.*, 1988), indicating that another factor may be involved. To investigate the possibility that the El Tor haemolysin may be this alternative diarrhoeagenic factor, further derivatives of these strains were made Hly$^-$ by deleting a small *Hpa*I - *Hpa*I fragment in the structural gene for the El Tor haemolysin. When these strains were tested, no relief from the mild diarrhoea was seen (Kaper *et al.*, 1986; Levine *et al.*, 1988).

This chapter describes the construction of a specific mutation in the *hlyA* gene, which has been introduced by recombination into the *V.cholerae* chromosome to produce isogenic *hlyA*$^+$ and *hlyA* strains. The location of the mutation within the gene

is such that both HlyA and the truncated HlyA* produced by Classical strains would not be produced so that their role in the disease process could be evaluated.

5.2 Results

5.2.1 Construction of *hlyA* mutants

5.2.1.1 Introduction of a Km^R cartridge into *hlyA*

The structural gene for the El Tor haemolysin, *hlyA*, has been cloned and sequenced. It has also been demonstrated that Classical strains possess an 11 bp deletion leading to the production of a truncated protein, HlyA*, of 27 kDa, compared to the 82 kDa wild type HlyA protein. In order to analyse the function of both the HlyA protein and the truncated product HlyA* seen in Classical 569B, a Km^R cartridge was introduced into the *Mlu*I site early in the gene such that neither HlyA or HlyA* could be produced. Figure 5.1 shows the stepwise formation of pPM2018 which contains the 3.5 kb *Pst*I - *Eco*RI fragment containing *hlyA* inactivated with the Km^R cartridge and converted to an *Eco*RI fragment with synthetic *Eco*RI linkers.

5.2.1.2 Subcloning into plasmid pPM1287

No generalized transformation system exists for the introduction of plasmid DNA into *V.cholerae*. Thus, conjugal mobilization is usually employed. Ditta *et al.* (1980) developed a broad host range cloning vehicle, pRK290, which can be mobilized at high frequency into various Gram negative bacteria using a helper plasmid. Plasmid pRK290 confers Tc^R and contains single cloning sites for the restriction endonucleases *Eco*RI and *Bgl*II. The mobilization (*mob*) region from RP4 was introduced into the *Bgl*II site of pRK290 to form plasmid pPM1287 (Figure 5.2). This plasmid can now be efficiently mobilized from the *E.coli* K-12 strains SM10 or S17-1, which contain the transfer genes of RP4 integrated into the chromosome (Simon *et al.*, 1983). The 5.1 kb *Eco*RI fragment containing the insertionally inactivated *hlyA*

Figure 5.1 Construction of the *hlyA*, Km^R plasmid pPM2018.

Plasmids pBR322 and pPM431 were digested with *Pst*I and *Eco*RI and the *hlyA* encoding fragment cloned into pBR322 to give plasmid pPM2011. The Km^R cartridge was removed from plasmid J225 as a *Pst*I fragment, blunt ended with Klenow fragment and synthetic *Mlu*I linkers added. Plasmid pPM2011 was cleaved with *Mlu*I and the Km^R cartridge inserted to give plasmid pPM2017. Plasmid pPM2017 was then digested with *Pst*I, blunt ended and synthetic *Eco*RI linkers added to produce plasmid pPM2018. This then allowed the inactivated *hlyA* gene to be removed as an *Eco*RI fragment.

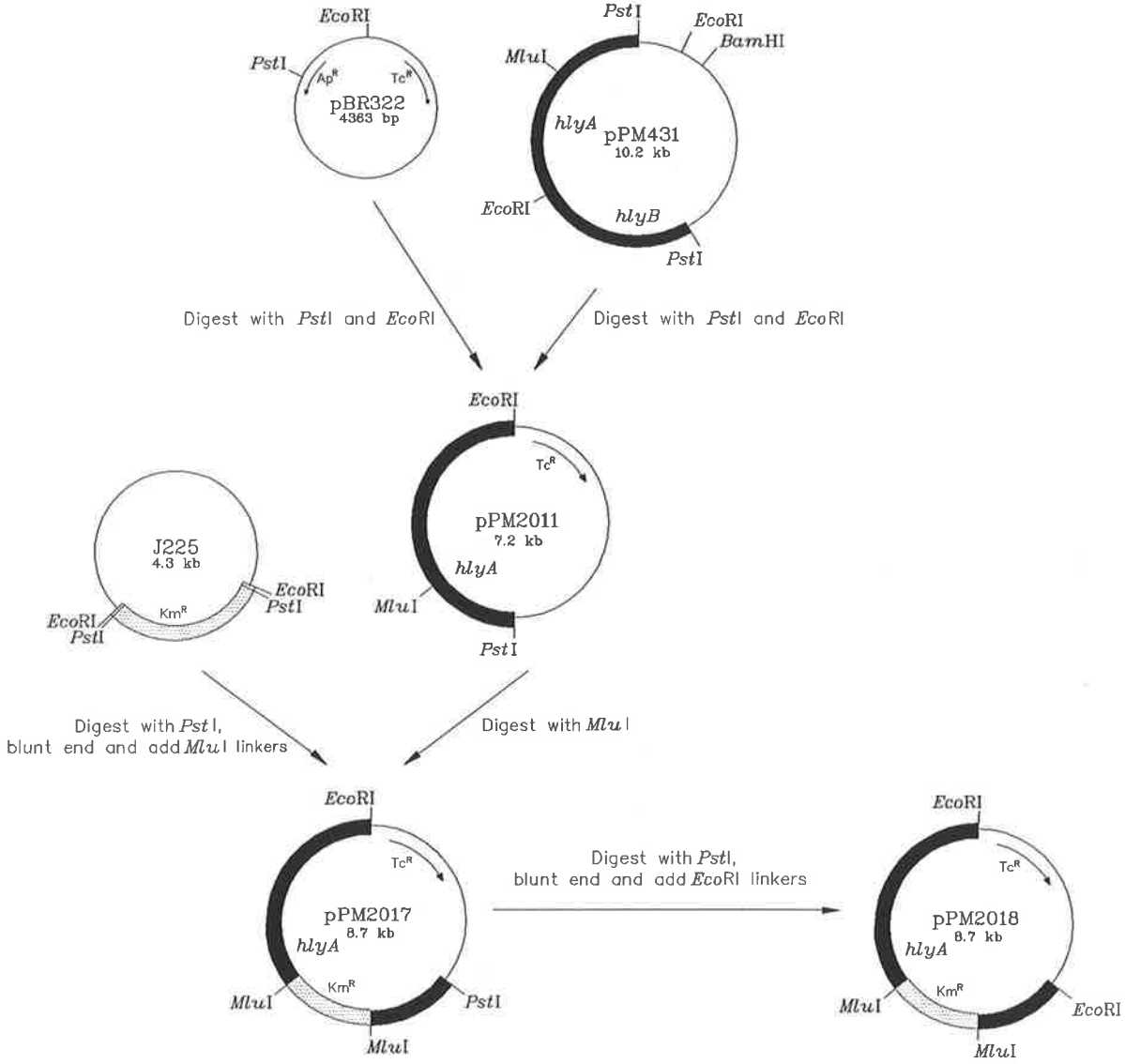
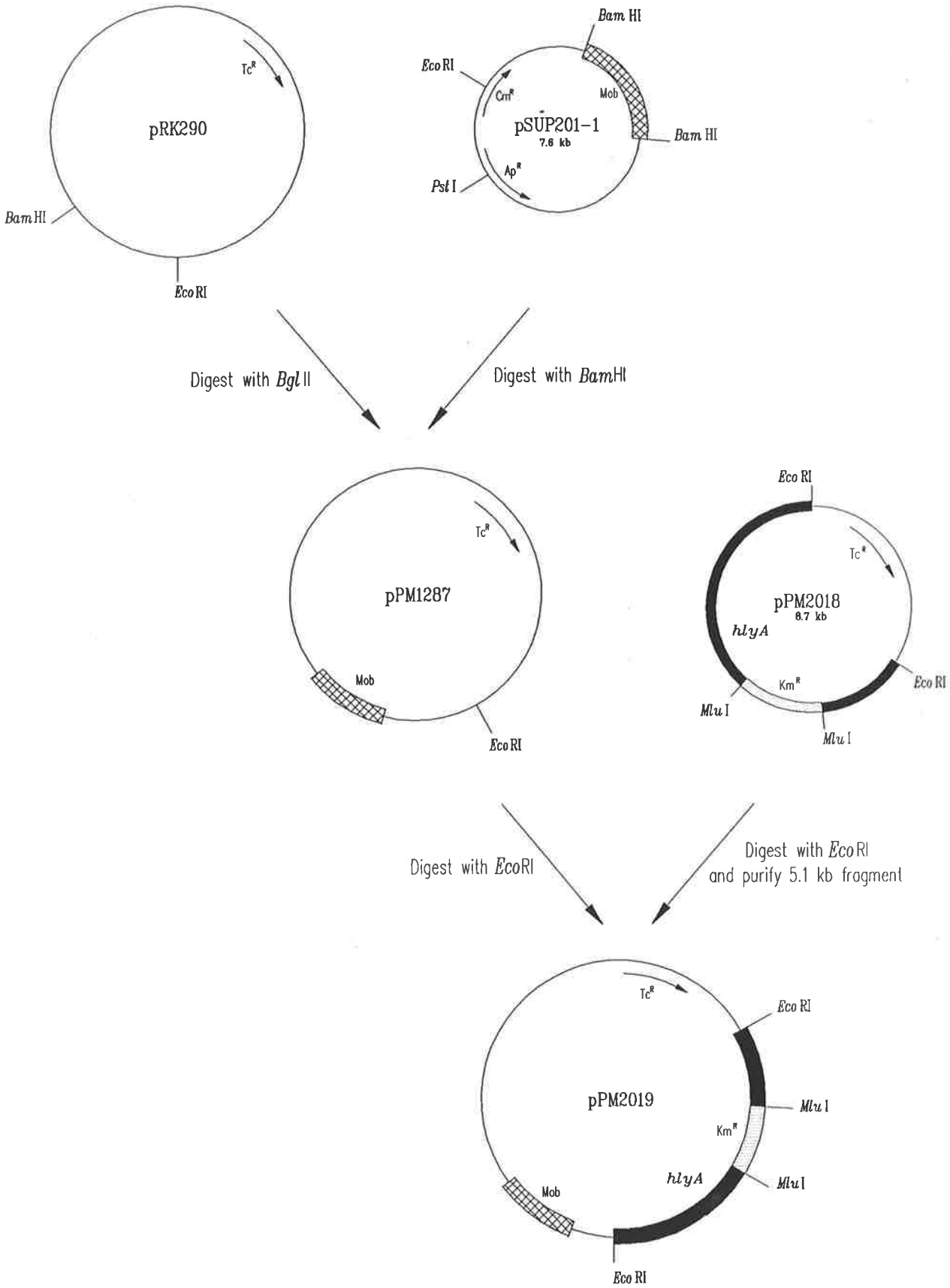

EcoRI
PstI
Ap^R
Tc^R
pBR322
4363 bp
PstI
EcoRI
BamHI
MluI
hlyA
pPM431
10.2 kb
EcoRI
hlyB
PstI
Digest with PstI and EcoRI
Digest with PstI and EcoRI
EcoRI
Tc^R
pPM2011
7.2 kb
hlyA
MluI
PstI
J225
4.3 kb
Km^R
EcoRI
PstI
EcoRI
PstI
Digest with PstI,
blunt end and add MluI linkers
Digest with MluI
EcoRI
Tc^R
pPM2017
8.7 kb
hlyA
Km^R
MluI
PstI
MluI
Digest with PstI,
blunt end and add EcoRI linkers
EcoRI
Tc^R
pPM2018
8.7 kb
hlyA
Km^R
MluI
EcoRI
MluI

Figure 5.2 Construction of the mobilizable plasmid pPM2019 harbouring a *hlyA*::Km^R mutation.

The mobilizable derivative of pRK290 (pPM1287) was constructed by cloning the *mob* region of RP4 from pSUP201-1 into the *Bgl*II site of the vector. The *mob* region isolated from pSUP201-1 was as a 1.9 kb *Bam*HI fragment and cloned directly into the *Bgl*II site of pRK290 with the resultant loss of both restriction endonuclease recognition sites, and the generation of pPM1287. The *hlyA*::Km^R gene was subcloned from pPM2018 on an *Eco*RI fragment into pPM1287 to generate plasmid pPM2019. The plasmid pPM2019 can be efficiently mobilized from *E.coli* strain S17-1 which contains the RP4 transfer genes.


pRK290
Tc^R
BamHI
EcoRI
pSUP201-1
7.6 kb
Bam HI
EcoRI
Cm^R
Mob
Bam HI
PstI
Ap^R
Digest with BglII
Digest with BamHI
pPM1287
Tc^R
Mob
EcoRI
pPM2018
8.7 kb
Eco RI
Tc^R
hlyA
Km^R
Eco RI
MluI
MluI
Digest with EcoRI
Digest with EcoRI
and purify 5.1 kb fragment
pPM2019
Tc^R
Eco RI
MluI
Km^R
hlyA
MluI
Mob
Eco RI

gene from pPM2018 was purified and ligated into the single *Eco*RI site of pPM1287. The resultant Tc^R, Km^R plasmid was designated pPM2019 (Figure 5.2).

5.2.1.3 Construction of *hlyA*::Km^R strains of *V.cholerae*

Plasmid construct pPM2019 was introduced into various *V.cholerae* strains by conjugation. *V.cholerae* strains O17 (El Tor, Ogawa), 569B (Classical, Inaba), N16961 (El Tor, Inaba), JBK70 (El Tor, Inaba, Δ*ctxA,B*) and the two non-toxigenic El Tor environmental strains 1074-78 and 1196-78 were made Sm^R by selection for spontaneous chromosomal mutations and Sm^R, Tc^R, Km^R transconjugants harbouring pPM2019 were selected. Plasmid pPH1JI which encodes Gm^R (Beringer *et al.*, 1978) belongs to the IncP incompatibility group, as does RK2, from which pRK290 and hence pPM1287 is derived. Thus, plasmids pPH1JI and pPM2019 are incompatible. After allowing reciprocal recombination with the chromosome to occur, pPM2019 was expelled from the *V.cholerae* strains by the introduction of pPH1JI by conjugation. Selection for both Km^R and Gm^R ensured that a double crossover recombination event had occurred replacing the intact *hlyA*$^+$ gene with the inactivated copy (Figure 5.3). The introduction of this mutation results in the loss of haemolytic activity in the O17 *hlyA*::Km^R mutant, V735, compared to the parent which can be readily detected after growth on blood agar (Figure 5.4). In contrast, both the Classical strain 569B, and its *hlyA*::Km^R mutant, V734, display a non-haemolytic phenotype. The loss of haemolytic activity was confirmed by assaying the ability of whole cells and culture supernatants to lyse sheep erythrocytes. Only O17 had any haemolytic activity.

To confirm that the *in vitro* constructed *hlyA* mutation had recombined into the *V.cholerae* chromosome as expected, whole genomic DNA of Gm^R, Km^R, Tc^S colonies was extracted, digested with *Pst*I and examined by Southern hybridization using the intact *hlyA*$^+$ gene present on the 3.5 kb *Pst*I - *Eco*RI fragment cloned into pGB2 (pPM2008) as a probe. Since the 1.5 kb Km^R cartridge contains no *Pst*I sites, the *Pst*I fragment in *V.cholerae* with the recombined mutation is 1.5 kb larger (Figure 5.5). The constructions have been confirmed by digestion with *Hin*dIII which yields two smaller fragments, not present in the wild-type *hlyA*$^+$ gene, as the Km^R cartridge

Figure 5.3 Introduction of a *hlyA* mutation into the *V.cholerae* chromosome.

Plasmid pPM2019 (Tc^R, Km^R) was mobilized from *E.coli* strain S17-1 into the Sm^R derivatives of the *V.cholerae* strains to be mutated (Panel A). The resulting transconjugants were Sm^R, Tc^R, Km^R. Recombination between the chromosome and pPM2019 was allowed to occur (Panel B). Plasmid pPH1JI (Gm^R), also belonging to the IncP incompatability group was conjugated into the *V.cholerae* strains containing pPM2019 (Panel C). Sm^R, Km^R, Gm^R colonies were selected for and screened for Tc^S (Panel D). The *V.cholerae* strains in which the *hlyA* mutation had apparently recombined into the chromosome were then analyzed further.

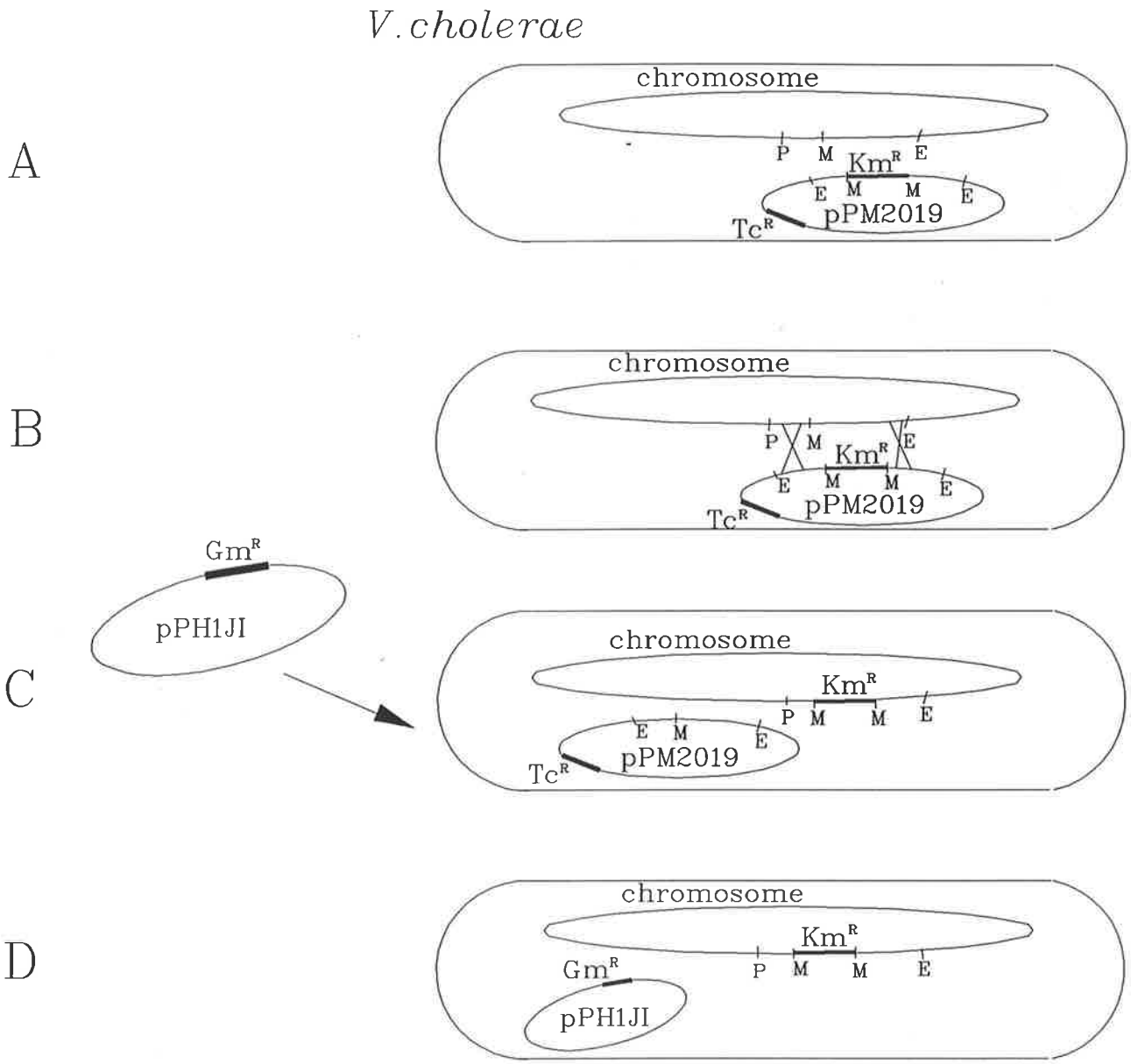

V. cholerae
A
chromosome
P
M
Km^R
E
E
M
M
E
pPM2019
Tc^R
B
chromosome
P
M
Km^R
E
E
M
M
E
pPM2019
Tc^R
Gm^R
pPH1JI
C
chromosome
Km^R
P
M
M
E
E
M
E
pPM2019
Tc^R
D
chromosome
Km^R
Gm^R
P
M
M
E
pPH1JI

Figure 5.4 Analysis of the putative *hlyA* mutations.

V.cholerae strains O17, 569B and their respective *hlyA*::Km^R mutants were plated onto NA containing 5% sheep erythrocytes. The loss of haemolytic activity compared to the wild-type in the O17 *hlyA*::Km^R mutant is evident. The Classical strain 569B and its mutant show the expected non-haemolytic phenotype.

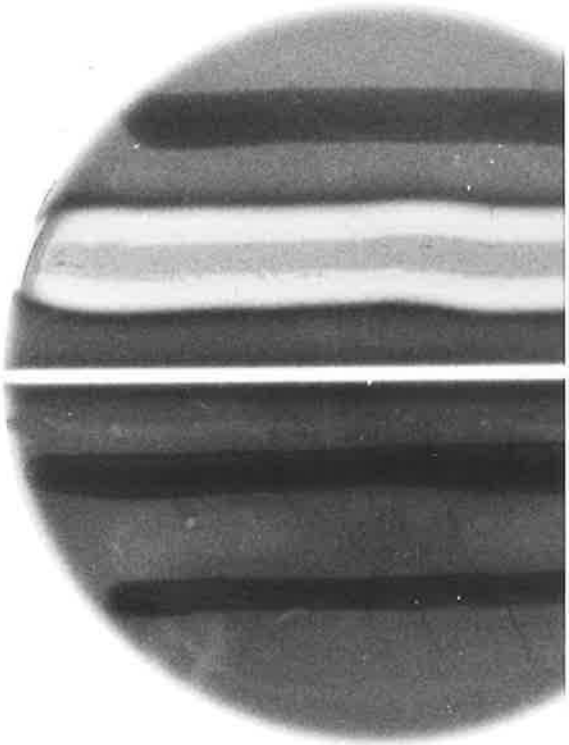
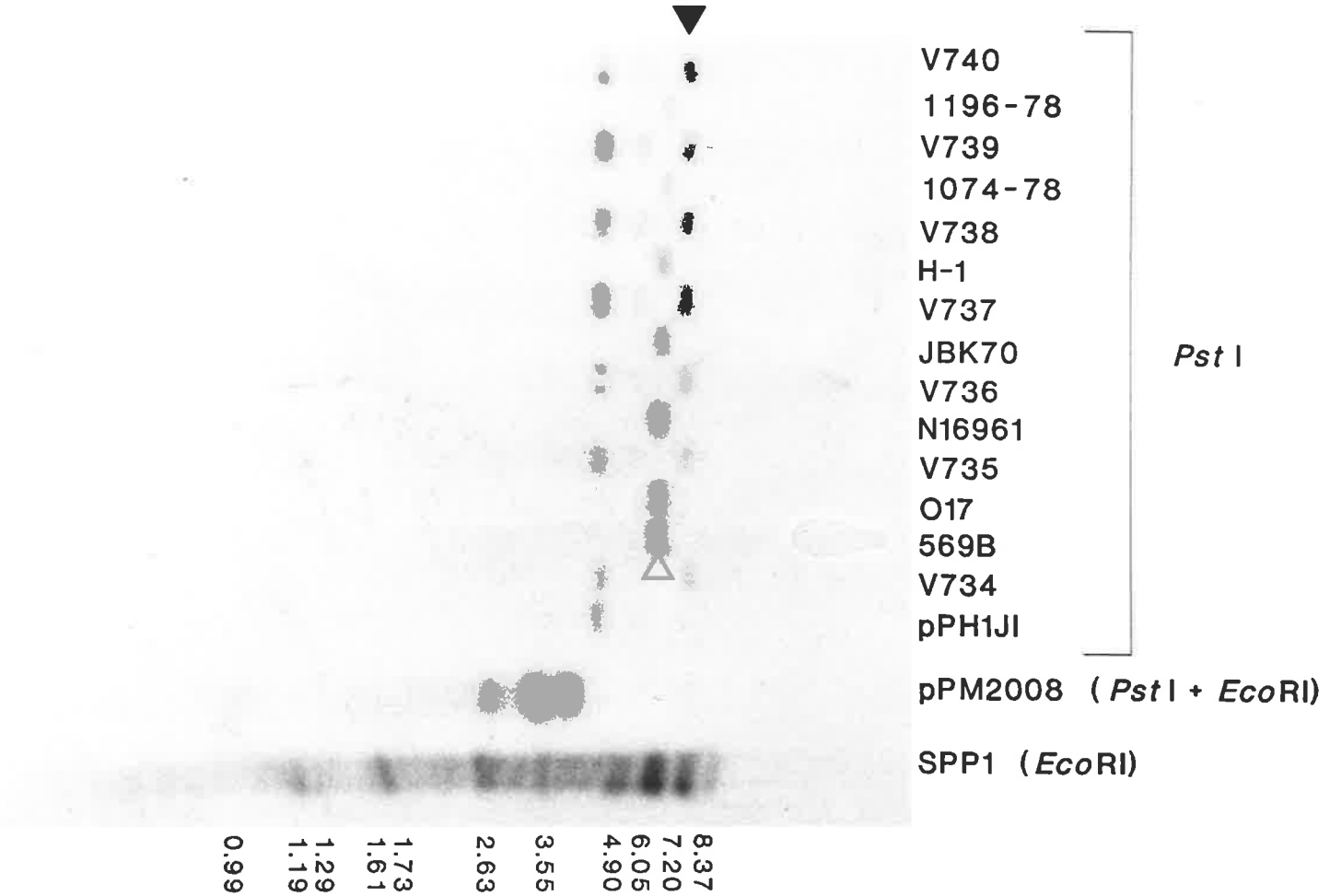

569B
O17
V734
V735

Figure 5.5 Southern hybridization analysis of *hlyA* mutants.

Whole genomic DNA of the *V.cholerae* strains and their *hlyA*::Km^R mutants were digested with *Pst*I and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α-[^{32}P]-dCTP nick translated pPM2008, washed and subjected to autoradiography. The 6.4 kb *Pst*I fragment harbouring the *hly* locus (open arrowhead) present in the wild-type strains is absent in all of the *hlyA*::Km^R mutants, but a new 7.9 kb fragment representing the 6.4 kb *Pst*I fragment containing the Km^R cartridge was detected (solid arrowhead). A cross-reacting band corresponding to pPH1JI can be detected in all of the mutants. SPP1 phage DNA digested with *Eco*RI was used as a standard and the size of the fragments generated in kb are shown on the right.


V740
1196-78
V739
1074-78
V738
H-1
V737
JBK70
V736
N16961
V735
O17
569B
V734
pPH1JI
Pst I
pPM2008 (Pst I + EcoRI)
SPP1 (EcoRI)
0.99
1.19
1.29
1.61
1.73
2.63
3.55
4.90
6.05
7.20
8.37

contains an internal *Hin*dIII site. The parental strains and their respective mutants were also subjected to Western blot analysis of trichloroacetic acid-precipitated culture supernatants using an antiserum to the purified haemolysin (Figure 5.6) and confirmed by haemolysis assays. These analyses and the altered restriction patterns of the *V.cholerae* Gm^R, Km^R, Tc^S mutants demonstrate the absence of both haemolytic activity and the HlyA protein as a result of substitution of the wild-type chromosomal *hlyA*$^+$ gene with an insertionally inactivated substitute.

5.2.2 Effect of the *hlyA*::Km^R mutation on virulence in the infant mouse model

Thus, in order to assess the affects of the *hlyA*::Km^R mutation on virulence it was necessary to expell pPH1JI from the constructed mutant strains. This was accomplished by introducing another IncP plasmid, pME305 which is temperature sensitive for replication (Rella *et al.*, 1985). This displaced pPH1JI and readily permitted the strain to be cured by growth at 42°C (Figure 5.7). Previous work in this laboratory has demonstrated that the presence of pPH1JI decreased the virulence of the wild type *V.cholerae* strains (R. Faast, personal communication).

V.cholerae strains O17 and 569B and their respective isogenic plasmid-free *hlyA*::Km^R mutants, V760 and V759 were tested for virulence in the infant mouse model. After intragastric inoculation with various doses of bacteria, the mice were checked regularly, their state of health noted and the effect of the strains on the survival of infant mice assessed over 48 hours (Figure 5.8). The hypertoxigenic Classical strain 569B began killing the mice slightly earlier than its *hlyA*::Km^R mutant. However, this difference was quite marked with the El Tor strain O17 which killed the animals 6-8 h earlier than its corresponding *hlyA*::Km^R mutant. This shift was most apparent at the lower dose of 5×10^6 organisms indicating that the loss of the haemolysin attenuates the strain for the mice. Since both 569B and O17 are affected this suggests that both HlyA and HlyA* have activity. At a higher dose of (5×10^7) the increased amounts of cholera toxin produced appears to mask the loss of the haemolysin.

Figure 5.6 Loss of HlyA protein in the *hlyA*::Km^R mutants.

Western blot analysis of trichloroacetic acid precipitated culture supernatants of wild type *V.cholerae* strains and their respective *hlyA*::Km^R mutants. The antiserum was generated against the 65 kDa form of the haemolysin. The 80 kDa HlyA protein is shown by an arrow and is present in the wild type strains but is not produced in the *hlyA*::Km^R mutants nor 569B.

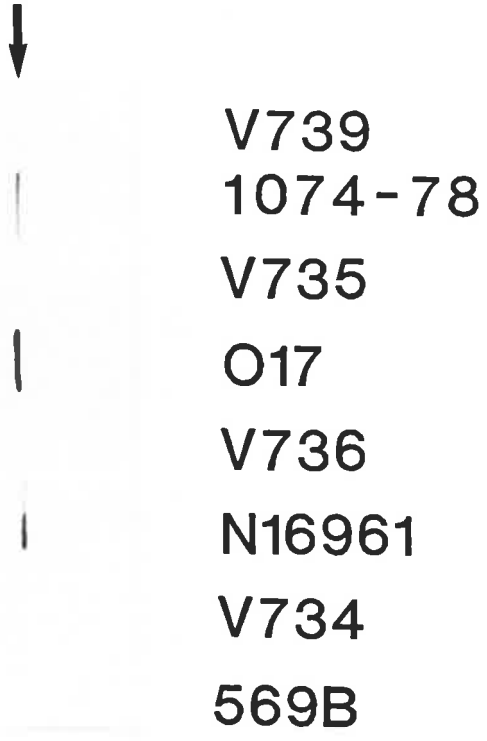

V739
1074-78
V735
O17
V736
N16961
V734
569B

Figure 5.7 Removal of plasmid pPH1JI.

Plasmid pME305, a temperature sensitive replication mutant of RP4, was introduced into the *V.cholerae hlyA*::Km^R mutant strains by conjugation (panel A). The transconjugants were selected by Km^R, Tc^R and screened for Gm^S to ensure the loss of pPH1JI (panel B). The strains were then grown at 42°C to cure plasmid pME305 and produce isogenic plasmid-free strains except for the *hlyA*::Km^R mutation (panel C).

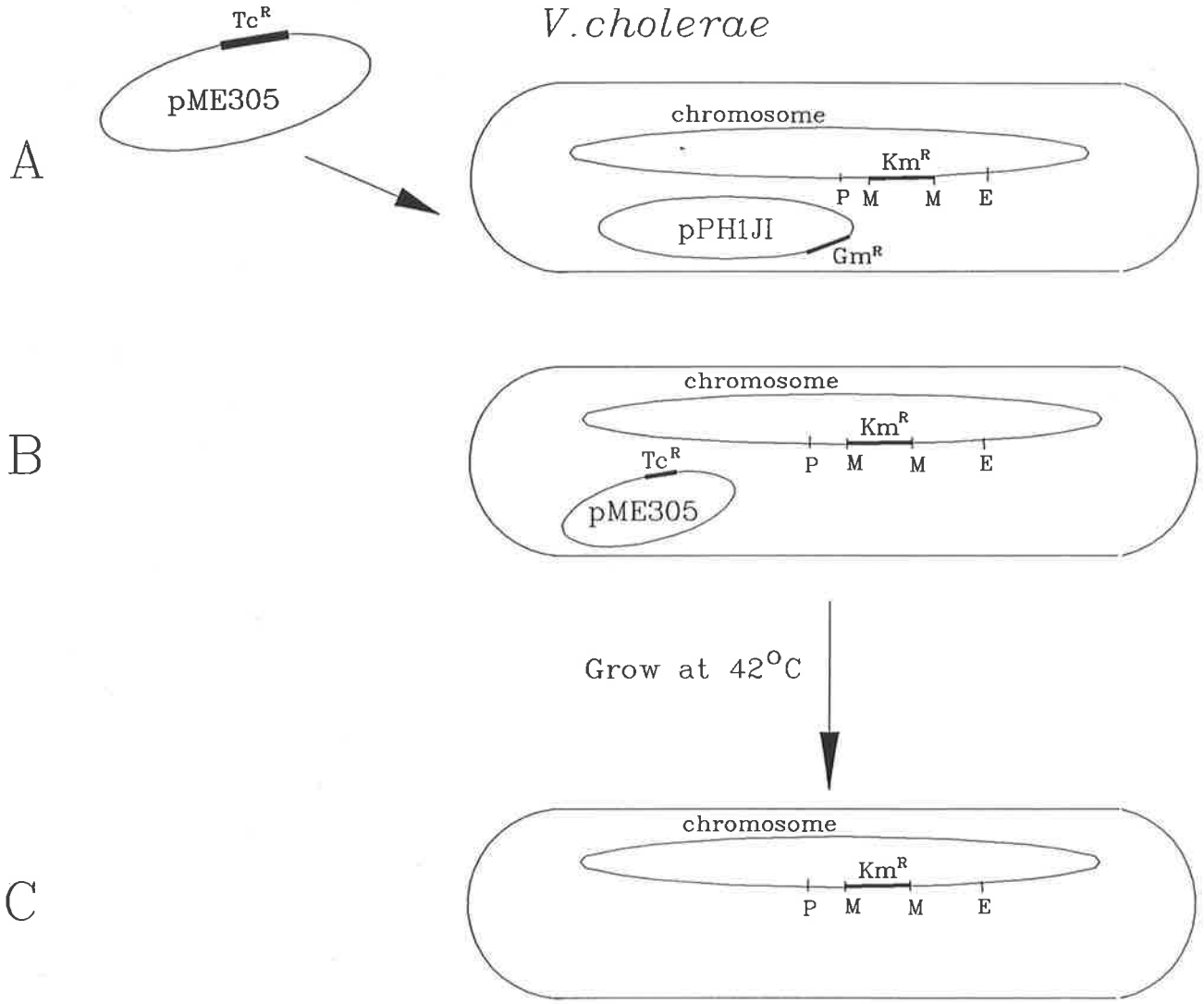
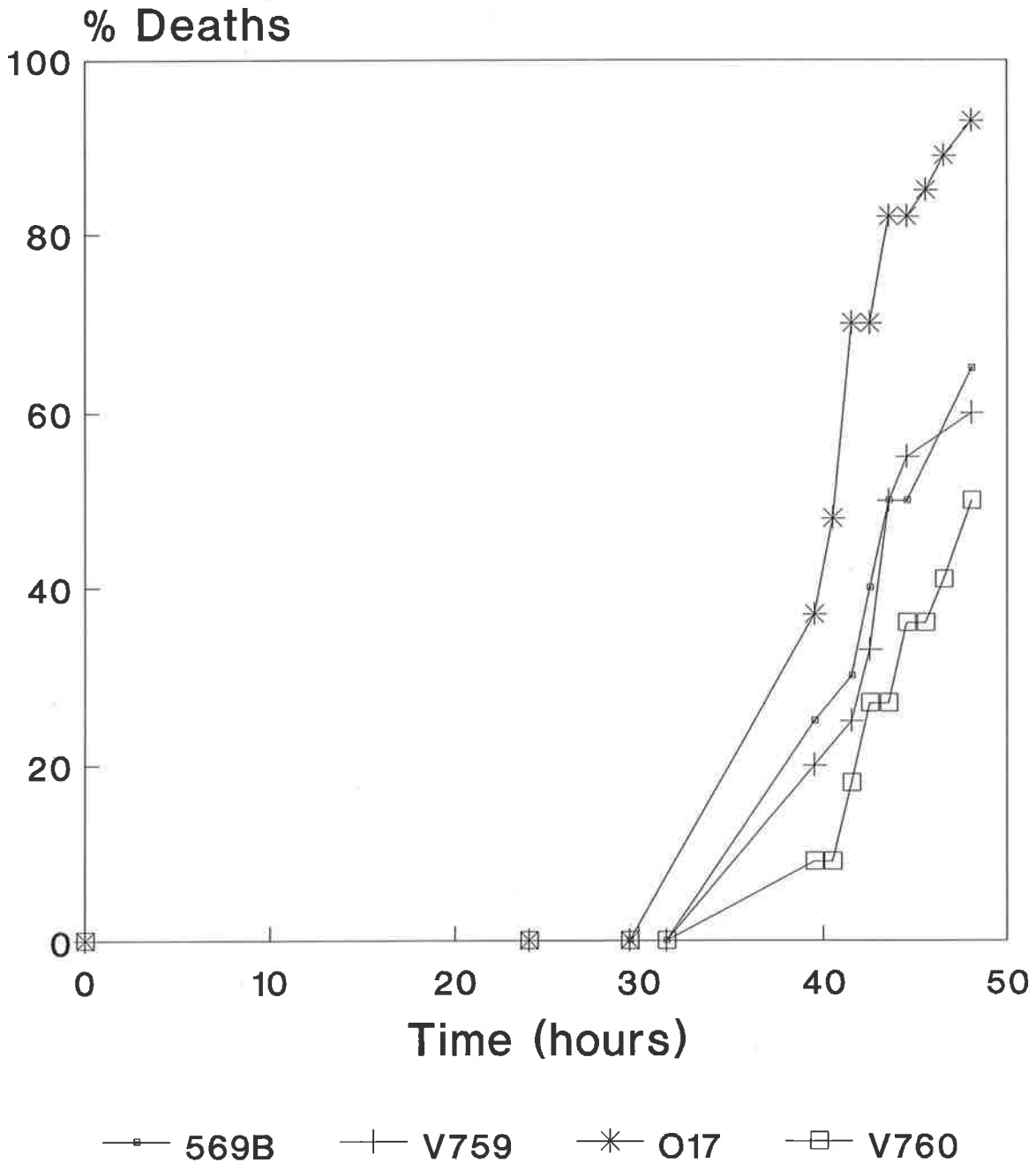

A
Tc^R
pME305
V.cholerae
chromosome
Km^R
P M M E
pPH1JI
Gm^R
B
chromosome
Km^R
Tc^R
P M M E
pME305
Grow at 42°C
C
chromosome
Km^R
P M M E

Figure 5.8 Effect of the *hlyA*::Km^R mutation on virulence in the infant mouse cholera model.

The *V.cholerae* strains O17, 569B and their respective *hlyA*::Km^R mutants were administered intragastrically to infant mice. The number of surviving mice at each time point was then recorded. The strains were administered with $5x10^6$ organisms. Although strain 569B is hypertoxigenic, it is also non-motile which reduces its LD_{50} significantly.


% Deaths
100
80
60
40
20
0
0
10
20
30
40
50
Time (hours)
569B
V759
O17
V760

At the time of death, the intestines of the infant mice were removed and prepared for histological examination. Marked histological differences were detected in the intestines of mice infected with 569B, O17, their *hlyA*::Km^R mutants compared with uninfected or mock-infected controls (Figure 5.9). Both 569B and O17 showed significant sloughing off of the epithelium compared to their mutants. This damage was most significant with O17, but was absent in both V759 and V760. In all cases the villi of the *V.cholerae* infected mice were swollen to almost fill the lumen and this was most marked with the hypertoxigenic strain 569B and its derivative V759.

5.2.3 Effect of the HlyA protein on cultured cells

5.2.3.1 ^{51}Cr release *in vitro* cytotoxicity assay

The marked intestinal epithelial damage corresponding to the presence of both HlyA and $HlyA^*$ led us to further investigate the cytolytic activity of the haemolysin. A mouse antigen presenting tumour cell line (P815) was internally labelled with ^{51}Cr. Culture supernatants from exponentially growing *V.cholerae* cells were incubated with the ^{51}Cr-labelled P815 for 4 h and then the amount of ^{51}Cr released was measured. The supernatants of these strains were diluted and checked for lytic activity (Table 5.1). This provides us with an indication of the percentage of ^{51}Cr-P815 cells that had been lysed. Culture supernatants of the haemolytic strain O17 and the nontoxigenic environmental strain 1074-78 readily lysed the cultured cells, whereas their respective *hlyA*::Km^R mutants had lost this ability. The cholera toxin deleted candidate vaccine strain, JBK70, used by Levine *et al.* (1988) and its parent strain Inaba N16961, displayed reduced lytic activity. However, when plated on blood agar they produced a smaller zone of haemolysis than O17 (Figure 5.10). This result was confirmed by performing haemolysis assays on culture supernatants, where it was noted that the haemolytic activity of JBK70 and Inaba N16961 was significantly less than that of O17. If the haemolysin was responsible for lysis of P815 cells, then the lytic activity of these strains would be expected to be much less than that of O17. The Classical strain 569B and a non-haemolytic El Tor strain H-1 did not show any significant lysis.

Figure 5.9 Histological examination of infant mouse intestines.

The intestines of the infant mice were removed at the time of death, preserved in formalin, set in resin and thin sections cut. The sections were stained with haematoxylin and eosin and examined by light microscopy. The marked histological differences between saline (panel A), uninfected (panel B), 569B (panel C), 569B *hlyA*::Km^R (panel D), O17 (panel E) and O17 *hlyA*::Km^R (panel F) are apparent. Panels E and C display marked swelling of the intestinal ~~lumen~~ villi whereas the mutants lack this obvious swelling. The epithelial border in panel E has also been degenerated when compared to the mutant strain in panel F, and this effect is attributable to the presence of the HlyA protein.

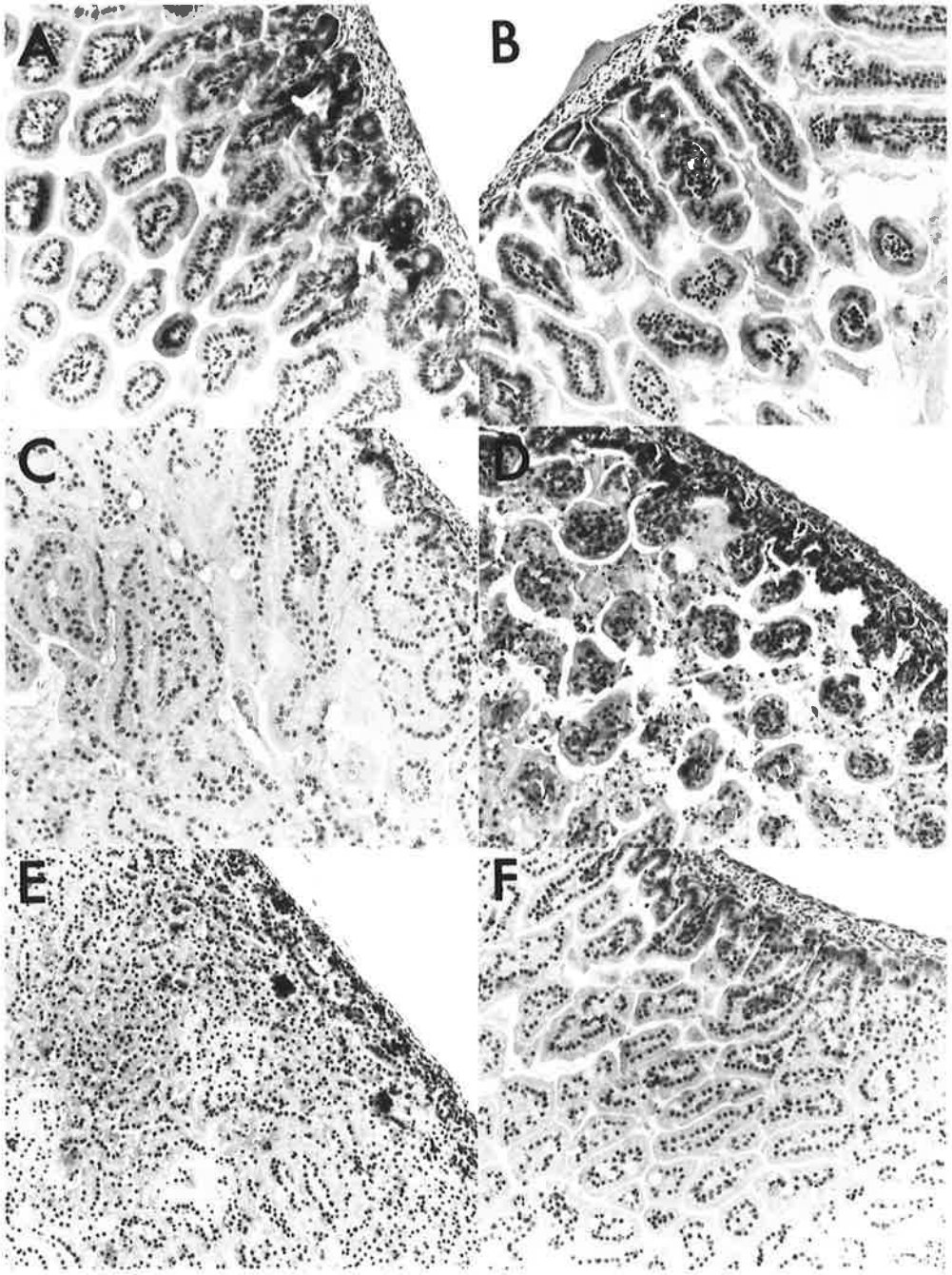

A
B
C
D
E
F

Table 5.1

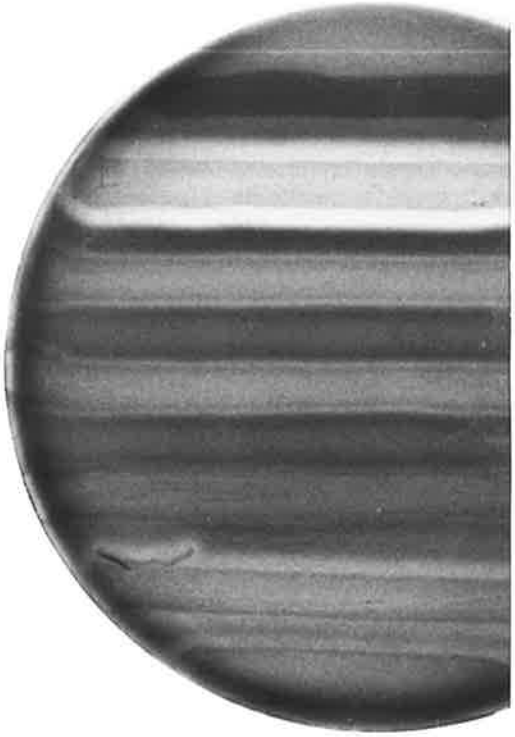
Percentage ^{51}Cr release from culture supernatants

Strain	Percentage released (Std. Dev.)		
	1/2	1/4	1/32
O17	77.1 (5.3)	81.4 (0.5)	75.0 (1.7)
O17*hlyA*::Km^R	0.8 (3.2)	0.2 (2.2)	
1074-78	51.1 (6.6)	26.9 (1.3)	4.5 (1.9)
1074-78*hlyA*::Km^R	0.6 (1.7)	1.2 (2.2)	
N16961	36.1 (3.3)	11.6 (5.0)	
N16961*hlyA*::Km^R	-1.8 (2.4)	-6.5 (1.9)	
JBK70	18.3 (4.6)	1.4 (3.9)	
JBK70*hlyA*::Km^R	-4.5 (1.0)	-2.5 (2.5)	
569B	-1.0 (3.03)	-0.76 (3.2)	
569B*hlyA*::Km^R	-4.7 (1.2)	Not determined	
H-1	-1.0 (3.0)	Not determined	
H-1*hlyA*::Km^R	1.2 (1.1)	Not determined	

The culture supernatants were diluted 1/2, 1/4 or 1/32 and the amount of spontaneous release has been deducted.

Figure 5.10 Haemolytic activity of *V.cholerae* strains.

The *V.cholerae* strains JBK70, N16961, O17, 569B, 1074-78 and 1196-78 were grown on NA containing 5% sheep erythrocytes. It can be seen that N16961 and its Δ*ctxAB* mutant, JBK70 are far less haemolytic than O17 while H-1 and 569B are clearly non-haemolytic. The two environmental strains (1074-78 and 1196-78) are only slightly less haemolytic than O17.



569B

O17

N16961

JBK70

H-1

1074-78

1196-78

The haemolytic activities of the strains parallels the results observed with ^{51}Cr-P815 cell lysis.

5.2.3.2 Cytotoxicity in HEp-2 cells

Monolayers of cultured HEp-2 cells were prepared and incubated with *V.cholerae* strains and their *hlyA* mutants. After incubation for 15 min at 37°C with O17, the HEp-2 cell monolayers were disrupted and significant cell degradation and lysis had occurred whereas its *hlyA*::Km^R mutant, V760, showed very little damage (Figure 5.11). Both the non-hemolytic Classical strain 569B and its *hlyA*::Km^R mutant did not produce any HEp-2 cell lysis after the incubation, however, there appeared to be changes in the morphology of the HEp-2 cells in the presence of 569B with the cells becoming elongated. However, this cell elongation was not seen with the 569B *hlyA*::Km^R mutant (Figure 5.11).

5.2.4 Fluid accumulation in ligated rabbit ileal loops

V.cholerae strains O17, 569B and JBK70 and their respective *hlyA*::Km^R mutants were introduced into ligated ileal segments of adult rabbits. The amount of fluid secreted into the rabbit gut was examined after 5 h and 12 h (Table 5.2). After 5 h fluid accumulation was detected with strain 569B but not with its *hlyA*::Km^R mutant. Only after 12 h did both 569B and its *hlyA*::Km^R mutant produce similar amounts of fluid accumulation (Figure 5.12). The El Tor strain O17 produces a very viscous mucoid fluid after 12 h and the fluid is visibly red when compared to the non-haemolytic Classical strain. However, the O17 *hlyA*::Km^R mutant caused no fluid accumulation after 5 h and the fluid seen after 12 h was less viscous and not noticeably bloody. At 12 h the nontoxigenic strain JBK70 causes weak fluid accumulation that corresponds to the mild diarrhoea seen in volunteers that had received this strain as a vaccine (Levine *et al.*, 1988). However, the JBK70 *hlyA*::Km^R mutant showed no fluid accumulation.

Figure 5.11 Cytotoxicity on cultured HEp-2 cells.

Monolayers of cultured HEp-2 cells, a human epithelial cell line were incubated with *V.cholerae* strains O17 and 569B and their *hlyA*::Km^R mutants, V760 and V759. Panel A represents an uninfected monolayer, whereas Panels B and C are monolayers infected with strains O17 and V760, respectively. The cells in Panel B show extensive lysis and only cell debris can be seen after microscopic examination. Panels D and E are monolayers infected with strains 569B and V759, respectively. No significant lysis is seen with the Classical strain nor its *hlyA*::Km^R mutant, although cell elongation is seen in panel D but not in panel E.

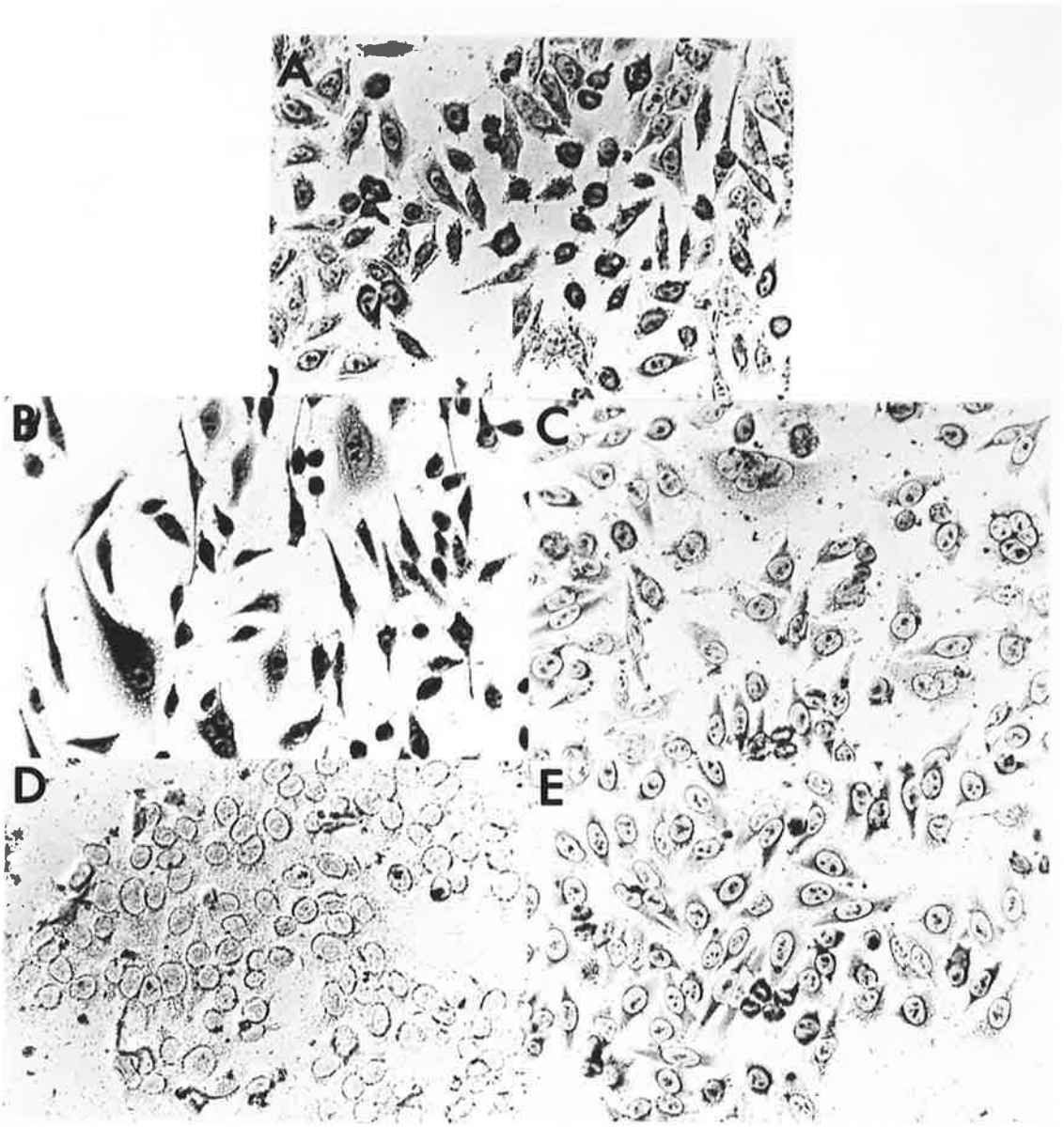

A
B
C
D
E

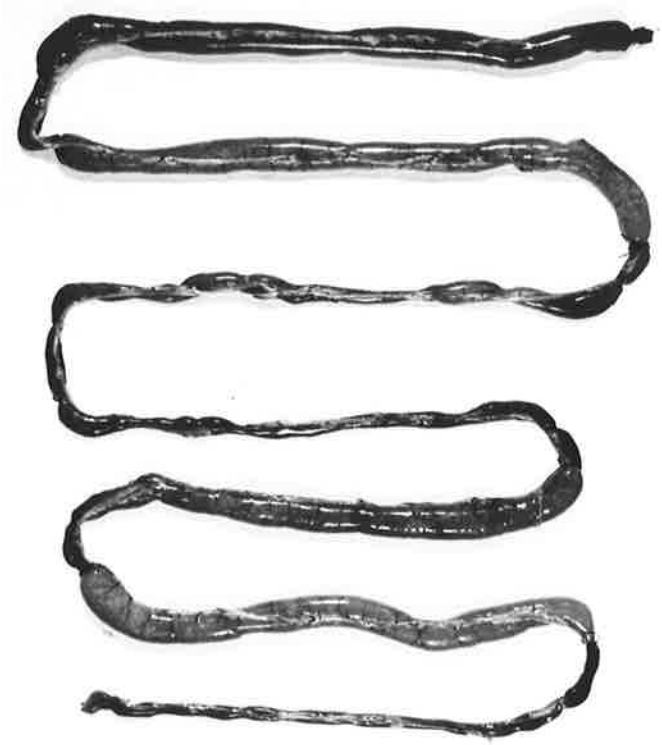
Table 5.2

Fluid Accumulation in Rabbit Ileal Loops

Strain	Time (hr) in animal	Fluid recovered(ml)
Luria broth	5	Nil
	12	Nil
O17	5	2.1
	12	12.24
O17*hlyA*::Km^R	5	Nil
	12	13.22
569B	5	7.1
	12	14.00
569B*hlyA*::Km^R	5	Nil
	12	14.55
JBK70	12	2.1
JBK70*hlyA*::Km^R	12	Nil

Figure 5.12 Fluid accumulation in ligated ileal loops of rabbits.

1 ml of the bacterial cultures (2 x 10^8 organisms) were introduced into ligated segments of the ileum in an adult rabbit. After 12 hours the rabbit was sacrificed and the contents of the ligated loops examined. The amount of fluid in the segments is listed in Table 5.2.



569B

569B *hlyA*::Km^R

JBK70

JBK70 *hlyA*::Km^R

O17

O17*hlyA*::Km^R

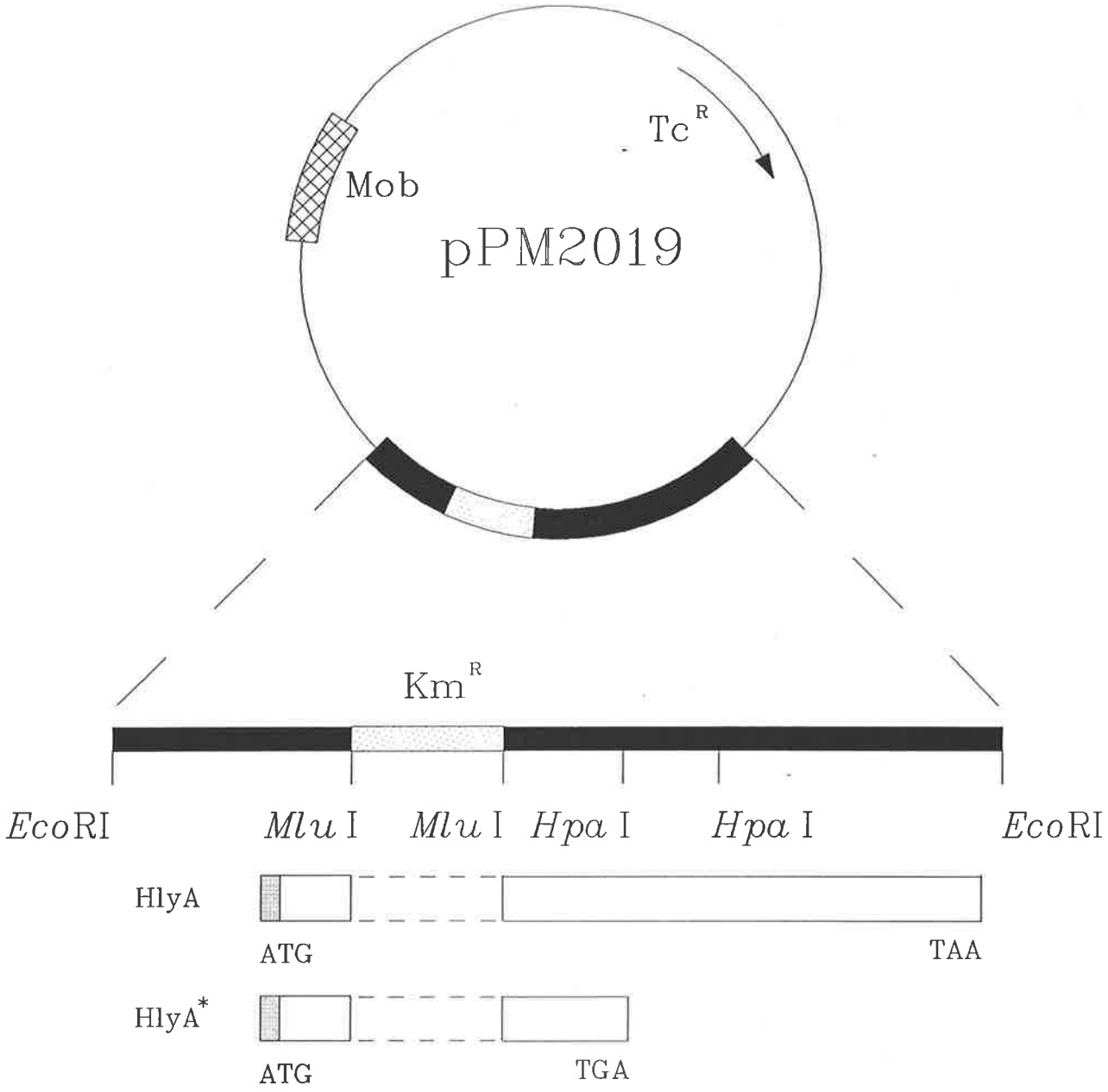
LB

5.3 Summary and Conclusions

It has previously been shown that a cytotoxin seen in *V.cholerae* of both the O1 and non-O1 serotypes stimulates fluid secretion in the rabbit ileal loop assay, and that this cytotoxin appears identical to the El Tor haemolysin (Madden *et al.*, 1984). In order to ascertain whether the haemolysin was responsible for these activities as well as being an alternative diarrhoeagenic factor in non-toxigenic candidate vaccine strains, a specific mutant in the structural gene, *hlyA*, was constructed. An insertion mutation was introduced into the *Mlu*I site since this would not only prevent the HlyA protein being made but also the truncated HlyA* product seen in the Classical *V.cholerae* strain 569B (Figure 5.13). The experiments reported here clearly indicate that the El Tor haemolysin, HlyA, of *V.cholerae* has both cytotoxic (enterotoxic) and cytolytic (haemolytic) activity. This is in agreement with observations by Yamamoto *et al.* (1986) using purified haemolysin. However, the cytolytic activity is not associated with the truncated protein, HlyA*, produced by Classical strains, typified here by strain 569B. This is consistent with previous observations (Chapter 3) which suggest that the ability to lyse sheep erythrocytes rests in the C-terminal portion of HlyA, corresponding to the region not expressed in HlyA* of Classical strains. Together with other data our observations imply that the N-terminal region of HlyA which is conserved in Classical strains in the form of HlyA* has enterotoxic activity.

Figure 5.13 Construction of the *hlyA* mutation.

Plasmid pPM2019 has the Km^R cartridge inserted in the *Mlu*I restriction site of the *hlyA* gene. This mutant would fail to produce both the HlyA and $HlyA^*$ product. The Hly^- mutant described by Kaper *et al.* (1986) was constructed by deleting the 398 base pair *Hpa*I fragment which results in a non-haemolytic phenotype but may not completely inactivate the truncated $HlyA^*$ product.


Tc R
Mob
pPM2019
Km R
EcoRI
Mlu I
Mlu I
Hpa I
Hpa I
EcoRI
HlyA
ATG
TAA
HlyA*
ATG
TGA

CHAPTER 6

CHARACTERIZATION OF THE *hlyB* GENE AND ITS ROLE IN HAEMOLYSIN PRODUCTION

6.1 Introduction

Some Gram negative bacteria possess the ability to actively excrete proteins into the extracellular milieu. This necessitates the translocation of these molecules across both the inner and outer membranes from one hydrophilic environment (cytoplasm) to another (external medium) without being trapped in the periplasmic space. The processes involved are poorly understood, however, models have been developed for several proteins and in most cases require the function of accessory genes.

Escherichia coli associated with extra-intestinal infections in humans commonly excrete an α-haemolysin (Hughes *et al.*, 1985; Minshew *et al.*, 1978), and its secretion into the extracellular medium is controlled by a group of genes that may be located either on the chromosome or on transmissible plasmids (Goebel *et al.*, 1974). The passage of HlyA from the cytoplasm across the cytoplasmic and outer membranes and the release from the cell surface requires the function of two cistrons, *hlyB* and *hlyD* and mutations in either gene leads to accumulation of intracellular haemolysin (Wagner *et al.*, 1983). The gene products of the *hlyB* and *hlyD* genes have been localised in the cytoplasmic membrane of *E.coli*. The lack of a signal sequence on HlyA or a periplasmic form of this protein has led to a number of models being proposed for the secretion of the haemolysin (Mackman *et al.*, 1986; Oropeza-Wekerle, 1989; see Section 1.5.1).

The production and excretion of haemolysin by *Serratia marcescens* requires two genes *shlA* and *shlB* (Braun *et al.*, 1987). ShlA is the actual haemolysin, while ShlB is required both for activation and for the release of the active protein from the cell surface (Poole *et al.*, 1988).

Another example of an excreted protein is the IgA protease produced by pathogenic *Neisseriae*, such as *N. gonorrhoeae* (Pohlner *et al.*, 1987). The mature enzyme has been reported to possess a size of 106 kDa, whereas nucleotide sequence analysis demonstrates that the IgA protease is transcribed as a 169 kDa precursor. This large precursor has been shown to be composed of three domains. Firstly, there is the typical leader or signal sequence that is commonly seen with excreted proteins, and this is thought to initiate the transport of the protein across the inner membrane barrier. The second domain is the IgA protease itself and the last domain is a 'helper' region that is located at the carboxy terminal end of the precursor that is involved in the extracellular excretion of the mature protease (Pohlner *et al.*, 1987). This 'helper' domain has been proposed to act as a pore that traverses the outer membrane through which the protease can travel. The IgA protease is postulated to fold into an active conformation during extracellular transport through this pore, and is then released autoproteolytically into the extracellular medium from the periplasm (Pohlner *et al.*, 1987).

When expressed in *E.coli* K-12, the *V.cholerae* haemolysin, HlyA, remains localized in the periplasmic space (Mercurio and Manning, 1985) suggesting that although the structural component had been cloned, additional information was required for the protein to be exported from the cell. In additon to *hlyA*, Manning *et al.* (1984) identified two further cistrons, *hlyB* and *hlyC*, which also affected the production of the haemolysin. The absence of *hlyB* in the clone of Goldberg and Murphy (1984) probably accounts for the long incubation times required to detect activity in *E.coli* K-12.

This chapter analyses the *hlyB* gene and seeks to describe the role of its product in synthesis and export of HlyA by constructing specific *hlyB*::Km^R mutants in the *V.cholerae* chromosome.

6.2 Results

6.2.1 Construction of *hlyB* mutants of *V.cholerae*

6.2.1.1 Introduction of a Km^R cartridge into *hlyB*

In order to analyse the function of the *hlyB* gene product, a specific mutation in this gene needed to be constructed in *V.cholerae*. Plasmid pPM431 was digested with *Pst*I and *Eco*RI, and synthetic *Eco*RI linkers were added so that the *hlyB* gene could be cloned into pBR325 on a *Eco*RI fragment to generate plasmid pPM2014 (Figure 6.1). This was followed by the insertion of a Km^R cartridge on a *Bam*HI fragment from plasmid J225 into the unique *Bam*HI restriction site within *hlyB*. Plasmid pPM2014 was partially digested with *Bam*HI so that the Tc^R gene was not inactivated. Km^R, Tc^R transformants were then selected, resulting in the isolation of plasmid pPM2015. This plasmid provided both a non-functional *hlyB* gene, and also a powerful selection (Figure 6.1). The inactivated *hlyB*::Km^R gene was subsequently inserted into the single *Eco*RI cloning site within pPM1287 (Section 5.2.1.2) to generate plasmid pPM2016.

6.2.1.2 Introduction of pPM2016 into *V.cholerae*

Plasmid pPM2016 was introduced into the *V.cholerae* strains O17 and 569B by conjugation from *E.coli* strain S17-1. After allowing recombination, this construct was then expelled from the *V.cholerae* strains by the introduction via conjugation of an incompatible plasmid, pPH1JI. Selection for both Km^R and the Gm^R encoded by pPH1JI ensured that the *V.cholerae* cell had undergone a double crossover recombination event that had replaced its effective copy of the *hlyB* gene with the inactivated substitute (Figure 6.2).

To confirm the presence of the *hlyB* mutation in the chromosome of *V.cholerae* strain O17, whole genomic DNA was extracted and digested with either *Eco*RI or *Pst*I + *Eco*RI and examined by Southern hybridization using pPM2009

Figure 6.1 Construction of the *hlyB* negative, Km^R, mobilizable plasmid pPM2016.

The *Eco*RI - *Pst*I fragment containing *hlyB* was cloned from pPM431 into the *Eco*RI site of pBR325 after synthetic *Eco*RI linkers were added to generate pPM2014. The Km^R cartridge from J225 was removed on a *Bam*HI fragment and inserted into the unique *Bam*HI site in *hlyB* yielding pPM2015. The *Eco*RI fragment containing the inactivated *hlyB*::Km^R gene was then cloned into the unique *Eco*RI site of pPM1287 to give pPM2016.

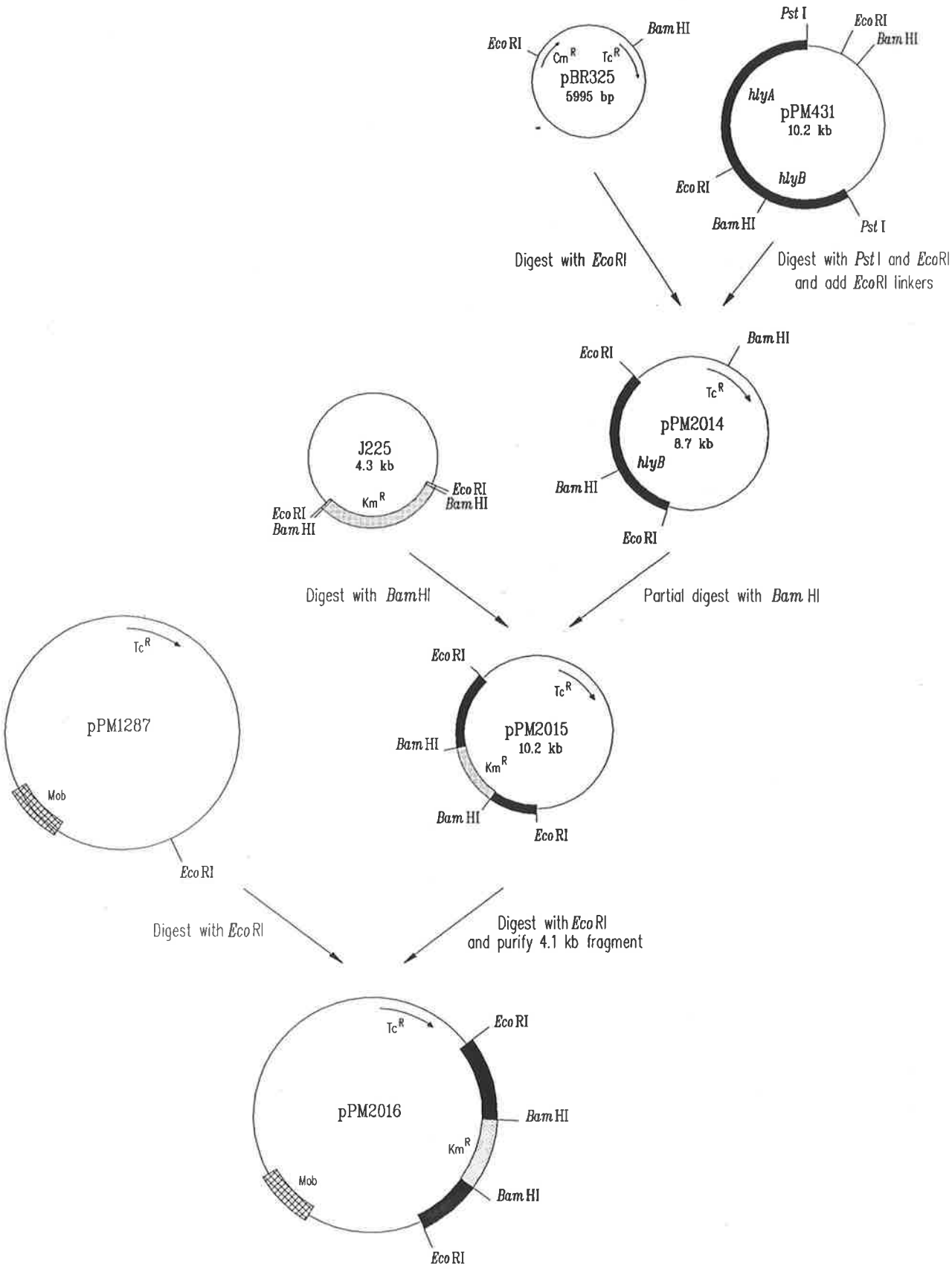
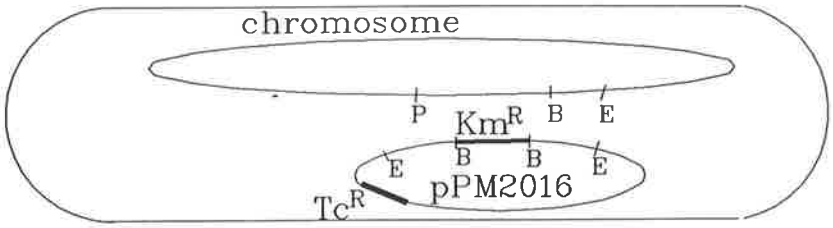

Eco RI
Bam HI
Cm R
Tc R
pBR325
5995 bp
Pst I
Eco RI
Bam HI
hlyA
pPM431
10.2 kb
hlyB
Eco RI
Bam HI
Pst I
Digest with Eco RI
Digest with Pst I and Eco RI
and add Eco RI linkers
Eco RI
Bam HI
Tc R
pPM2014
8.7 kb
hlyB
Bam HI
Eco RI
J225
4.3 kb
Km R
Eco RI
Bam HI
Eco RI
Bam HI
Digest with Bam HI
Partial digest with Bam HI
Tc R
pPM1287
Mob
Eco RI
Eco RI
Tc R
pPM2015
10.2 kb
Bam HI
Km R
Bam HI
Eco RI
Digest with Eco RI
Digest with Eco RI
and purify 4.1 kb fragment
Tc R
Eco RI
pPM2016
Bam HI
Km R
Mob
Bam HI
Eco RI

Figure 6.2 Introduction of a *hlyB*::Km^R mutation into the chromosome of *V.cholerae*.

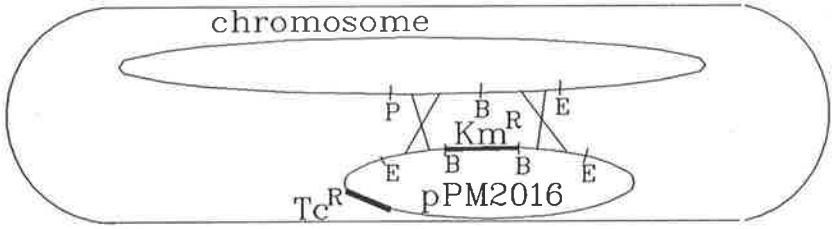
Plasmid pPM2016 (Tc^R, Km^R) was mobilized from *E.coli* strain S17-1 into the *V.cholerae* strains O17 and 569B (panel A). The resulting transconjugants were Sm^R, Tc^R, Km^R. Recombination between the chromosome and pPM2016 was allowed to occur (panel B). Plasmid pPH1JI (Gm^R), also belonging to the IncP incompatability group was introduced via conjugation into the *V.cholerae* strains containing pPM2016 (panel C). Sm^R, Km^R, Gm^R colonies were then selected and screened for Tc^S (panel D). The *V.cholerae* strains in which the *hlyB*::Km^R mutation had apparently recombined into the chromosome were isolated and analysed further.

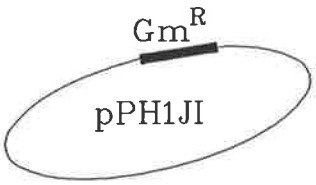
V. cholerae

A

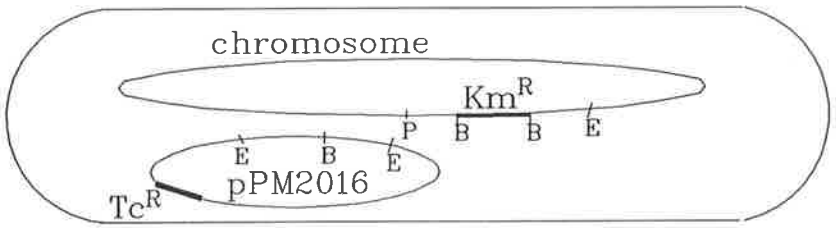


B

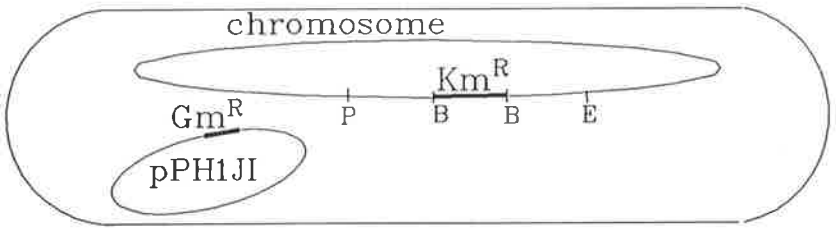




C



D



(*Eco*RI - *Pst*I fragment encoding *hlyB* cloned into pGB2) as a probe (Figure 6.3). There are no *Eco*RI sites within the Km^R cartridge once it has been removed on a *Bam*HI fragment, however, there are two *Pst*I sites at each end of the cartridge which are still present. Thus, digestion of the potential mutants with *Eco*RI results in an increase in size of 1.5 kb of a chromosomal *Eco*RI fragment carrying *hlyB* due to the Km^R cartridge (Figure 6.3). Insertion of the Km^R cartridge into the wild-type 2.9 kb fragment introduces two new *Pst*I sites (Figure 6.4). Upon digestion with *Eco*RI and *Pst*I, the *hlyB* probe now hybridizes with the homologous fragments on either side of the *Bam*HI site, but with the Km^R cartridge fragment not reacting (Figure 6.4). Plasmid pPH1JI was also included to ascertain whether any cross-reaction would occur. It can be seen in the mutant strains that some common bands within pPH1JI do hybridize to the probe. These cross-reacting bands are probably due to the Sp^R gene carried by both pPH1JI and pGB2.

6.2.2 Effect of the *hlyB* mutation on haemolysin production in *V.cholerae* strain O17

The *V.cholerae* O17 *hlyB* mutant, V745, was then assayed in order to determine the effect of the mutation on haemolytic activity. Cells were taken at different stages of growth, incubated with washed sheep erythrocytes, and samples taken at 20 min intervals to follow the development of haemolysis with bacterial growth (Figure 6.5). It can be seen that there is a significant lag in the rate of development of haemolytic activity with the *hlyB*::Km^R mutant. This seems to indicate that in the mutant, the haemolysin is only released into the extracellular medium when the cells enter stationary phase and are subject to autolysis.

This proposal, that the haemolysin was trapped within the cell during growth, and only released due to cell lysis in stationary phase was examined. Cultures of both O17 and its *hlyB*::Km^R mutant were taken at various stages of growth, and the cells separated from the extracellular medium by centrifugation. The cells were then sonicated in fresh medium to release intracellular contents and both cellular and extracellular fractions were assayed for haemolytic activity (Figure 6.6). This provided an indication as to the relative haemolytic activity and its distribution at a particular

Figure 6.3 Southern hybridization analysis of *hlyB*::Km^R mutants.

Whole genomic DNA of the *V.cholerae* parents and their *hlyB*::Km^R mutants were digested with *Eco*RI or *Eco*RI + *Pst*I and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α-[^{32}P]-dCTP nick translated pPM2009, washed and subjected to autoradiography.

The 1.5 kb larger *Eco*RI fragment in the *hlyB*::Km^R mutants compared to the parents is evident, whereas with the *Eco*RI + *Pst*I digest the wild-type fragment is split into two smaller fragments due to the introduction of two *Pst*I sites at the ends of the Km^R cartridge. The cross reaction between plasmid pPH1JI and pPM2009, due probably to the Sp^R encoded by both is evident. SPP1 phage DNA digested with *Eco*RI was used as a standard and the size of the fragments generated in kb are shown on the right.

8.37
7.20
6.05
4.90
3.55
2.63
1.73
1.61
1.29
1.19
0.99
SPP1 - EcoRI
DH1[pPH1JI]
569B
569B hlyB::Km^R
O17
O17 hlyB::Km^R
EcoRI
DH1[pPH1JI]
569B
569B hlyB::Km^R
O17
O17 hlyB::Km^R
EcoRI + PstI

Figure 6.4 Diagrammatic representation of the fragments detected in Southern hybridization of the *V.cholerae* O17 *hlyB* region.

The fragments detected by Southern hybridization (Figure 6.3) can be explained diagrammatically. Digestion of whole genomic DNA of *V.cholerae* O17 and its *hlyB*::Km^R mutant would give the red fragments upon digestion with *Eco*RI and the blue fragments upon digestion with *Eco*RI + *Pst*I. The insertion of the Km^R cartridge into the *Bam*HI site would effectively disrupt the *hlyB* coding sequence, and increase the size of the *Eco*RI fragment by 1.5 kb. The *Eco*RI - *Pst*I fragment would be split into two smaller fragments due to the *Pst*I sites at the ends of the Km^R cartridge.

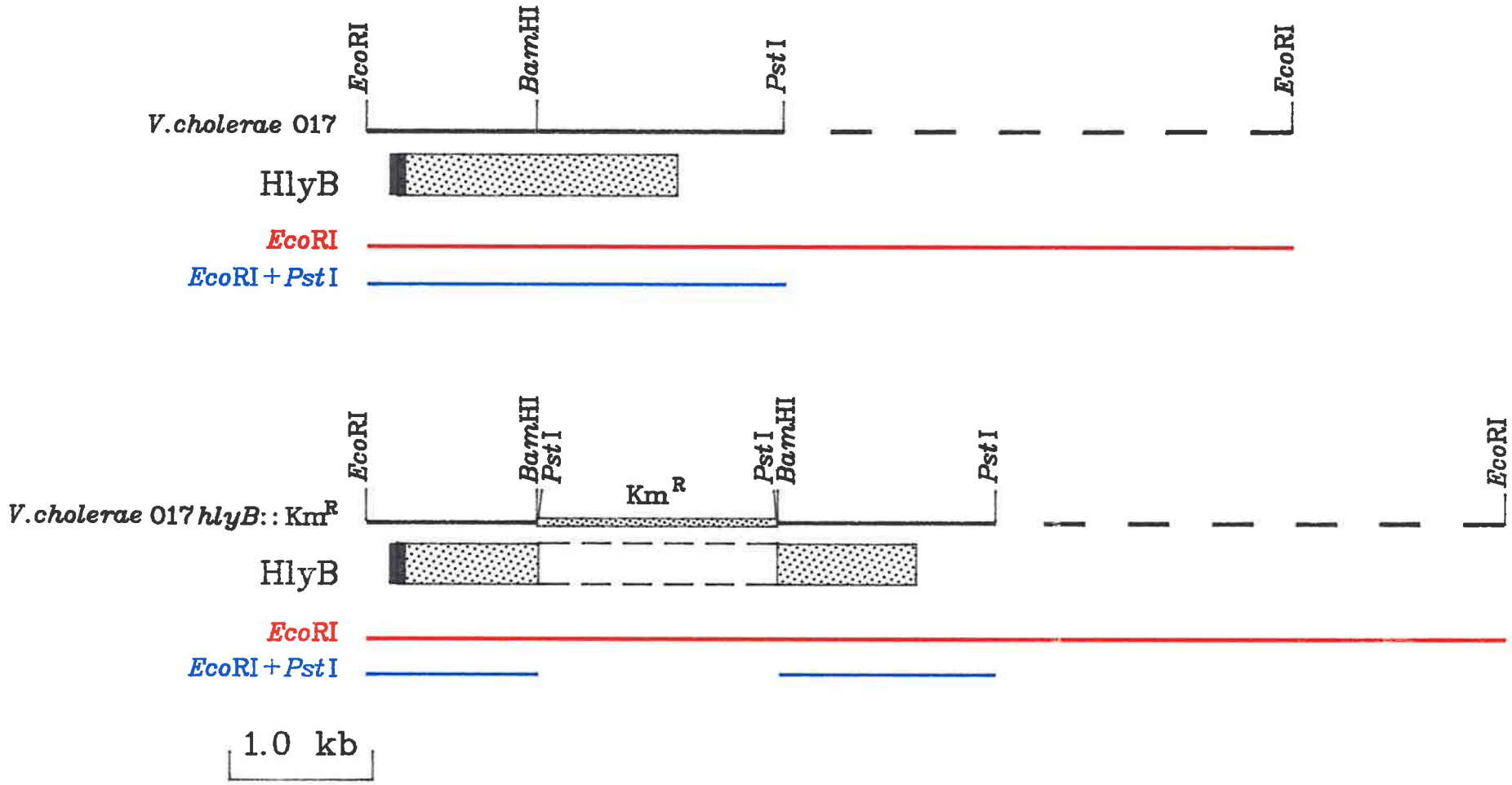

EcoRI
BamHI
PstI
EcoRI
V.cholerae 017
HlyB
EcoRI
EcoRI + PstI
EcoRI
BamHI
PstI
PstI
BamHI
PstI
EcoRI
V.cholerae 017 hlyB::KmR
KmR
HlyB
EcoRI
EcoRI + PstI
1.0 kb

Figure 6.5 Haemolytic activity of *V.cholerae* strains O17 and O17 *hlyB*::Km^R.

The haemolytic *V.cholerae* strain O17 and its *hlyB*::Km^R mutant were sampled at various stages of exponential growth and incubated with sheep erythrocytes and the development of haemolysis was measured over time.

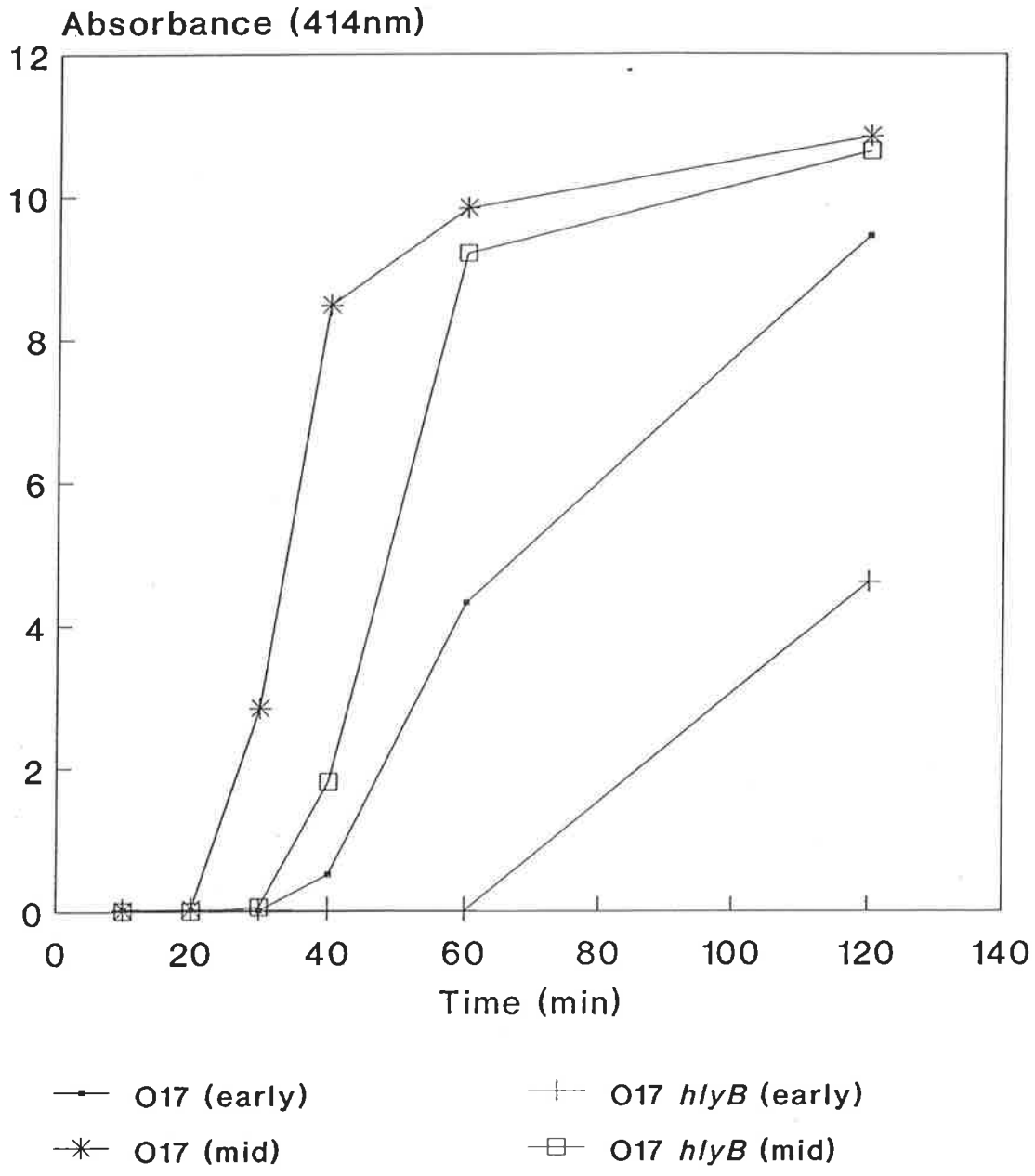
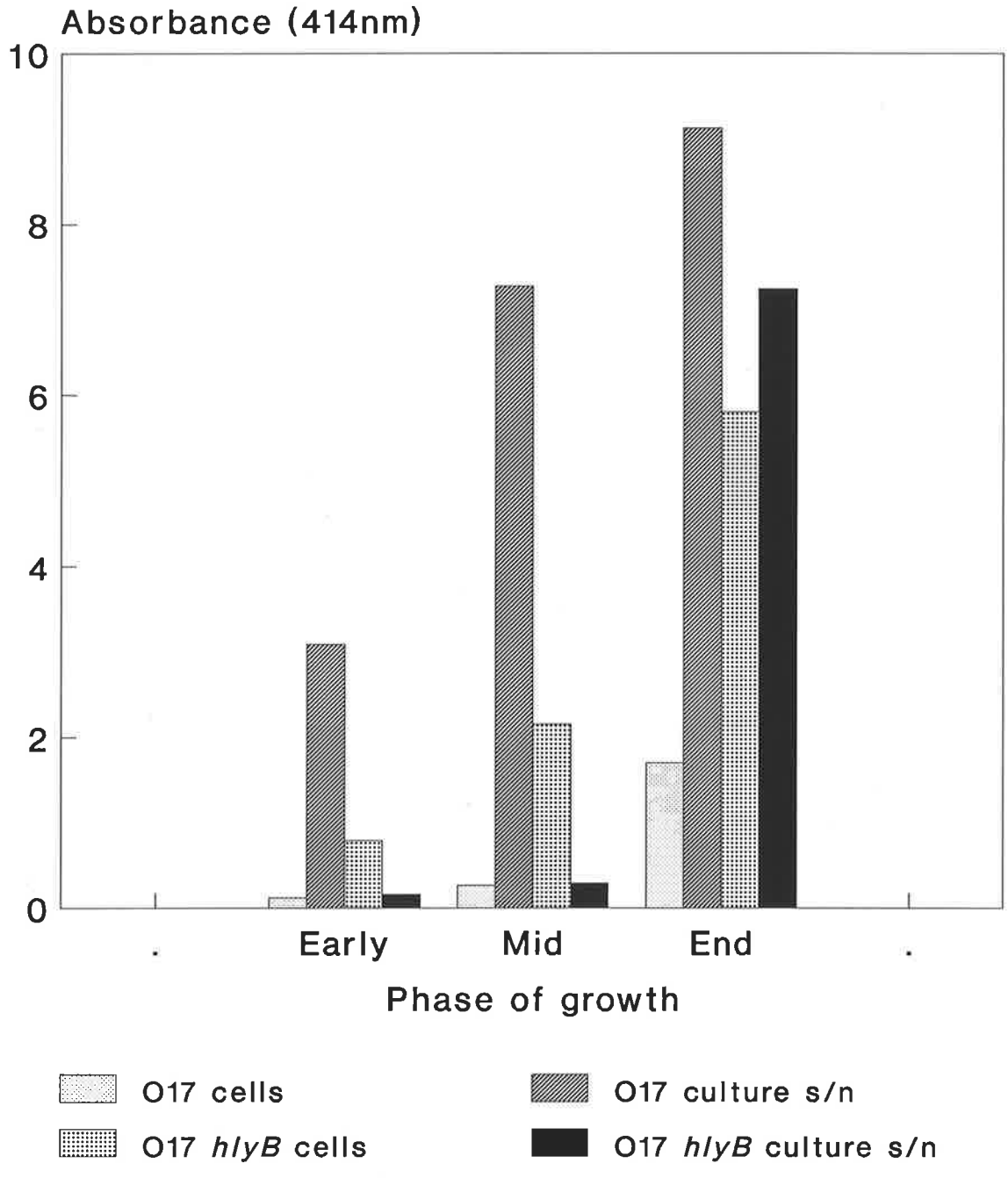

Absorbance (414nm)
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Time (min)
O17 (early)
O17 (mid)
O17 hlyB (early)
O17 hlyB (mid)

Figure 6.6 Haemolytic activity of culture supernatants and cell contents of *V.cholerae* strains O17 and O17 *hlyB*::Km^R.

V.cholerae strains O17 and O17 *hlyB*::Km^R were sampled at various stages of exponential growth and the culture supernatant separated from the bacterial cells. The cells were then sonicated to release any internal proteins and both fractions tested for haemolytic activity after 2 h.


Absorbance (414nm)
10
8
6
4
2
0
Early
Mid
End
Phase of growth
O17 cells
O17 hlyB cells
O17 culture s/n
O17 hlyB culture s/n

stage of growth. In the case of the O17 *hlyB*::Km^R mutant (V745), the haemolytic activity was trapped within the cell during growth, and very little could be detected in the culture supernatant. This contrasted with the parental strain, O17, in which the majority of the haemolytic activity is seen in the extracellular medium. Only in stationary phase is there significant haemolytic activity in the supernatant of V745. Thus, HlyB would appear to be necessary for the release of active haemolysin during exponential growth in *V.cholerae*.

6.2.3 Nucleotide sequence determination and analysis of *hlyB* from *V.cholerae* O17

The 2.9 kb *Pst*I - *Eco*RI fragment that carries the *hlyB* gene was cloned from pPM431 and ligated into M13mp18 and M13mp19. Sequencing was carried out using the chain termination method of Sanger *et al.* (1977, 1980) using Sequenase enzyme. Universal primer was employed to sequence from both directions and then synthetic oligodeoxynucleotides were made to extend the sequence. A map of the sequencing strategy showing the oligodeoxynucleotides used can be seen in Figure 6.7.

6.2.3.1 Nucleotide sequence analysis of *hlyB*

The sequence of the 2915 bp *Eco*RI - *Pst*I fragment encoding *hlyB* was determined in both directions (Figure 6.7) and is shown in Figure 6.8. Analysis of the sequence revealed the presence of an ORF beginning 135 bp after the *Eco*RI site. When translated, this ORF would give rise to a 548 aa protein of 60.3 kDa. However, Manning *et al.* (1984) had estimated the size of HlyB to be 71 kDa by SDS-PAGE analysis. The reason for this size discrepancy will be investigated later (Section 6.2.5).

The regulatory signals that are detected before the ATG start codon resemble the consensus sequences (as discussed in Section 4.2.2.1). A potential promoter can be detected 5' to the *hlyB* gene by virtue of the homology displayed between this region and the known *E.coli* promoter consensus sequences. The "-10 region" or "Pribnow box" located between nt 92 and 97 (Figure 6.7) is **TATAGG**, in which 4 of the 6 nucleotides match the consensus sequence TATAAT. However, the "-35 region", located between nt 73 and 78 (Figure 6.7) is **CTGACA**. This is in excellent

Figure 6.7 Strategy used for dideoxy sequencing of *hlyB*.

The *hlyB* gene was cloned into both M13mp18 and M13mp19. The 2.9 kb *Eco*RI - *Pst*I fragment was sequenced with universal primer. Synthetic oligonucleotides were constructed to extend the sequence in both directions. The strategy for sequencing the M13mp18 clone is shown in blue while the M13mp19 strategy is displayed in red. The 2.9 kb fragment was also sequenced with the -35 primer to accurately determine the extreme ends at the junction of the polylinker. The regions indicated with an asterisk were generated by double stranded sequencing of *hlyB*::Tn*1725* insertions.

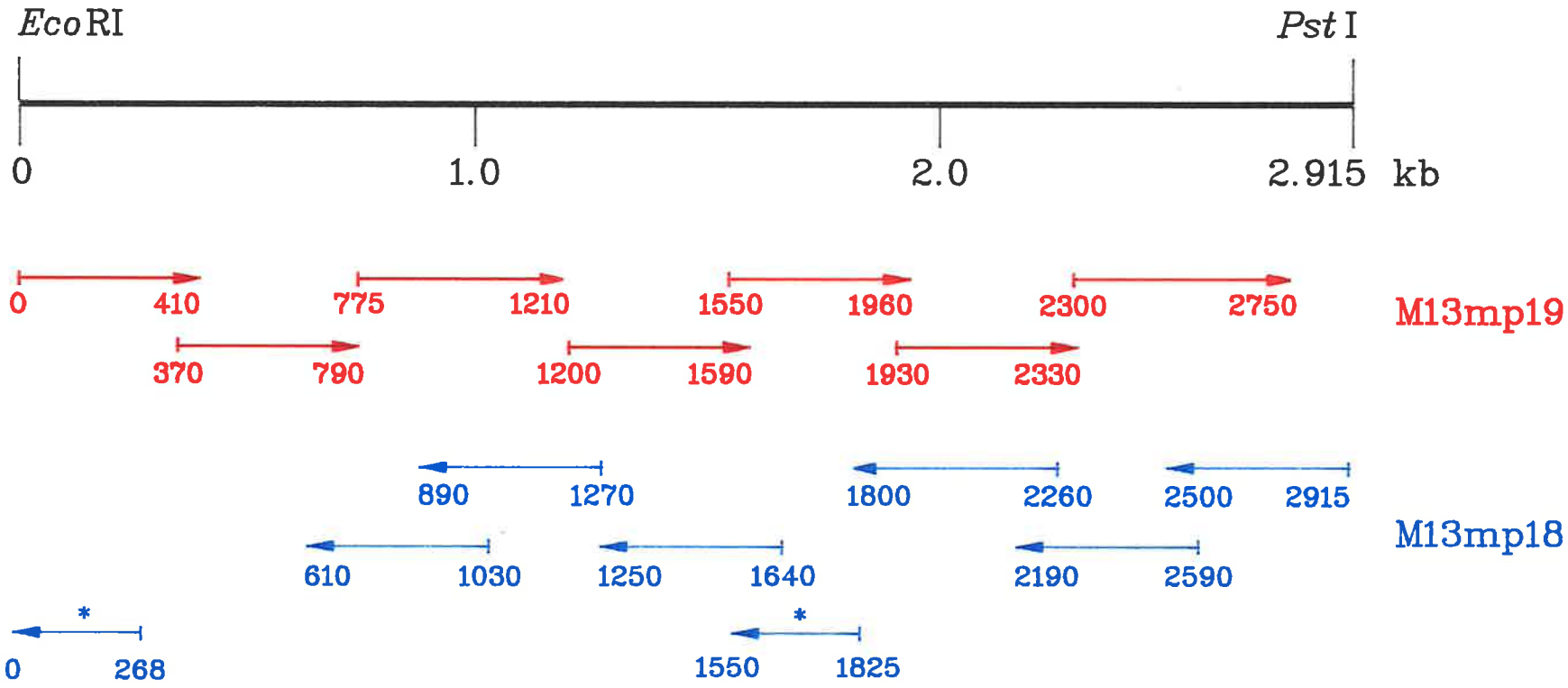

EcoRI
PstI
0
1.0
2.0
2.915 kb
0
410
775
1210
1550
1960
2300
2750
M13mp19
370
790
1200
1590
1930
2330
890
1270
1800
2260
2500
2915
M13mp18
610
1030
1250
1640
2190
2590
*
0
268
*
1550
1825

Figure 6.8 Nucleotide sequence of the 2.9 kb *Eco*RI - *Pst*I fragment of cloned *V.cholerae* DNA.

The nucleotide sequence of the 2915 base-pair region containing the *hlyB* gene (nt 135 to 1781). The *E.coli* consensus promoter sequences for the "Pribnow box" and the "-35 region" and the Shine-Dalgarno sequence are shown under the putative *hlyB* promoter. The putative signal sequence site after Ala_{17} is indicated with an upward arrow. The transcriptional terminator (nt 1847 to 1868) is indicated by head to head arrows. The nucleotides are numbered on the right whereas the amino acids within the open reading frame that codes for HlyB are numbered below the sequence beginning at the initiation codon (ATG-Met). The points of Tn*1725* insertions in the respective derivatives of pPM431 (Manning *et al.*, 1984) are indicated by arrowheads above the sequence and are discussed in Section 6.2.5. The second ORF situated 3' to *hlyB* lies between nt 2048 and 2560. The potential promoter sequences are shown with respect to the consensus sequences.

EcoRI

GAATTCCCTT TCACATTAGG GTAAAAGTAC GCAATCACAA TACTAAGCGC GAGACAGCGT AAGTCCAGTT TGCTGACATC CCTACAACCC ATATAGGTCA 100
TTGACA TATAAT

GCAGACTCTT TTTTCTGAAC GATAGGTACG AGAC ATG ATC ATC AAT AAA TTT TCC CTT AAA TGG ATG TTG GCT ATT GCC GTC GCC ATC 188
AGGT
Met Ile Ile Asn Lys Phe Ser Leu Lys Trp Met Leu Ala Ile Ala Val Ala Ile
1 10
pPM698

CCT GCG ATA CGA CTG TTG TTT GTG GCT TTC ACC AGT CTA AAC ACC ATG TCA GTG ATG CAA GCG CAG TCC AAC AGC TTG TAT GCC 272
Pro Ala Ile Arg Leu Leu Phe Val Ala Phe Thr Ser Leu Asn Thr Met Ser Val Met Gln Ala Gln Ser Asn Ser Leu Tyr Ala
20 30 40

AAC ACG GCT GCA CCA ATG CGT GCC ATG GCT GAA GCA ACC TCA CGT ATT CCT CGG ATG CGT GTC GGT ATC GAT ATG ATG CTA CTG 356
Asn Thr Ala Ala Pro Met Arg Ala Met Ala Glu Ala Thr Ser Arg Ile Pro Arg Met Arg Val Gly Ile Asp Met Met Leu Leu
50 60 70

CAA GAA ACG GCG CTC AAA GAT GCG AAA GGG GTC CTC AAA CGA GTC GAA GAG GCA AGA ACC GAA GAT ATC CCA GAA ATG CGT CAA 440
Gln Glu Thr Leu Ala Lys Asp Ala Lys Gly Val Leu Lys Arg Val Glu Glu Ala Arg Thr Glu Asp Ile Pro Glu Met Arg Gln
80 90 100

CGA ATG CAA GTT GCG GTT GAT TCT CAG GTT AAT CCG GAA CTC AAA GAG CAG GCA CGC AAA CTT CAA GCT CGT TTT GAA CAA ATG 524
Arg Met Gln Val Ala Val Asp Ser Gln Val Asn Pro Glu Leu Lys Glu Gln Ala Arg Lys Leu Gln Ala Arg Phe Glu Gln Met
110 120 130

GTA CGT GAA GAG TTA GAG CCT ATG CTG CAA GCC TTC GCC AAT AAC GAT ATG ACC ACG GCA CAA AAC ATT TAC CGC GAT AAA TAC 608
Val Arg Glu Glu Leu Glu Pro Met Leu Gln Ala Phe Ala Asn Asn Asp Met Thr Thr Ala Gln Asn Ile Tyr Arg Asp Lys Tyr
140 150

GCG CCG ACC TAT GGT GAA ATG CGT AAA CAA GCC AAC CAG ATC CTC GAT ACG CTT TTG CAG CAA GCG GAC AGG CAA AAC CAT GCC 692
Ala Pro Thr Tyr Gly Glu Met Arg Lys Gln Ala Asn Gln Ile Leu Asp Thr Leu Leu Gln Gln Ala Asp Arg Gln Asn His Ala
160 170 180

AGT GTG GAA AGC TTC GAA GCA GGA CGC ACC AAG CAA ATG GTG ATC ATT GCA GCA GGC TTG ATC ATT TCA TTC ATC ACT TCA CTG 776
Ser Val Glu Ser Phe Glu Ala Gly Arg Thr Lys Gln Met Val Ile Ile Ala Ala Gly Leu Ile Ile Ser Phe Ile Thr Ser Leu
190 200 210

GTT ATC ATA ACG AAC TTA CGT AGC CGA GTG GCT TAC CTG AAA GAT CGT ATG AGT TCT GCG GCG GCG AAT CTT TCA CTG CGT ACT 860
Val Ile Ile Thr Asn Leu Arg Ser Arg Val Ala Tyr Leu Lys Asp Arg Met Ser Ser Ala Ala Ala Asn Leu Ser Leu Arg Thr
220 230 240

CGA TTG GAG TTG GAT GGT AAC GAT GAA CTG TGT GAC ATC GGT AAA AGC TTC AAT GCG TTC ATT GAT AAA GTG CAT CAC TCG ATT 944
Arg Leu Glu Leu Asp Gly Asn Asp Glu Leu Cys Asp Ile Gly Lys Ser Phe Asn Ala Phe Ile Asp Lys Val His His Ser Ile
250 260 270

GAA GAA GTG GCA GAA AAC TCA AAA GAG CTG GCG ACG ATG GCC TCT AGT GTG TCG CAG CGC GCG CAC ATG ACG CAA TCT AAC TGT 1028
Glu Glu Val Ala Glu Asn Ser Lys Glu Leu Ala Thr Met Ala Ser Ser Val Ser Gln Arg Ala His Met Thr Gln Ser Asn Cys
280 290
pPM657

GCT TCG CAG CGA GAT AGA ACA GTG CAA GTT GCG ACG GCG ATT CAT GAG CTT GGT GCC ACC GTA TCC GAA ATC GCT TCC AAT GCG 1112
Ala Ser Gln Arg Asp Arg Thr Val Gln Val Ala Thr Ala Ile His Glu Leu Gly Ala Thr Val Ser Glu Ile Ala Ser Asn Ala
300 310 320

GCC ATG GCT CGC GAT GTC GCG AAC GAA GCG ACG CTG CAT TCT GGT GAA GGG AAA AAA GTG GTA GGC GAA GTG CAA AAT CGG ATC 1196
Ala Met Ala Arg Asp Val Ala Asn Glu Ala Thr Leu His Ser Gly Glu Gly Lys Lys Val Val Gly Glu Val Gln Asn Arg Ile
330 340 350

CAA ACA CTG GTC AAT GAA CTC GAT AAT GCC ACT CAA GTT GTC TCA TCA CTG GCG ACC CAA ATT AAC GGT ATT AGC TCA ACA CTT 1280
Gln Thr Leu Val Asn Glu Leu Asp Asn Ala Thr Gln Val Val Ser Ser Leu Ala Thr Gln Ile Asn Gly Ile Ser Ser Thr Leu
360 370 380

GAT ACC ATT CGC AGT ATT TCT GAG CAA ACG AAC CTA TTG GCG CTC AAC GCT GCG ATT GAA GCT GCG CGA GCG GGT GAA CAA GGT 1364
Asp Thr Ile Arg Ser Ile Ser Glu Gln Thr Asn Leu Leu Ala Leu Asn Ala Ala Ile Glu Ala Ala Arg Ala Gly Glu Gln Gly
390 400 410
pPM610

CGT GGT TTT GCG GTG GTG GCG GAT GAA GTT CGC ACA TTA GCA AGT CGT TCA GCG GCA TCG ACG GAA GAG ATC CAG CAA GTC ATT 1448
Arg Gly Phe Ala Val Val Ala Asp Glu Val Arg Thr Leu Ala Ser Arg Ser Ala Ala Ser Thr Glu Glu Ile Gln Gln Val Ile
420 430

AAT CGC CTT CAA ACG GAG TCA ACT CGC GCA GTA GAA GCA ATG GAA AAA GGT CGC TCG CAA AGT GAT GTG GTG GTT GAG TTT TCC 1532
Asn Arg Leu Gln Thr Glu Ser Thr Arg Ala Val Glu Ala Met Glu Lys Gly Arg Ser Gln Ser Asp Val Val Val Glu Phe Ser
440 450 460
pPM685

GCT AAA GCG AAC CAA TCT CTC ACA GAG ATC AAC AGC CAA ATT GAT CAG ATT AAT GAT CAA AAT ATT CAA GTT GCG ACC GCG ACA 1616
Ala Lys Ala Asn Gln Ser Leu Thr Glu Ile Asn Ser Gln Ile Asp Gln Ile Asn Asp Gln Asn Ile Gln Val Ala Thr Ala Thr
470 480 490

GAG GAA CAA TCA ACG GTG GTG GAA GAC ATT AAT CGC AAC GTT GAA GAC ATC AAC CAA CTG ACG ACA GAA ACC TCG CAT GTT GCG 1700
Glu Glu Gln Ser Thr Val Val Glu Asp Ile Asn Arg Asn Val Glu Asp Ile Asn Gln Leu Thr Thr Glu Thr Ser His Val Ala
500 510 520

GAT GAG TTA AGC CGA GCC AGT GCA AGC TTG CAA CGT CTC TCT TCG CAA CTG GAT AAA CTG GTG GGC AGT TTT GAA CTT TAA 1781
Asp Glu Leu Ser Arg Ala Ser Ala Ser Leu Gln Arg Leu Ser Ser Gln Leu Asp Lys Leu Val Gly Ser Phe Glu Leu ***
530 540
pPM642

CCAATGACTA CGCTAAGCAA GCATTCATGT CGCTTGCTGA TGACGTTGAT ATTCTATTTC TCACTAAGCG GCCTTTCTAG GCCGCTTTTT TATCGCTCTT 1881
CGCCTTTTTT ATGTTGCCTC AACCAAAGTT GGGGTTGTAC ACATTTTGTG TTAATGAAAT GTTATTAATT TTATGTTTTG TTCACATTTT GACCTTTTGA 1981
TTGA

GATTCGGAAA TCCGCCACTT CTCTTCTAGC TTAATTAACG GTAAGAGGTC TGACTTCTAC CGAGTA ATG ACC CTG TTT TCA GTT AAT GAG ACT 2074
CA TATAAT GAGGT
Met Thr Leu Phe Ser Val Asn Glu Thr
1

CAC GCC ACT TCG TGT GGT TCC AAG ACT GAA GAC AAG AAC GAG AAG GAA TAT CCC TTG AAT AAA ATC ATT ATA TTA ATC GCT CTC 2158
His Ala Thr Ser Cys Gly Ser Lys Thr Glu Asp Lys Asn Glu Lys Glu Tyr Pro Leu Asn Lys Ile Ile Ile Leu Ile Ala Leu
10 20 30

TCA CTG TTT TCT TCC TCA ATC TGG GCC GGA ACC AGT CGT CAC GCG TTA TCG CAA CAA GGC TAT ACC CAA ACC CGT TAT CCA ATT 2242
Ser Leu Phe Ser Ser Ser Ile Trp Ala Gly Thr Ser Arg His Ala Leu Ser Gln Gln Gly Tyr Thr Gln Thr Arg Tyr Pro Ile
40 50 60

GTC TTA GTT CAT GGC CTA TTT GGC TTC GAC ACC TTG GCG GGG ATG GAT TAC TTT CAC GGC ATT CCA CAA TCA CTG ACC CGA GAT 2326
Val Leu Val His Gly Leu Phe Gly Phe Asp Thr Leu Ala Gly Met Asp Tyr Phe His Gly Ile Pro Gln Ser Leu Thr Arg Asp
70 80 90

GGC GCT CAA GTG TAT GTG GCA CAG GTT TCA GCG ACC AAC AGC TCC GAG CGC CGA GGT GAG CAG TTA TTG GCT CAG GTG GAA TCG 2410
Gly Ala Gln Val Tyr Val Ala Gln Val Ser Ala Thr Asn Ser Ser Glu Arg Arg Gly Glu Gln Leu Leu Ala Gln Val Glu Ser
100 110 120

CTA CTG GCG GTA ACC GGA GCG AAA AAA GTG AAC TTG ATT GGG CAT AGT CAT GGT GGC CCC ACC ATT CGC TAT GTG GCA TCA GTG 2494
Leu Leu Ala Val Thr Gly Ala Lys Lys Val Asn Leu Ile Gly His Ser His Gly Gly Pro Thr Ile Arg Tyr Val Ala Ser Val
130 140

CGT CCG GAT TTA GTC GCC TCA GTG ACC AGT ATT GGC GGG GTG CAT AAA GGC TCT GCG TTG CCG ACT TAG TGCGTGGT GTGATTCCAT 2581
Arg Pro Asp Leu Val Ala Ser Val Thr Ser Ile Gly Gly Val His Lys Gly Ser Ala Leu Pro Thr ***
150 160 170

CGGGTTCCGT GAGTGAGCAG GTAGCGGTTG GGTTAACCCA AGGCTTGGTG GCTTTGATTG ATCTGCTCTC TGGCGGAAAA GCGCATCCAC AAGATCCCCT 2681
TGCTTCGCTG GCGGCGCTCA CCACCGAAGG ATCGCTGAAA TTCAATCAGT ATTATCCTGA AGGTGTTCCC ACATCAGCGT GTGGAGAGGG GGCGTACCAA 2781
GTGAATGGGG TACGTTACTA CTCATGGAGT GGCGCTGCGA CCGTCACCAA TATTCTTGAT CCAAGCGATG TGGCGATGGG GTTGATTGGG TTGGTCTTTA 2881
ATGAGCCGAA TGATGGCTTG GTCGCAACCT GCAG 2915
PstI

agreement with the consensus sequence TTGACA, where 5 of the 6 nucleotides are identical. The spacing between these two hexamers is 14 nt which is not optimal for maximum promoter strength (Hawley and McClure, 1983). Furthermore, as with the putative *hlyA* promoter, the hexamer of A's centered around position -43 described by Gentz and Bujard (1985) is not seen. The lack of-this region coupled with the 14 nt spacing between the -10 and -35 regions suggests that this promoter, like the *hlyA* promoter, is not highly expressed or is subject to regulation (Gentz and Bujard, 1985).

A Shine-Dalgarno sequence or ribosome binding site is homologous to the free 3' end of the 16S RNA (AAGGAGGU), and the average spacing between this sequence and the AUG initiation codon is 7 nt (Gold *et al.*, 1981; Kozak, 1983). The potential Shine-Dalgarno sequence before *hlyB*, AGGU, is found 7 nt prior to the AUG codon.

Beyond the UAA termination codon, there is a 65 nt spacer region which is followed by a potential Rho-independent transcriptional terminator (Figure 6.9). This region of dyad symmetry would form a stable base-paired stem loop structure with a free energy value of -24.4 kCal/mol (Tinoco *et al.*, 1973). This is followed by a stretch of four U's typical of a Rho-independent terminator (Rosenberg and Court, 1979).

6.2.3.2 Codon and Base Usage

A summary of the codon usage within the coding region of the *hlyB* mRNA is shown in Table 6.1. Table 6.2 shows the % codon usage in *hlyB* as compared with the average usage in *V.cholerae* based on the sequences of known genes (Section 4.2.2.2).It can be seen that the preferred codon usage within *hlyB* conforms well with that of other sequenced *V.cholerae* genes. The GC content of the 2.9 kb *Eco*RI - *Pst*I fragment is 48.4% which is typical of *V.cholerae* which is usually 47-49% (Bauman and Schubert, 1984). The *hlyB* coding region has a GC content of 48.6% and the non-coding regions in this fragment are not atypical as seen prior to the *hlyA* gene.

Figure 6.9 Transcriptional terminator.

A typical Rho-independant transcriptional terminator exists after *hlyB* corresponding to bp 1847 to 1868 in Figure 6.7. It has a potential free energy of -24.4 kCal/mol (Tinoco *et al.*, 1973). The UAA termination codon is indicated by asterisks followed by a spacer region of 65 bases.

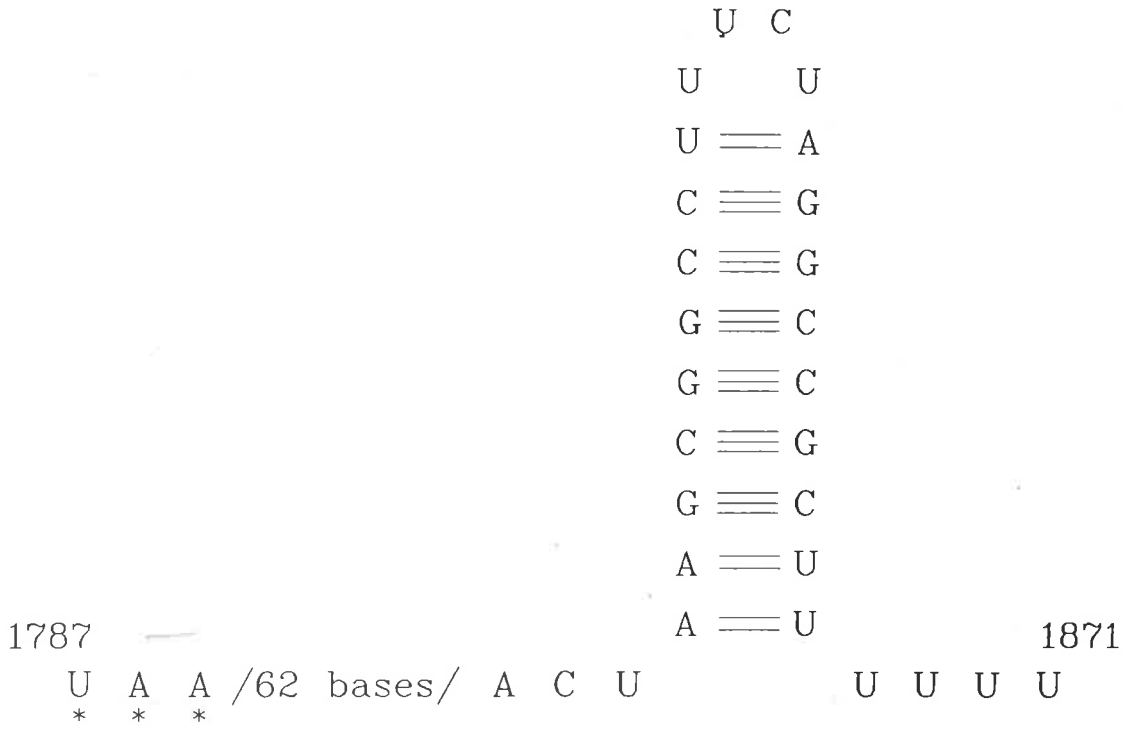

U C
U U
U A
C G
C G
G C
G C
C G
G C
A U
A U
1787
1871
U A A /62 bases/ A C U U U U U
* * *

Table 6.1 Codon usage within *hlyB*

Codon	Number	Codon	Number
UUU-Phe	6	UAU-Tyr	2
UUC-Phe	6	UAC-Tyr	3
UUA-Leu	4	UAA-ochre	1
UUG-Leu	9	UAG-amber	0
CUU-Leu	8	CAU-His	5
CUC-Leu	8	CAC-His	2
CUA-Leu	3	CAA-Gln	31
CUG-Leu	14	CAG-Gln	9
AUU-Ile	18	AAU-Asn	13
AUC-Ile	16	AAC-Asn	19
AUA-Ile	2	AAA-Lys	18
AUG-Met	21	AAG-Lys	1
GUU-Val	11	GAU-Asp	21
GUC-Val	8	GAC-Asp	4
GUA-Val	4	GAA-Glu	31
GUG-Val	18	GAG-Glu	14
UCU-Ser	8	UGU-Cys	2
UCC-Ser	5	UGC-Cys	0
UCA-Ser	12	UGA-opal	0
UCG-Ser	7	UGG-Trp	1
CCU-Pro	3	CGU-Arg	13
CCC-Pro	0	CGC-Arg	11
CCA-Pro	2	CGA-Arg	8
CCG-Pro	2	CGG-Arg	2
ACU-Thr	4	AGU-Ser	9
ACC-Thr	13	AGC-Ser	8
ACA-Thr	7	AGA-Arg	2
ACG-Thr	13	AGG-Arg	1
GCU-Ala	12	GGU-Gly	11
GCC-Ala	13	GGC-Gly	3
GCA-Ala	14	GGA-Gly	1
GCG-Ala	30	GGG-Gly	2

Table 6.2 Comparison of *hlyB* gene codon usage with the codon usage amongst sequenced *V.cholerae* genes

Codon	% *hlyB*	% *V.cholerae*	Codon	% *hlyB*	% *V.cholerae*
UUU-Phe	50.0	67.1	GCU-Ala	17.4	27.8
UUC-Phe	50.0	32.9	GCC-Ala	18.8	18.8
			GCA-Ala	20.3	25.6
UUA-Leu	8.7	25.3	GCG-Ala	43.5	27.8
UUG-Leu	19.6	19.5			
CUU-Leu	17.4	13.0	UAU-Tyr	40.0	56.9
CUC-Leu	17.4	9.6	UAC-Tyr	60.0	43.1
CUA-Leu	6.5	11.7			
CUG-Leu	30.4	20.9	CAU-His	71.4	70.5
			CAC-His	28.6	29.5
AUU-Ile	50.0	48.3			
AUC-Ile	44.4	35.8	CAA-Gln	77.5	69.1
AUA-Ile	5.6	15.9	CAG-Gln	22.5	30.9
AUG-Met	100.0	97.3	AAU-Asn	40.6	52.0
GUG-Met	0.0	2.7	AAC-Asn	39.4	47.0
GUU-Val	26.8	30.8	AAA-Lys	94.7	74.7
GUC-Val	19.5	17.2	AAG-Lys	5.3	25.3
GUA-Val	9.8	17.6			
GUG-Val	43.9	34.4	GAU-Asp	84.0	76.8
			GAC-Asp	16.0	23.2
UCU-Ser	16.3	13.2			
UCC-Ser	10.2	8.7	GAA-Glu	68.9	64.5
UCA-Ser	24.5	22.9	GAG-Glu	31.1	35.5
UCG-Ser	14.3	12.9			
AGU-Ser	18.4	26.0	UGU-Cys	100.0	67.5
AGC-Ser	16.3	16.3	UGC-Cys	0.0	32.5
CCU-Pro	42.8	23.8	UGG-Trp	100.0	100.0
CCC-Pro	0.0	11.4			
CCA-Pro	28.6	31.5	CGU-Arg	35.1	38.2
CCG-Pro	28.6	33.3	CGC-Arg	29.7	21.3
			CGA-Arg	21.6	15.2
ACU-Thr	10.9	26.1	CGG-Arg	5.4	5.1
ACC-Thr	35.1	28.5	AGA-Arg	5.4	15.7
ACA-Thr	18.9	22.2	AGG-Arg	2.8	4.5
ACG-Thr	35.1	23.2			
			UAA-ochre	100.0	28.6
GGU-Gly	64.7	47.0	UAG-amber	0.0	35.7
GGC-Gly	17.6	30.0	UGA-opal	0.0	35.7
GGA-Gly	5.9	12.7			
GGG-Gly	11.8	10.3			

6.2.4 Analysis of the HlyB protein

6.2.4.1 The signal sequence

The extreme N-terminus of the predicted aa sequence of HlyB was analysed. There are two positively charged Lys residues at postions 5 and 9. These are followed by a stretch of 8 residues representing the hydrophobic core. The predicted cleavage site is after Ala_{17}. This site conforms well to the (-3, -1) rule of von Heijne (1984), with an Ala residue in both positions. Furthermore, this cleavage would place the Pro_{19} residue in the +2 position, a configuration that is not uncommon. The absence of a helix-breaking residue (Pro or Gly) in the hydrophobic core of the leader peptide has previously been seen with the OmpV and DNase signal sequences (Pohlner *et al.*, 1986; Focareta and Manning, 1987).

6.2.4.2 The mature HlyB protein

A hydropathy plot according to Kyte and Doolittle (1982) of the aa sequence (Figure 6.10) reveals that the HlyB protein contains several interesting features. A region within the N-terminal portion from aa 23 to 52, and a stretch from position 198 to 220 show marked hydrophobicity which is indicative of membrane associated domains, as the uninterrupted hydrophobic stretch permits insertion into the lipid bilayer. These two regions and a third somewhat less hydrophobic domain (aa 363 to 382) are associated with continuous β-sheet structures as predicted by a Chou and Fasman (1978) analysis (Figure 6.11). This sort of folding is indicative of potential membrane associated domains forming a transmembrane channel, which in turn could be lined with the other major β-sheet regions (aa 238 to 267 and 474 to 501) which contain a number of hydrophilic residues. There are three large regions (aa 90 to 142, 164 to 195 and 517 to 548) which consist predominantly of hydrophilic residues. These regions and the lesser hydrophilic region, aa 304 to 339, are all extended regions of α-helix.

Figure 6.10 Hydropathic nature of HlyB.

The amino acid sequence of the precursor form of the HlyB protein was analyzed according to Kyte and Doolittle (1982) using a window of nine amino acids and averaging each group of three residues.

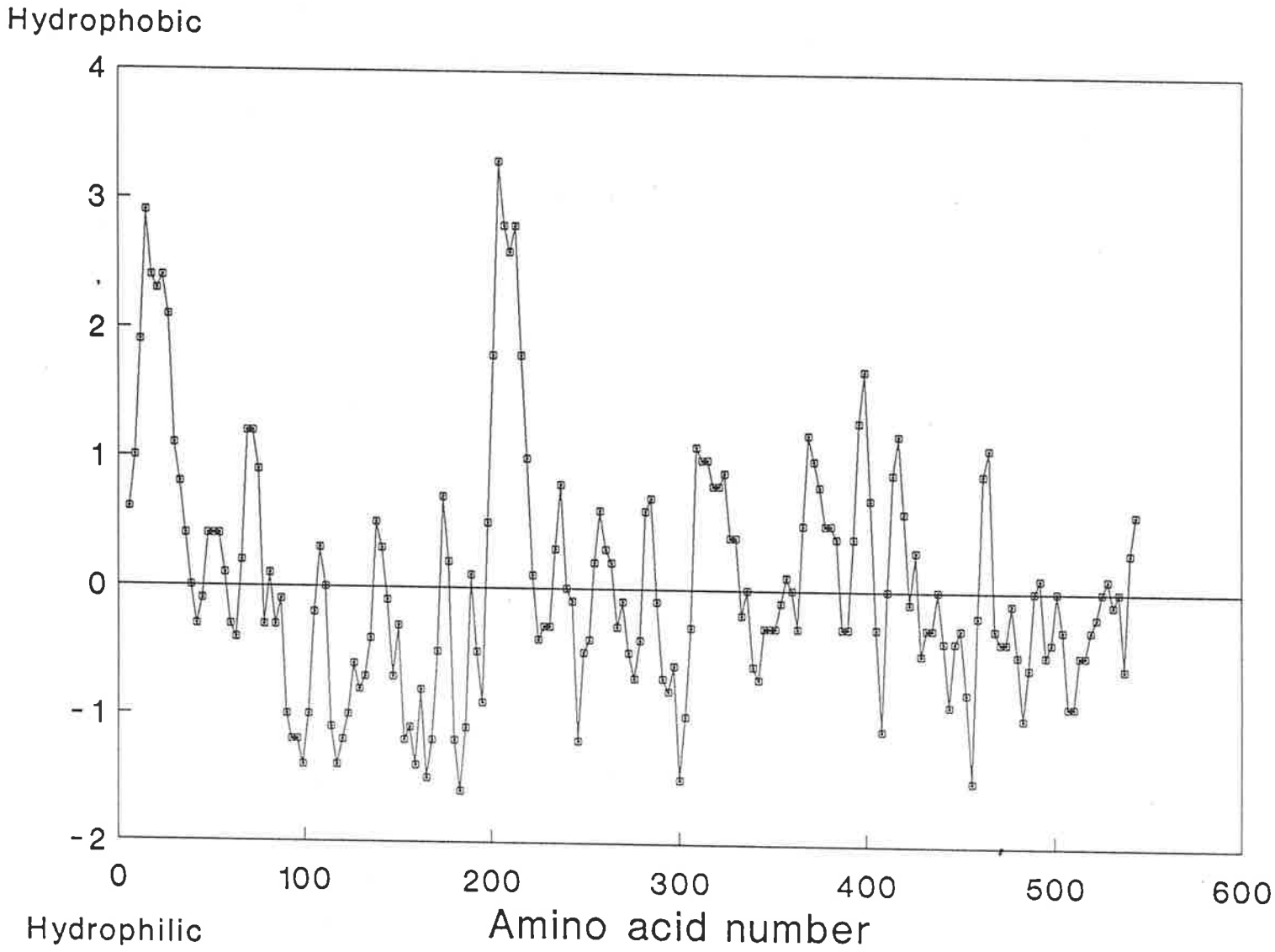

Hydrophobic
4
3
2
1
0
-1
-2
0
100
200
300
400
500
600
Hydrophilic
Amino acid number

Figure 6.11 Predicted secondary structure of HlyB.

The amino acid sequence of the HlyB protein was subjected to analysis using the algorithm of Chou and Fasman (1978). The various regions are represented as follows:

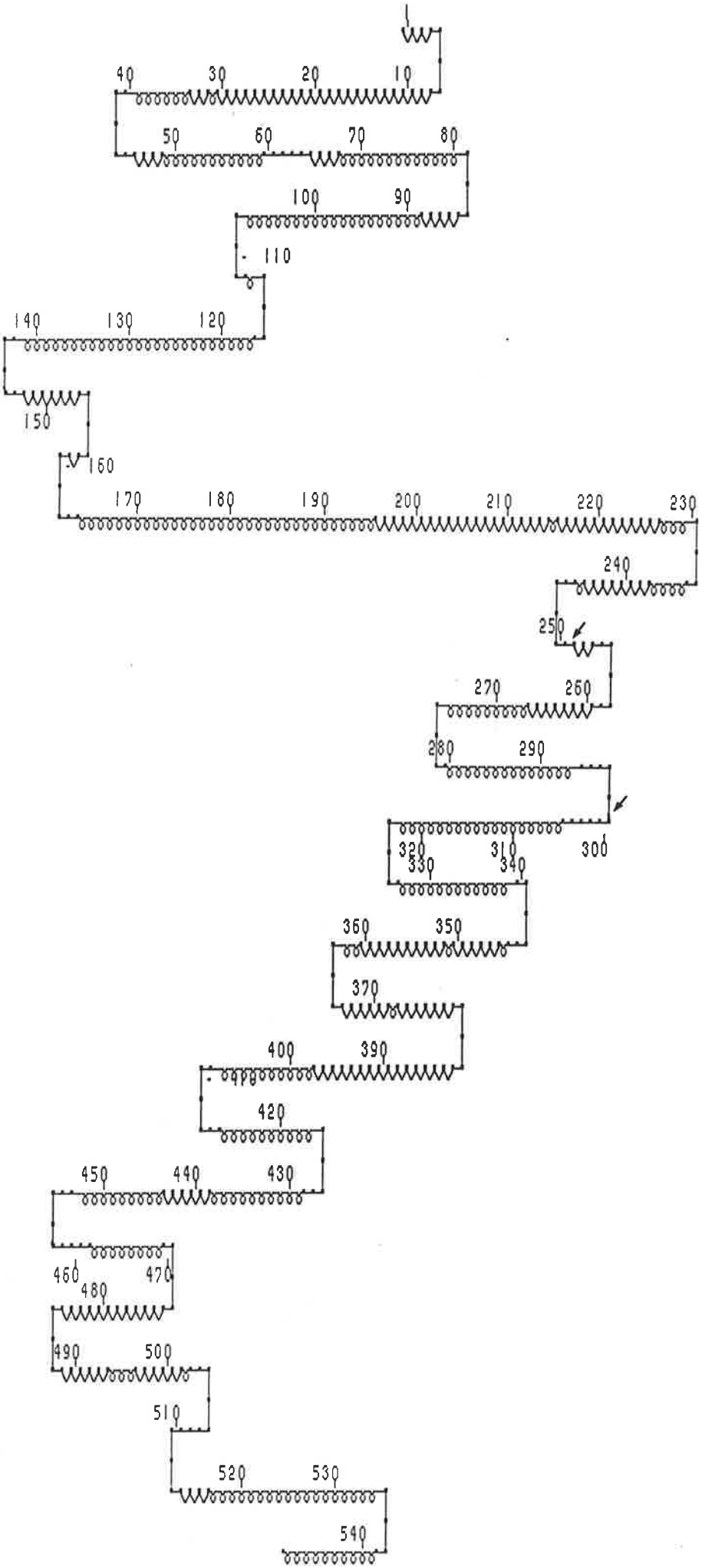
α-helix : 

β-sheet : 

β-turn : 

random coil :

The position of the Cysteine residues are indicated by arrow heads.


40
30
20
10
50
60
70
80
100
90
110
140
130
120
150
160
170
180
190
200
210
220
230
240
250
270
260
280
290
320
310
300
330
340
360
350
370
400
390
420
450
440
430
460
470
480
490
500
510
520
530
540

6.2.4.3 Localization of HlyB

The presence of a potential signal sequence at the N-terminus and of numerous hydropathic domains suggest that HlyB is an outer membrane protein. In an attempt to localize HlyB, *E.coli* minicells harbouring pPM431 were fractionated and the membranes extracted with Sarkosyl. HlyB was clearly membrane associated and sarkosyl insoluble suggesting an outer membrane location (Figure 6.12).

6.2.5 Confirmation of the location of *hlyB* by analysis of *hlyB*::Tn*1725* insertions

As mentioned previously, Manning *et al.* (1984) estimated the size of HlyB to be 71 kDa. In contrast, the size predicted from the nucleotide sequence is significantly smaller at only 60.3 kDa. In order to account for this large discrepancy in size, a number of transposon insertion mutants producing truncated HlyB proteins were analysed.

Transposon insertion plasmids pPM698, pPM657, pPM610, pPM685, pPM642 and the parent plasmid pPM431 were digested with *Eco*RI (Figure 6.13). These transposon insertion plasmids were also analyzed in *E.coli* minicells (Figure 6.14) in an attempt to correlate the size of the truncated HlyB proteins with the point of Tn*1725* insertion. However, the error in estimating the *Eco*RI fragments on an agarose gel was too large and so the sites of insertion of Tn*1725* in each of the plasmids was precisely mapped by double-stranded sequencing using oligodeoxynucleotide primers that bind prior to the inverted repeats at the ends of Tn*1725* (Figures 6.8 and 6.15). Plasmids pPM698 and pPM657 have the transposon located 268 and 1089 bp from the *Eco*RI site, respectively, and do not show any indication of a truncated protein product in *E.coli* minicells. Minicell analysis demonstrates that plasmid pPM610 produces a truncated peptide of 41.3 kDa, and the transposon is inserted 1395 bases from the *Eco*RI site, whereas plasmid pPM685 which results in a truncated product of 46.3 kDa has the transposon inserted 1559 bases from the *Eco*RI site. In both cases the size of the protein detected is consistent with the point of insertion within the sequence allowing for cleavage of the predicted signal sequence. However, the site of insertion in plasmid pPM642, which does not affect the size of the HlyB protein, maps at

Figure 6.12 Localization of HlyB.

E.coli minicells harbouring pPM431 were purified on sucrose gradients and labelled with [^{35}S]-methionine. The minicells were then sphaeroplasted and sonicated and the membranes separated from the periplasm and cytoplasm by ultracentrifugation (Achtman *et al.*, 1979). The membranes were then extracted with Sarkosyl. The HlyA and HlyB proteins are indicated.

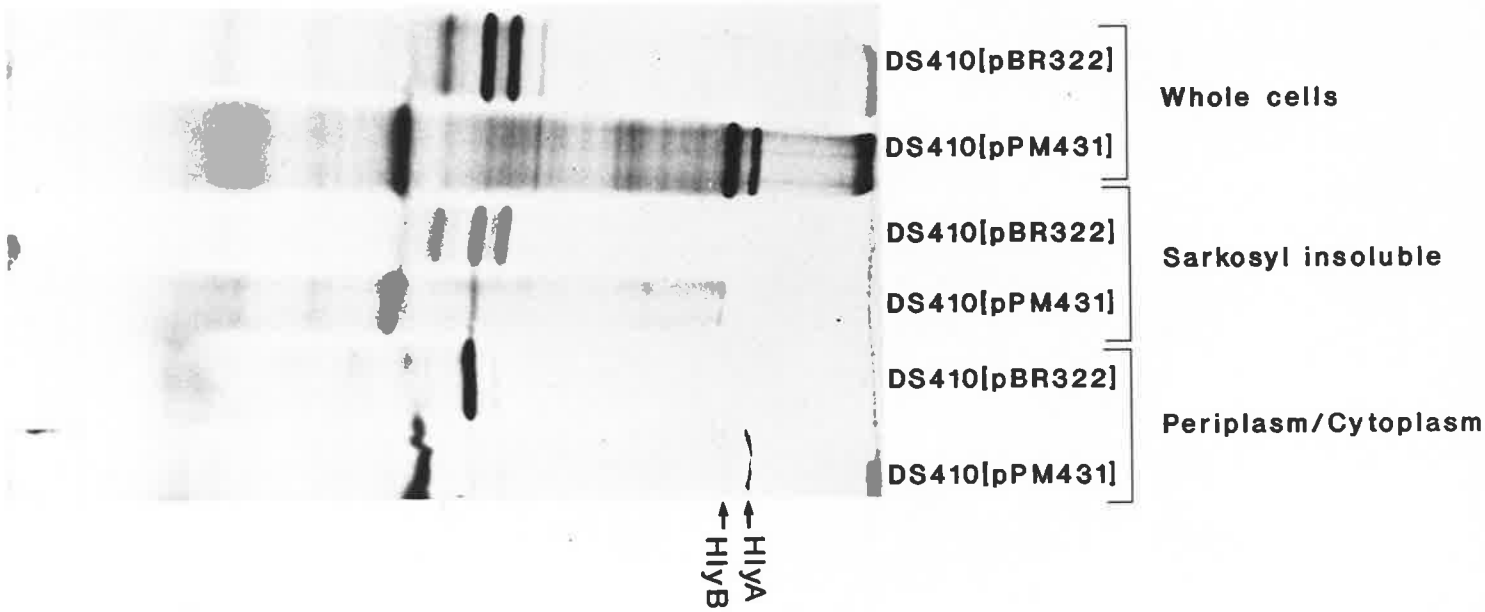
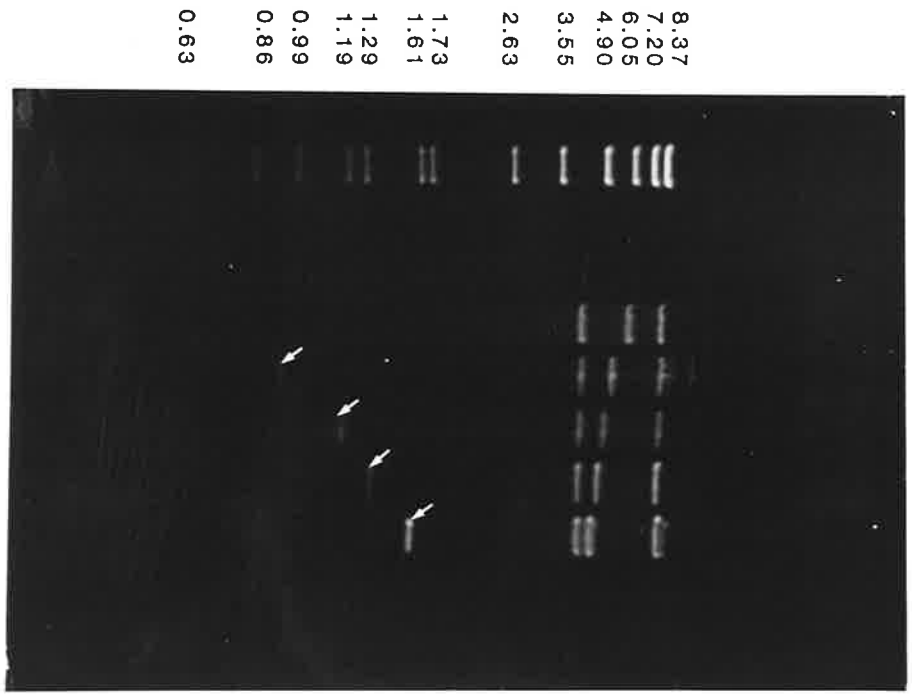

DS410[pBR322]
DS410[pPM431]
Whole cells
DS410[pBR322]
DS410[pPM431]
Sarkosyl insoluble
DS410[pBR322]
DS410[pPM431]
Periplasm/Cytoplasm
HlyA
HlyB

Figure 6.13 Restriction analysis of Tn*1725* insertion plasmids.

The plasmids were digested with *Eco*RI in an attempt to map where the transposon had inserted. The band in each track indicated by an arrow is the *Eco*RI fragment that represents the distance from before the *hlyB* gene to the inverted repeat within the transposon. SPP1 phage DNA digested with *Eco*RI was used as a standard and the size of the fragments generated in kb are shown on the right.



SPP1 (*Eco* RI)

pPM431
pPM698
pPM657
pPM610
pPM685
pPM642

(*Eco* RI)

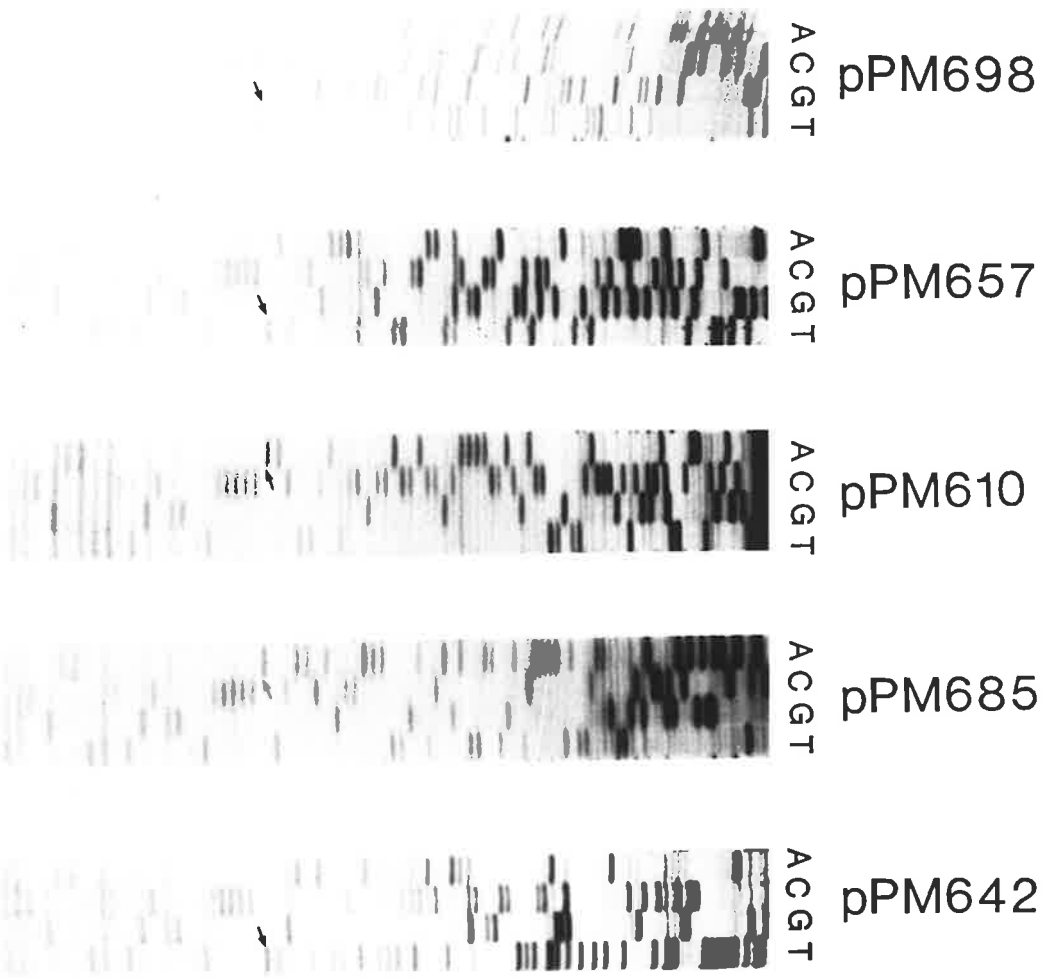
Figure 6.14 Plasmid encoded proteins in *E.coli* minicells.

Minicells harboring the various plasmids were purified on sucrose gradients, labelled with [^{35}S]-methionine, solubilized in sample buffer and subjected to SDS-PAGE followed by autoradiography of the dried gel. The HlyA, HlyB, 18.3 kDa and chloramphenicol acetyltransferase (Cat) proteins are shown. The truncated HlyB proteins in pPM610 and pPM685 are indicated by arrows. Plasmid pPM642 encodes a HlyB protein indistinguishable from the wild-type.

DS410(pPM698)
DS410(pPM657)
DS410(pPM610)
DS410(pPM685)
DS410(pPM642)

Figure 6.15 Determination of Tn*1725* insertion points by double stranded sequencing.

A synthetic oligodeoxynucleotide that binds to the end of transposon Tn*1725* was used to sequence plasmid DNA of the various transposon insertion mutants. The first base within the cloned 2.9 kb *Eco*RI - *Pst*I fragment beyond the end of Tn*1725* is indicated by the arrows.


A C G T
pPM698
A C G T
pPM657
A C G T
pPM610
A C G T
pPM685
A C G T
pPM642

position 1825, only 45 bp after the translational stop codon. Clearly the size difference between the protein products of pPM685 and pPM642 of 24.7 kDa could not be encoded by the 219 bp between the point of insertion in pPM685 and the translational stop codon. Thus, within the C-terminal end of HlyB there must be an unusual aa sequence or composition which is responsible for the abnormal movement of the protein through a polyacrylamide gel system. A possible explanation is that this region is very atypical in its distribution of charged aa's: there are 14 negatively charged aa's compared to 4 positively charged. This C-terminal region also has a relatively low pI (4.8) when compared to the remainder of the protein (5.9).

Upon insertion of a transposon, a small sequence within the target DNA is repeated on each end of the transposon. Sequence analysis of the *hlyB*::Tn*1725* insertions with oligodeoxynucleotide primers that read out of both ends of the transposon reveals a 5 bp target sequence.

6.2.6 Analysis of the 3' *hlyB* region

Another ORF exists after the HlyB coding region beginning at nt 2048 with a UAG termination codon at nt 2560 (Figure 6.7). This ORF codes for 171 aa's, which, when translated would give rise to a 18.3 kDa protein. The function of this protein, and any possible role it plays in the synthesis or export of the *V.cholerae* haemolysin is unknown. However, based on both hydropathy plots and predicted secondary structure of the aa sequence, it would be expected to be a cytoplasmic membrane protein. The gene is preceeded by a sequence (AGAGGU) that displays excellent homology to the known Shine-Dalgarno sequence. However, the 17 nt spacing from the initiation codon is very atypical (Gold *et al.*, 1981; Kozak, 1983), and it seems unlikely that this region acts as a translation initiation point. However, good promoter consensus sequences with a 19 bp spacing are seen prior to the AUG start codon.

6.3 Summary and Conclusions

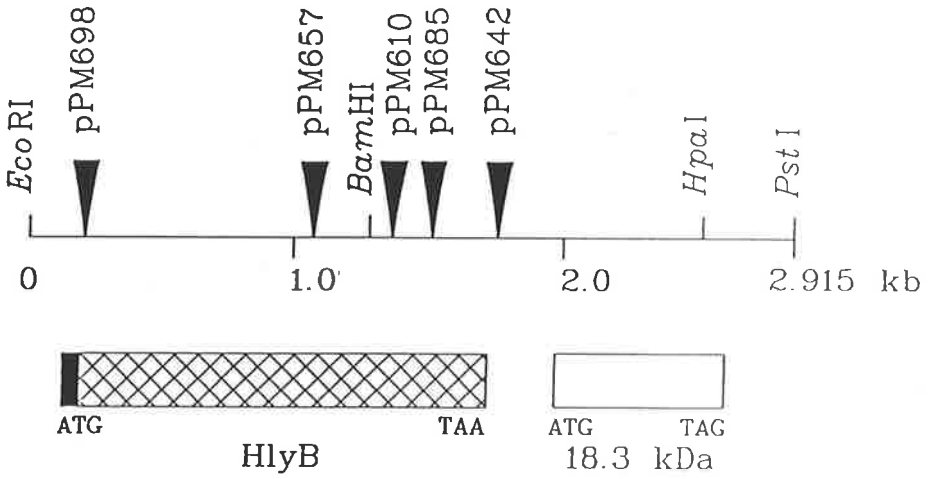
The genetic organization of the *hly* locus of *V. cholerae* is summarized in Figure 7.1. It is clear that *hlyA* encodes the structural gene for the haemolysin, and that *hlyB* is encoded in a separate transcriptional unit and its product has an accessory function in haemolysin production.

The data presented here support the notion that the *hlyB* gene product aids in the excretion of the *V.cholerae* haemolysin, as mutants in this gene have been shown to lead to intracellular accumulation of active haemolysin. When O17 *hlyB*::Km^R cells were fractionated during various stages of growth, and analysed for haemolytic activity, it could be seen that the haemolysin was being trapped within the O17 *hlyB*::Km^R cells until they approached stationary phase at which stage it was released presumably due to cell lysis. Coupled with the data that O17 cells produced active haemolysin in the supernatant during all phases of growth, this indicates that the *hlyB*::Km^R mutation was inhibiting the excretion of the haemolysin during active growth of the *V.cholerae* cells. In this way the *hlyB* genes of both *V.cholerae* and *E.coli* and the *shlB* gene of *S. marcescens* seem to have similar roles in the release of the respective bacterial haemolysins. These proteins are all distinct and we know that the export genes for the *E. coli* haemolysin cannot functionally replace those of *V. cholerae* (T. Focareta and P.A. Manning, unpublished data). Comparison of the haemolysin determinants of these species does not reveal any significant homology at either the DNA or protein sequence levels. Thus, the export of the *V. cholerae* El Tor haemolysin seems unique.

Analysis of a Kyte and Doolittle (1982) hydropathy plot and a Chou and Fasman (1978) predicted structure of the HlyB protein makes it tempting to speculate that the HlyB protein in some way forms a pore through which the haemolysin can be exported. The *hlyB* gene product possesses a characteristic signal sequence seen on secreted proteins which indicates that it may be localized in the outer membrane.

Figure 6.16 Genetic organization of the 2.9 kb *Eco*RI - *Pst*I fragment.

The upper line shows the restriction pattern of the *Eco*RI - *Pst*I fragment which encodes *hlyB* and the gene for the 18.3 kDa protein. The lower part of the figure shows the location of the coding regions of these genes. The sites at which Tn*1725* is inserted in the various derivatives of pPM431 are shown.


EcoRI
pPM698
pPM657
BamHI
pPM610
pPM685
pPM642
HpaI
PstI
0
1.0
2.0
2.915 kb
ATG
TAA
HlyB
ATG
TAG
18.3 kDa

CHAPTER 7

DISCUSSION

7.1 Introduction

V.cholerae of the O1 serotype are subdivided into two biotypes, Classical and El Tor (Feeley, 1965; Sen, 1969), both of which are capable of causing human cholera. These biotypes differ in several characteristics, with the El Tor strains originally being identified as haemolytic, whereas Classical strains were not. However, further differences such as resistance to polymyxin B and Mukerjee's group IV bacteriophages have subsequently been identified (Monsur *et al.*, 1965).

V.cholerae, like other Vibrionaceae, excrete a plethora of extracellular proteins including cholera toxin (Finkelstein, 1969), proteases (Schneider *et al.*, 1981), haemagglutinins (Hanne and Finkelstein, 1982), neuraminidase (Vimr *et al.*, 1988), DNases (Focareta and Manning, 1987; Tsan, 1978) and haemolysins (Manning *et al.*, 1984; Richardson *et al.*, 1986). All of these proteins are potentially important for either colonization or the pathogenic effects of the organism. The studies undertaken here have involved characterization of the *hly* locus determining production of the El Tor haemolysin of *V.cholerae* O1, and the role of this protein in the pathogenesis of this organism.

7.2 The *hly* locus in *V.cholerae* O1

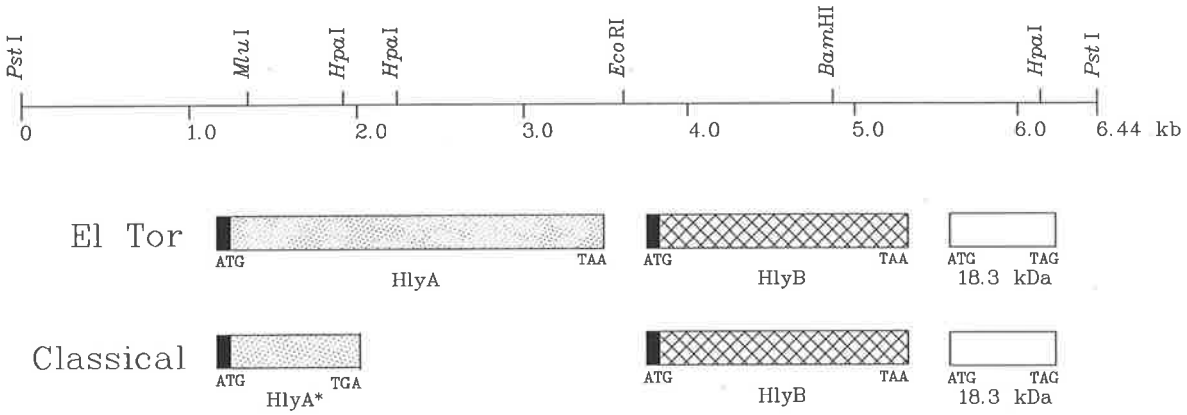
The structural gene for the El Tor haemolysin, *hlyA*, and the surrounding genes were cloned from *V.cholerae* strain O17 into *E.coli* K-12 to generate plasmid pPM431 (Manning *et al.*, 1984). The plasmids pPM431 (Manning *et al.*, 1984) or pPM2005 (Section 3.2.5) were used as radiolabelled probes in Southern hybridization

analysis for the presence of homologous DNA in a variety of O1 and non-O1 *V.cholerae* strains. These results confirm previous observations (Brown and Manning, 1985) that non-haemolytic strains belonging to both biotypes of *V.cholerae* O1 still contain DNA homologous to that which encodes the haemolysin from strain O17. This implies that the genes are present but not expressed in these strains. In order to explain why the *hly* locus is retained in non-haemolytic Classical strains, a series of experiments were carried out using strain 569B as an example. Strain 569B was shown not to be defective in its ability to excrete HlyA into the extracellular medium as the introduction of pPM2005 (*hlyA*$^+$ only) results in the haemolysin being efficiently transported to the extracellular medium. After introduction of plasmid pPM2001 into 569B, and permitting a non-reciprocal recombination event to occur between pPM2001 and the 569B chromosome, plasmid pPM2002 was isolated. The *Pst*I - *Eco*RI DNA fragment that carries the *hlyA* gene was recloned into pBR322 to generate pPM2012. This plasmid displayed an identical restriction pattern to that seen by Goldberg and Murphy (1985) who independently cloned the *hlyA* genes from *V.cholerae* strains RV79 (El Tor, Hly$^+$) and 569B (Classical, Hly$^-$) (Goldberg and Murphy, 1984, 1985). They demonstrated by restriction enzyme analysis with *Sau*3A and *Taq*I that the 569B *hlyA* gene carried a small deletion, rendering it non-haemolytic. This implies that pPM2012 harboured the same defect seen in 569B. Subsequent analysis of pPM2002 in *E.coli* minicells revealed not only the expected absence of the 80 kDa HlyA protein but also the presence of a 27 kDa truncated peptide, designated HlyA*. The HlyA* protein is conserved probably because it functions as an enterotoxin (Section 7.5).

Nucleotide sequence determination of the 6,442 bp *Pst*I fragment harbouring the *hly* locus from *V.cholerae* O17 revealed the presence of 3 ORFs which have the potential to encode proteins of size 82.0 kDa, 60.3 kDa and 18.3 kDa (Figure 7.1). The 82.0 kDa HlyA protein is the haemolysin proper and its properties are discussed below (Section 7.3), whereas the 60.3 kDa HlyB protein effects release of HlyA during mid-exponential growth (Section 7.4). The 18.3 kDa protein probably corresponds to the HlyC product described by Manning *et al.* (1984). The function of HlyC and whether or not it plays any role in the production, regulation or export of

Figure 7.1 The *hly* locus.

The upper line shows the restriction endonuclease pattern of the *Pst*I fragment which encodes the *hly* locus. The position of the ORF's encoding HlyA, HlyB and the 18.3 kDa protein are shown for the El Tor strain O17. The position of HlyA* in Classical strain 569B is also indicated. The solid regions in HlyA, HlyA* and HlyB represent the N-terminal signal sequences.


Pst I
Mlu I
Hpa I
Hpa I
Eco RI
BamHI
Hpa I
Pst I
0
1.0
2.0
3.0
4.0
5.0
6.0
6.44 kb
El Tor
ATG
TAA
HlyA
ATG
TAA
HlyB
ATG
TAG
18.3 kDa
Classical
ATG
TGA
HlyA*
ATG
TAA
HlyB
ATG
TAG
18.3 kDa

HlyA is unknown. The 18.3 kDa protein is relatively hydrophobic, does not possess a characteristic signal sequence and would therefore likely to be in the cytoplasmic membrane. The initial suggestion that the *hly* locus exists as an operon can now be refuted as powerful Rho-independent transcriptional terminators exist after both the *hlyA* and *hlyB* genes. Thus, the effects seen by Manning *et al.* (1984) with Tn*1725* insertions are possibly due to alterations in the copy number of the plasmid rather than polarity caused by Tn*1725* as previously suggested.

The *hlyA* and *hlyB* genes are preceeded by typical promoter-like regions based on their homology with the known consensus sequences for *E.coli* promoters (Rosenberg and Court, 1979; Hawley and McClure, 1983), however, in both cases the spacing between the -10 and -35 regions is not optimal and a -43 region is lacking suggesting that these promoters are not highly expressed or are subject to some form of regulation (Gentz and Bujard, 1985). Both genes possess regions just prior to the AUG start codons that exhibit excellent homology with the Shine-Dalgarno sequence (Shine and Dalgarno, 1974).

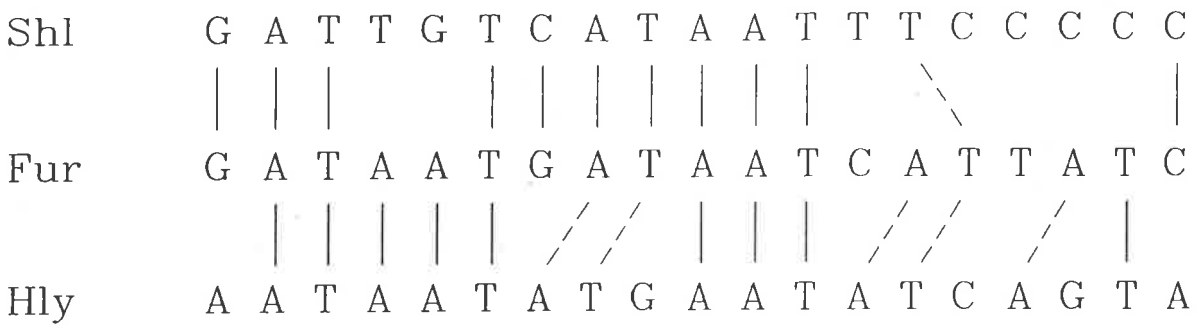
Analysis of the 5' pre-coding region of *hlyA* reveals an extremely AT-rich region. The GC ratio of this 1140 bp region is only 36% which is significantly less than the average of 47-49% for *V.cholerae*. This region was analysed for repeated sequences. Prior to the start of the cholera toxin operon (*ctxAB*) are multiple tandem repeats (TTTTGAT) which are recognised by the DNA-binding protein, ToxR (Miller *et al.*, 1987). The binding of ToxR to these sequences results in the activation of transcription. Copies of this sequence are not found 5' to *hlyA*, implying that *hlyA* is not regulated by ToxR in a similar manner. But, not all ToxR regulated genes contain copies of this repeat sequence in their control regions. Peterson *et al.* (1988) has demonstrated that this sequence is not present prior to the start of the Tcp operon, yet the expression of this pilus is regulated by ToxR. However, three copies of the sequence TATTTTA are present upstream of the initiation codon for *hlyA*, as well as five copies of TAAATTA. The role of these sequences are unknown but it is tempting to speculate that they may serve a similar function to the ToxR binding site in the *ctxAB* system and act as a binding site for a positive regulatory factor. A gene that

regulates haemolysin production in *V.cholerae* has been mapped in the vicinity of *toxR* (von Mechow *et al.*, 1985). This gene has been designated *hlyR* and is widely separated from the *hly* locus on the chromosome. However, the differentiation of *hlyR* from *toxR* rests on a single recombinant colony from a conjugation experiment (von Mechow *et al.*, 1985). One possiblity is that *hlyR* actually represents *toxS*, the second component of the *toxR,S* system (Miller *et al.*, 1989).

Iron regulated proteins of *E.coli* often possess areas of dyad symmetry in their promoter regions. The product of the *fur* locus acts here to result as a negative regulator of transcription (Hantke, 1982). Poole and Braun (1988) demonstrated that haemolytic activity of *S.marcescens* could be dramatically increased by restricting the amount of iron present in the growth medium. These workers also described a possible Fur-binding site 5' to the *S.marcescens* haemolysin operon (*shlAB*) (Calderwood and Mekalanos, 1987; Poole and Braun, 1988). The haemolytic activity of *V.cholerae* O17 is also markedly increased under iron-starved conditions. However, if a Fur-like protein is involved in the regulation of the haemolysin, it clearly must be different from that of *E.coli* since the activity of DH1[pPM431] does not increase under iron stress. Analysis of the 5' precoding region reveals a sequence that displays homology to the consensus "Fur-binding" site (Figure 7.2). The possibility that this region somehow regulates expression of *hlyA* under conditions of low-iron could help explain the role of a haemolysin in pathogenesis, by facilitating the release of essential iron from erythrocytes. However, the Fur-binding sites are usually present in the promoter/operator region of the regulated genes. The site 5' to *hlyA* is 480 bp upstream of the AUG start codon. Therefore, either the regulation system is different to that described for *E.coli* and *S.marcescens* or the real *hlyA* promoter is present there. Site-directed or deletion mutagenesis to change or remove the potential "Fur" binding site, and its resultant effect on haemolysin expression under iron starved condidtions could provide an indication of its importance. There are good potential promoter sequences present in this region that incorporate the "Fur" binding site and mRNA primer extension analysis using an oligodeoxynucleotide that binds to the N-terminal region of *hlyA* has localized the transcriptional initiation point between nt 721 and 750

Figure 7.2 Potential "Fur-binding" site 5' to *hlyA*.

A possible "Fur-binding" site in the *hly* locus of *V.cholerae* is shown. The nt sequence of the Fur-binding consensus sequence in *E.coli* (Calderwood and Mekalanos, 1987) and the region upstream of the *shl* haemolysin determinant in *S.marcescens* (Poole and Braun, 1988) have been reported previously. Solid lines indicate the identical matches with the consensus sequence.


Shl G A T T G T C A T A A T T T C C C C C
Fur G A T A A T G A T A A T C A T T A T C
Hly A A T A A T A T G A A T A T C A G T A

(Figure 4.2) (S. Williams, personal communication). Analysis of this region reveals a potential -10 region AAGTAT between nt 706 and 711. The putative -35 region between nt 682 and 687 reads TAGAAA. However, the potential "Fur" binding site is still 100 bases upstream. Closely linked to this potential promoter are five closely related hexamers (ATTTTAC). Furthermore, three of these hexamers are in tandem directly 5' to the predicted -35 region. These tandem repeats may function as binding sites for the transcriptional regulatory element, HlyR, in the same way that ToxR functions for the *ctxAB* operon.

Nucleotide sequence analysis of the Classical 569B *hlyA* revealed an 11 bp deletion which accounts for the slight difference in restriction pattern in this region seen by Goldberg and Murphy (1985). This change results in a truncated *hlyA* product of 27 kDa as predicted from the size of HlyA* which was detected in *E.coli* minicells. Using a 19 nt synthetic oligodeoxynucleotide probe that spans this deleted region, a wide range of Vibrio strains were analysed. The probe reacted with all El Tor strains, regardless of their haemolytic phenotype, whereas all Classical strains failed to do so. This indicates that the genetic basis for the non-haemolytic phenotype seen among Classical strains and El Tor strains is different, and that the 19 nt oligodeoxynucleotide is an effective probe to distinguish the two biotypes of *V.cholerae* O1. Other commonly used methods are less reliable and often difficult to interpret. Thus, the reason why some El Tor isolates display a non-haemolytic phenotype remains unknown. Recently, Rader and Murphy (1988) determined the sequence of the *hlyA* genes from El Tor strains RV79 (Hly^{+}) and RV79 (Hly^{-}) and found the coding regions to be identical. However, these workers have only sequenced from the *Bst*EII site (position 926; Figure 4.2) to the *Eco*RI site. With the knowledge that the region upstream of this *Bst*EII site harbours the *hlyA* promoter as well as the AT-rich region including the tandem repeats, suggests that this may be the area of difference. Alternatively, another regulatory protein or element may be responsible for the difference between Hly^{+} and Hly^{-} El Tor strains.

7.3 The HlyA protein

The *hlyA* ORF codes for the 82.0 kDa HlyA protein which is produced with an N-terminal hydrophobic stretch corresponding to a signal sequence. Such N-terminal extensions are a common feature among excreted proteins, and are thought to initiate the transport of the protein across the cytoplasmic membrane barrier. The HlyA signal sequence conforms well to the rules defined by von Heijne (1984, 1985). It has 2 positively charged residues (Lys_3 and Arg_6) within the N-terminal 6 aa followed by a hydrophobic core and a good cleavage site after Ala_{25}. This cleavage site was determined by amino acid sequencing of the N-terminus of the mature 80 kDa protein. It places Pro_{21} at position -5 which is optimal for a helix-breaking residue. The truncated $HlyA^*$ peptide produced in Classical *V.cholerae* strains also still possesses the signal sequence necessary to initiate its transport out of the cytosol.

During purification of the 65 kDa extracellular form of HlyA from culture supernatants of *V.cholerae*, Yamamoto *et al.* (1986) identified a number of smaller, immunoreactive peptides which were suggested to be proteolytically derived epitopes of HlyA. The HlyA protein is extremely unstable and the effects of ammonium sulphate precipitation have been discussed in Chapter 3. Western blot analysis indicated that the 80 kDa form of HlyA was being degraded while trapped in the *E.coli* periplasm, where the concentration of HlyA molecules is higher than in the extracellular medium. These data are consistent with the HlyA protein being autoproteolytic, however, it would appear that any protease activity that is associated with HlyA must be relatively specific, since there is no detectable hydrolysis when DH1[pPM431] is grown on casein agar. Furthermore, no change in protease activity could be seen with strains O17 and V760.

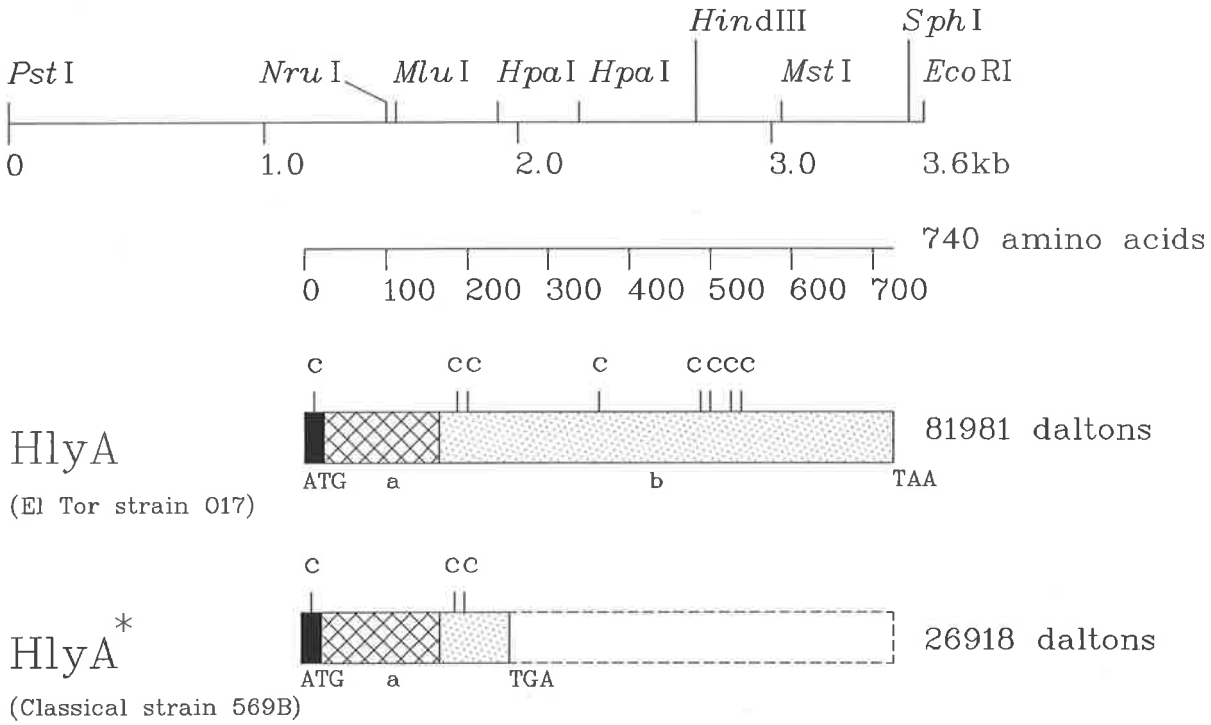
Truncated HlyA proteins which are produced in normal amounts but have lost less than 10 kDa from the C-terminus as a result of Tn*1725* insertion into the gene are haemolytically inactive (Manning *et al.*, 1984). In addition, the haemolysin purified by Yamamoto *et al.* (1986) has lost approximately 15 kDa but still retains its haemolytic activity whereas $HlyA^*$ detected in Classical strains is non-haemolytic. These data imply that the active site of the haemolysin resides in the C-terminal

domain and that the 65 kDa form of HlyA is the result of cleavage at the N-terminus (Figure 7.3). A possible cleavage site representing the junction between the 15 kDa N-terminal region (a) and the 65 kDa C-terminal region (b) may be near residue Arg_{168}. This directly follows a region that includes the most hydrophobic domain of the protein. Yamamoto *et al.* (1986) assayed haemolytic activity during purification of the 65 kDa form of HlyA. Although haemolytic activity could be detected in the periplasm of *E.coli* DH1[pPM431], proteolytic cleavage to the 65 kDa form also occurred. Hence, because of the ready degradation of the 80 kDa protein into smaller fragments, it has been difficult to determine whether the 80 kDa form of HlyA is haemolytically active. One possibility is that the N-terminal domain (region a : 15 kDa; Figure 7.3) needs to be cleaved off to activate the protein. One method attempted to determine the relative activities of the two forms was to separate them on a polyacrylamide gel under non-denaturing conditions and then overlay the gel onto blood agar. The proteins would diffuse from the gel and if active, form zones of haemolysis on the blood agar. The difficulties encountered while attempting this were that no SDS could be used anywhere in the gel system as this caused lysis of the erythrocytes. Even extensive washing could not remove all traces of the detergent. Additionally, the isoelectric points of the 65 kDa and 80 kDa forms are similar and under non-denaturing conditions would migrate too closely to be able to detect distinct zones of haemolysis on the blood agar. If the cleavage of the 80 kDa protein could be prevented by site-directed mutagenesis of the potential cleavage sites or by using protease inhibitors, then it may be possible to determine whether both the 80 kDa and 65 kDa forms are active.

Cysteine residues are known to be involved in tertiary structure of proteins, and often hold the protein in a conformational state such that an active site is formed. The haemolysins of *Streptococcus pneumoniae* (pneumolysin; Walker *et al.*, 1987), *Streptococcus pyogenes* (streptolysin O; Kehoe *et al.*, 1987), *Clostridium perfringens* (perfringolysin; Tweten, 1988) and *Listeria monocytogenes* (listeriolysin; Mengaud *et al.*, 1987) all contain a unique Cys residue in a region of conserved aa homology. Although this residue was considered essential, the precise role that it

Figure 7.3 The HlyA protein.

Comparison of the *hlyA* locus from *V.cholerae* strains O17 (El Tor, haemolytic) and 569B (Classical, non-haemolytic). The position of the *hlyA* gene with respect to the surrounding restriction nuclease cleavage sites is shown. The HlyA protein is produced as a precursor with a N-terminal signal peptide (solid region) and upon release from the cell, a further fragment is cleaved (a: crosshatched) to produce the b (dotted) fragment. The Classical strain 569B has an 11 bp deletion leading to early termination of translation and a truncated protein, $HlyA^{*}$. It is not known whether the cleavage occurs after 'a' in $HlyA^{*}$. The position of the cysteine residues (C) are also indicated.


HindIII
SphI
PstI
NruI
MluI
HpaI
HpaI
MstI
EcoRI
0
1.0
2.0
3.0
3.6kb
740 amino acids
0 100 200 300 400 500 600 700
c
cc
c
cccc
HlyA
81981 daltons
ATG
a
b
TAA
(El Tor strain 017)
c
cc
HlyA*
26918 daltons
ATG
a
TGA
(Classical strain 569B)

played in activity was unclear. Oligonucleotide mediated site directed mutagenesis was used to alter the unique Cys residue to Ser, Gly or Ala (pneumolysin) and Ser or Ala (SLO) (Saunders *et al.*, 1989; Pinkney *et al.*, 1989). The modified toxins were indistinguishable from the wild-types in terms of haemolytic activity, lytic and inhibitory effects on human polymorphonuclear leukocytes and binding and insertion into the erythrocyte membranes (Saunders *et al.*, 1989). In addition, the modified SLO mutants showed only slightly reduced activity levels (Pinkney *et al.*, 1989). Furthermore, it has been demonstrated that the IgA protease of *N.gonorrhoeae* does not require its two Cys residues for activity (Halter *et al.*, 1989). These results indicate that the widely held assumption that the *in vitro* activity of these proteins requires the "essential" Cys residues is not true. However, in contrast, Cys residues are critical for both structure and activity of the *E.coli* enterotoxin ST (Garièpy *et al.*, 1987). Mature HlyA has 7 Cys residues after Cys_7 is removed with the signal sequence. There are three pairs of residues (Cys_{182}/Cys_{200}; Cys_{496}/Cys_{510}; Cys_{536}/Cys_{548}) that are close to each other and may be involved in the tertiary structure of HlyA, whereas Cys_{384} is relatively isolated and may be associated with activity. However, the construction of specific mutants that alter the Cys residues is necessary to determine whether any of them are critical in the cytotoxic or cytolytic activities of HlyA.

7.4 Export of HlyA

HlyA remains localized in the periplasm when expressed in *E.coli* K-12, but is primarily extracellular in *V.cholerae*. The inability therefore of *E.coli* to excrete the haemolysin beyond the periplasm may be due to the absence of additional determinants concerned with release. However, excretion can be mimicked by introducing the cloned genes into *E.coli* K-12 *tol* mutants which leak periplasmic proteins due to changes in their outer membranes (Bernstein *et al.*, 1972; Anderson *et al.*, 1979).

The *hlyB* gene exists in a separate transcriptional unit and encodes a 60.3 kDa protein. By constructing a *hlyB* mutation in *V.cholerae*, it was demonstrated that this protein is involved in the release of HlyA during the mid-exponential phase of

growth. When cells of the mutant were fractionated during various stages of growth, and analysed for haemolytic activity, it could be seen that the haemolysin was being trapped within the cells until they approached stationary phase when it was released presumably due to cell lysis. This contrasts with the wild-type cells which produced active haemolysin in the culture supernatant during-all stages of growth, indicating that the *hlyB*::Km^R mutation was inhibiting the excretion of the haemolysin during active growth of the *V.cholerae* cell. The HlyA protein, due to its intrinsic ability to initiate transport across the cytoplasmic membrane via its signal sequence remains in the periplasm in *hlyB* mutants of both *E.coli* and *V.cholerae* suggesting that the presence of HlyB is irrelevant in *E.coli*. However, minicell analysis imply that HlyB is associated with the outer membrane.

The HlyB protein possesses a N-terminal stretch of aa's that conform to the rules of a signal sequence (von Heijne, 1984, 1985), with the predicted cleavage site after the Ala_{17} residue. This conforms with the (-3, -1) rule and places Pro_{19} in the +2 position. However, N-terminal analysis of the mature protein needs to be performed to confirm this. Analysis of a Kyte and Doolittle (1982) hydropathy plot and a Chou and Fasman (1978) predicted structure of the HlyB protein makes it tempting to speculate that the HlyB protein in some way forms a pore through which the haemolysin can be exported. There are a number of hydrophobic, β-pleated sheet regions that have the ability to span the membrane. Furthermore, there is a C-terminal β-sheet domain which is rich in negatively charged aa's. One possibility is that this region forms the inner wall of the pore structure. This negatively charged domain is probably responsible for the aberrent migration of HlyB in a polyacrylamide gel system.

V.cholerae produces a number of extracellular products of which HlyA is only one example, that fail to be transported across the outer membrane barrier when expressed in *E.coli*. This implies that *V.cholerae* possesses a specific export system for extracellular proteins that either has not been cloned or fails to function in *E.coli*. The HlyA protein cannot excrete itself from the *V.cholerae* cell with any efficiency, as extracellular haemolytic activity in a *V.cholerae hlyB*::Km^R mutant is only minimal until the cells enter stationary phase when the cells are subject to autolysis. In contrast, the

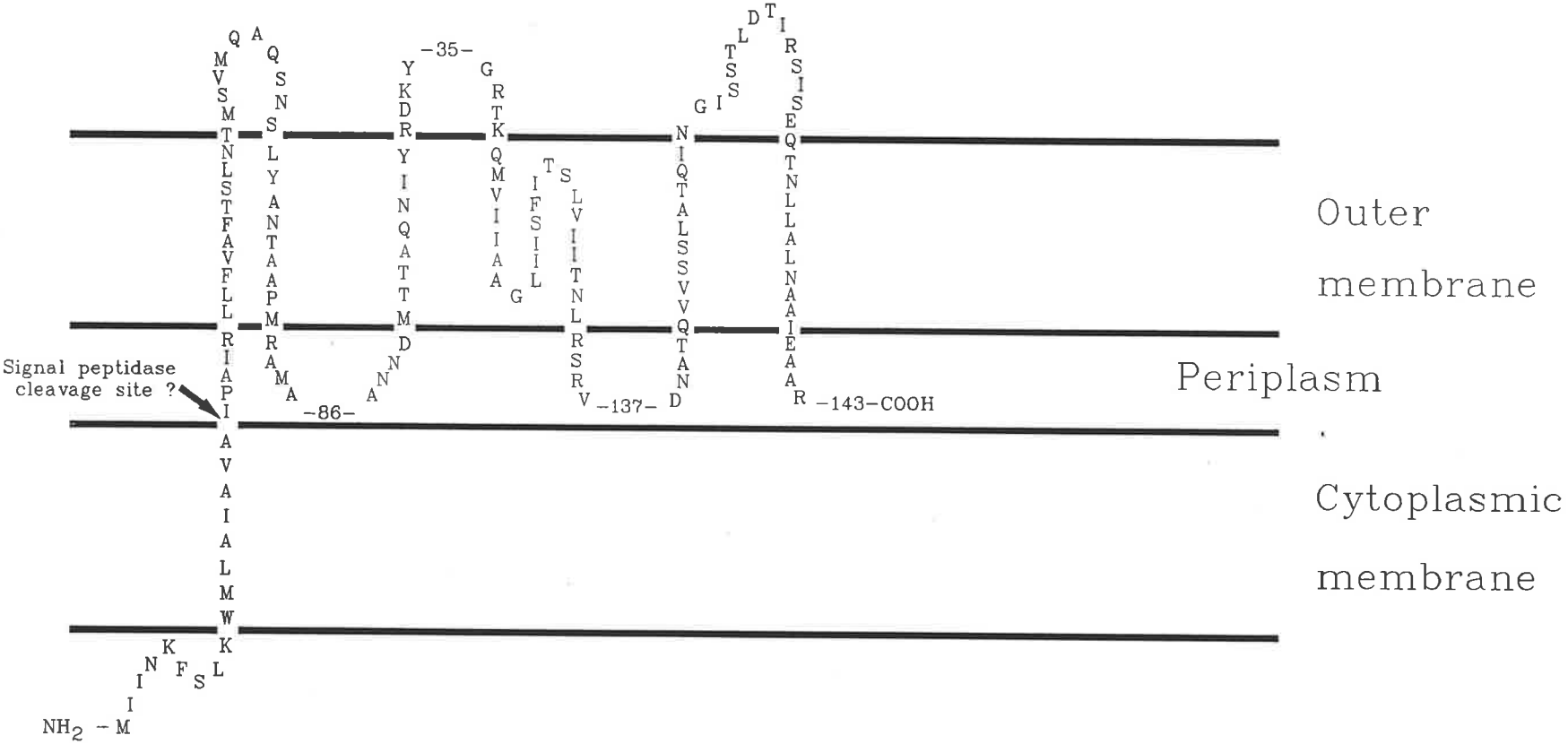
IgA protease of *N.gonorrhoeae* possesses the ability to initiate and complete excretion to the extracellular environment. Extensive banks of *V.cholerae* chromosomal Tn*5* and Tn*phoA* insertions using have been screened for the loss of a generalized transport system based on their ability to excrete HlyA and DNase. However, such mutants have not been detected. But, Tn*5* and its derivatives are known to "hot-spot" and this phenomenon may be the reason why the desired mutations have not been isolated. Alternatively such mutations are lethal to the bacterium due to the accumulation of intracellular products. A further possibility is that *V.cholerae* may possess individual excretion systems for each of its extracellular determinants.

Both HlyA and the DNase are excreted via a two-step process, as periplasmic intermediates are detected. In contrast, the α-haemolysin of *E.coli* is excreted directly from the cytosol to the extracellular environment without a periplasmic intermediate and without any N-terminal processing (Felmlee *et al.*, 1985a). The HlyB protein of *E.coli* does not possesses a signal sequence and associates with the cytoplasmic membrane (Felmlee *et al.*, 1985b). Mutations in the *E.coli hlyB* gene results in the cytoplasmic accumulation of haemolysin, and none is seen in the periplasm (Gray *et al.*, 1986; Oropeza-Wekerle *et al.*, 1989). The *E.coli* HlyB protein possesses hydrophobic transmembranous domains which cross the inner membrane, and are thought to form a pore through which the α-haemolysin can be exported. Interestingly, the HlyB protein sequence displays homology to the identical halves of the eukaryotic P-glycoprotein which crosses the lipid bilayer 12 times and acts to pump toxins out of the cell (Kartner and Ling, 1989).

A possible model to explain haemolysin excretion in *V.cholerae* and when the cloned genes are expressed in *E.coli* is as follows. The HlyA protein, via signal sequence translocation crosses the cytoplasmic membrane barrier to the periplasmic space. It is here that HlyA comes into contact with the membrane associated HlyB protein. HlyB possesses hydrophobic domains that are associated with β-pleated sheet structures that may cross the outer membrane and form a specific pore. Figure 7.4 illustrates the possible transmembrane organization of HlyB. The model maximizes the hydrophobic amino acid interaction with the membrane. Whether HlyA needs to

Figure 7.4 Model of the transmembrane organization of the HlyB protein.

The HlyB protein is proposed to be synthesized with a N-terminal signal sequence which gets cleaved and is localized in the outer membrane. Within the outer membrane there are several transmembrane β-sheet domains which are proposed to interact with the specific membrane environment to constitute a pore for release of the HlyA protein.


Signal peptidase cleavage site ?
-35-
-86-
-137-
-143-COOH
NH2 - M
Outer membrane
Periplasm
Cytoplasmic membrane

be in a certain configuration that allows specific interaction with HlyB is not known, however, further defined mutations in both HlyA and HlyB will aid in the understanding of this process. It would appear that HlyB can not function in *E.coli* and that lipopolysaccharide mutants of *V.cholerae* lacking the O-antigen side chains are defective in haemolysin excretion (U. Stroeher,- personal communication). This indicates that the HlyB protein may rely on a specific LPS interaction to function, an interaction that would not occur in *E.coli* and could thus account for the periplasmic location.

7.5 Role of HlyA in pathogenesis

It has been shown that a cytotoxin detected in *V.cholerae* of both the O1 and non-O1 serogroups stimulates fluid secretion in the rabbit ileal loop assay, and that this cytotoxin appears to be identical to the El Tor haemolysin (Madden *et al.*, 1984). In order to ascertain whether the haemolysin was responsible for these activities as well as being an alternative diarrhoeagenic factor in nontoxigenic vaccine strains, a *hlyA*::Km^R mutant was constructed. The site of this mutation was chosen specifically to be the *Mlu*I site as a mutation here would not only prevent the HlyA protein being made but also the $HlyA^*$ product seen in the Classical strain 569B would be inactivated. This enabled the following hypothesis to be tested; that the *hly* locus has been conserved in non-haemolytic Classical strains because the $HlyA^*$ protein has a alternative function.

The studies untertaken here confirm that the El Tor haemolysin possesses both cytotoxic and cytolytic properties. Several *V.cholerae* strains and their *hlyA*::Km^R mutants were tested in the infant mouse model after the *hlyA*::Km^R mutants had been cured of the plasmid pPH1JI. This was performed as it has been shown that the plasmid pPH1JI attenuated the strains in the infant mouse model. This may be due to inhibition of expression of the Tcp pilus (R. Faast, personal communication) and possibly other virulence determinants. Taylor *et al.* (1987a) isolated a number of *tcpA*::Tn*phoA* mutants still harbouring pPH1JI. Thus, the fact that they were less virulent may be due to the presence of pPH1JI. This plasmid must be removed before

any virulence assays can be performed. It was shown that the loss of the *hlyA* gene product did not totally attenuate the strains, ostensibly due to the production of cholera toxin. However, a significant effect on the virulence of the El Tor strain V760 was observed. Both the number of animals dead after 48 h, and also the rate of death were reduced in the mutant. Such a marked difference was not detected with the hypertoxigenic Classical strain 569B and this was probably due to the large amounts of cholera toxin as a result of the multiple copies of the *ctxAB* operon present. CT presumably has a masking effect on any alteration that may have been caused by the deletion of the HlyA* product. In order to eliminate the role of cholera toxin, nontoxigenic strains were also mutated in a similar manner.

The candidate vaccine strain JBK70 has been shown to induce a good immune response, significantly protect against challenge with wild type Inaba N16961 but cause mild diarrhoea in over 50% of recipients (Levine *et al.*, 1988). Kaper *et al.* (1986) investigated the role of the El Tor haemolysin in the diarrhoeal response of JBK70 by creating a small 400 bp deletion between two *Hpa*I sites within the *hlyA* gene. When this new recombinant strain, CVD104 was fed to volunteers, the same residual diarrhoea was still observed. Removal of this *Hpa*I fragment would only decrease the HlyA* product by 3 kDa. It seems likely that this may not eliminate any effects that HlyA* caused, unlike the mutation at the *Mlu*I site which deletes 19 kDa of the HlyA* product. This notion is supported by results obtained when these strains were tested in rabbit ileal loops. After 5 h, neither V759 nor V760 caused any fluid accumulation whereas the parent strains had already caused fluid secretion into the ligated ileum. When the rabbit was sacrificed after 12 h, V759 (569B*hlyA*::Km^R) and V760 (O17*hlyA*::Km^R) produced a comparable amount of fluid to their parent strains. The nontoxigenic strain JBK70, devoid of the major diarrhoeagenic factor, cholera toxin, still produced the slight diarrhoea seen by Levine *et al.* (1988). In contrast to their *hlyA* deletion mutant, the JBK70 *hlyA*::Km^R mutant described here (V736) caused no fluid accumulation at all in the ileal loop. These results seem to imply that early in *V.cholerae* infections the HlyA* product causes some diarrhoea, and this is masked later by the onset of the diarrhoea produced by cholera toxin. When the ability to

produce both cholera toxin and this 27 kDa HlyA* product is removed, no fluid secretion into the ligated ileum was observed.

It is tempting to speculate that the 15 kDa cleavage product that is derived from the N-terminal end of the mature HlyA protein contains the cytotoxic determinant. Thus, CVD104 still has the potential to produce this cytotoxic determinant, whereas V736 does not. Furthermore, Yamamoto *et al.* (1986) purified the haemolytically active extracellular product and estimated its size to be 65 kDa. On the basis of the observations made here and other emerging data, it seems likely that the El Tor haemolysin has dual functions, which are encoded by two distinct domains within the one protein. The N-terminal domain represents the cytotoxin or the residual diarrhoeagenic factor present in cholera toxin deleted strains. The larger C-terminal domain possesses the haemolytic activity. It is also interesting to speculate that these two domains are released as separate components following autoproteolysis.

To date, a general problem encountered with all immunogenic live attenuated *V.cholerae* vaccine candidates is the production of mild diarrhoea in a significant proportion of volunteers, making the vaccines unacceptable as a public health measure against cholera. The observations reported here suggest that HlyA* is expressed in Classical strains and of course its activity is also contained within HlyA in El Tor strains. This activity leads to early fluid accumulation in rabbit ileal loops but is masked later by the effects of cholera toxin, especially in hypertoxigenic strains such as 569B. It can be also readily envisaged that elimination of such an early effect by the introduction of a *hlyA* mutation could account for the time shift in the death curves of infected infant mice. Both the ability of JBK70 to produce HlyA and the mild fluid accumulation seen in rabbit ileal loops, are eliminated with the introduction of the *hlyA*::Km^R mutation. The results seen with strain 569B and its mutant, V759 indicate a cytotoxic/enterotoxic role for HlyA*. Therefore, HlyA* may be at least in part responsible for the residual toxicity and diarrhoea observed in the candidate vaccine strains constructed to date. Thus, the removal of the entire *hlyA* region may help in creating an attenuated *V.cholerae* vaccine strain that has the ability to stimulate high

levels of antitoxic antibodies, confer protection to rechallenge and yet not cause any significant adverse reactions.

7.6 Concluding Remarks

There are many documented examples of excreted bacterial proteins. However, little information is available on the mechanism of export from the cell or the sequences which are recognised within the protein during this process. Both the DNase and haemolysin of *V.cholerae* are excreted via two-step mechanisms, as periplasmic intermediates can be detected. By virtue of its signal sequence, the HlyA protein of *V.cholerae* possesses the ability to cross the inner membrane of *E.coli*. When the *hlyA* gene is expressed alone in *E.coli*, the active HlyA protein accumulates in the periplasmic space. This fact, coupled with the knowledge that the HlyB protein possesses a putative signal sequence and has been localized to the outer membrane of *E.coli* minicells, suggests that HlyB may form a channel composed of a series of β-sheet domains, through which HlyA can efficiently pass to the extracellular medium from the periplasmic space. The *hlyB*::Km^R mutants constructed in this study show a reduced efficiency to export HlyA to the culture medium in the exponential phase of growth. Furthermore, O-Antigen mutants of *V.cholerae* possess a similar inefficiency (U. Stroeher, personal communication). It is tempting to speculate that HlyB requires some form of interaction with the lipopolysaccharide (LPS) to construct this transmembranous channel, an interaction that would not be possible in *E.coli* K-12. The interaction between HlyB and LPS and its effect on haemolysin transport is a critical step in the understanding of protein export in *V.cholerae*. Site-directed mutagenesis is necessary to help define the active sites within HlyA and to elucidate the interactions between HlyA::HlyB and HlyB::LPS. This will entail analysis of:

(1) The importance of the Cys residues, not only in activity, but also in determining the conformation of HlyA so that it is able to be exported.

(2) The presence of specific topogenic sequences present in HlyA that may act as "recognition signals" for HlyB to initiate transport.

(3) The hydrophobic regions of HlyB and assessing whether the introduction of hydrophilic residues within the proposed transmembrane regions will affect pore formation, and

(4) HlyB itself, to determine whether it can function as a pore in the absence of HlyA.

A number of cytotoxins which also display cytolytic activity on erythrocytes have been documented. The experiments reported here suggest that the El Tor haemolysin (HlyA) has both cytotoxic (enterotoxic) and cytolytic activity. The C-terminal domain of HlyA possesses the cytolytic activity, whereas the N-terminal region also present in $HlyA^*$, the truncated derivative produced by Classical *V.cholerae* O1 strains contains the enterotoxic activity. These studies have significant implications in terms of cholera vaccine development. None of the attenuated strains constructed in other laboratories have eliminated the ability to produce $HlyA^*$ and all retained some diarrhoeagenic capacity. Inactivation of the *hlyA* coding region by inserting a Km^R resistance cartridge in a Tox^- strain resulted in no fluid accumulation in a rabbit ileal loop assay and a lack of the cytotoxic effect seen in HEp-2 cells. This suggests that a strain deleted for both the cholera toxin and haemolysin genes should be evaluated in human volunteers as a potential cholera vaccine.

The dual activities possessed by HlyA imply that there must be two discrete receptor recognition domains; one in the haemolysin (cytolytic C-terminus) and the other within $HlyA^*$ (cytotoxic N-terminus). This raises several questions:

(1) Are the receptors the same or are the two activities directed at different cell types?

(2) Is the proteolytic degradation observed in purification attempts an inbuilt mechanism to separate these two activities into distinct peptides, and is HlyA autoproteolytic?

(3) What are the modes of action of the two domains?

These unanswered questions provide a diversity of directions for further research on the *hly* locus of *V.cholerae* O1.

APPENDIX

Material contained in this thesis has been published or submitted for publication in the following papers:

Alm, R.A., Stroeher, U.H., and Manning, P.A. (1988) Extracellular proteins of *Vibrio cholerae*: nucleotide sequence of the structural gene (*hlyA*) for the haemolysin of the haemolytic El Tor strain O17 and characterization of the *hlyA* mutation in the non-haemolytic classical strain 569B. *Molec Microbiol* **2:** 481-488.

Alm, R.A., and Manning, P.A. (1990) Characterization of the *hlyB* gene and its role in the production of the El Tor haemolysin of *Vibrio cholerae* O1. *Molec. Microbiol.* in press.

Alm, R.A., and Manning, P.A. (1990) A biotype-specific probe for *Vibrio cholerae* O1. *J. Clin. Microbiol.* in press.

Alm, R.A., Mayrhofer, G., Kotlarski, I., and Manning, P.A. The amino terminal domain of the El Tor haemolysin of *Vibrio cholerae* is expressed in Classical strains and is cytotoxic. *Vaccine* submitted for publication.

In addition, some of the material has been accepted for a patent in association with F.H. Faulding and Co. Ltd.:

Alm, R.A., and Manning, P.A. (1989) Cholera vaccines.

BIBLIOGRAPHY

Achtman, M., Schwuchow, S., Helmuth, R., Morelli, G., and Manning, P.A. (1978) Cell-cell interactions in conjugating *Escherichia coli*: Con$^-$ mutants and stabilization of mating aggregates. *Mol. Gen. Genet.* **164**: 171-183.

Achtman, M., Manning, P.A., Edelbluth C., and Herrlich, P. (1979) Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor tra cistrons in *Escherichia coli* minicells. *Proc. Natl. Acad. Sci. USA* **76**: 4837-4841.

Al-Dujail, A.H., and Harris, D.M. (1975) *Pseudomonas aeruginosa* infection in hospital: a comparison between "infective" and "environmental" strains. *J. Hyg. Camb.* **75:** 195-201.

Alouf, J.E. (1980) Streptococcal toxins (streptolysin O, streptolysin S and erythrogenic toxin). *Pharm. Ther.* **11:** 661-717.

Andersen, B.R., and van Epps, D.F. (1972) Suppression of chemotactic activity of human neutrophils by streptolysin O. *J. Infect. Dis.* **125:** 353-359.

Anderson, J.J., Wilson, J.M., and Oxender, D.L. (1979) Defective transport and other phenotypes of a periplasmic "leaky" mutant of *Escherichia coli* K-12. *J. Bacteriol.* **140**: 351-358.

Asao, T., Kinoshita, Y., Kozaki, S., Uemura, T., and Sakaguchi, G. (1984) Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infect. Immun.* **46:** 122-127.

Asao, T., Kozaki, S., Kato, K., Kinoshita, Y., Otsu, K., Uemura, T., and Sakaguchi, G. (1986) Purification and characterization of an *Aeromonas hydrophila* hemolysin. *J. Clin. Microbiol.* **24:** 228-232.

Attridge, S.R., and Rowley, D. (1983a) The role of the flagellum in the adherence of *Vibrio cholerae*. *J. Infect. Dis.* **147**: 864-872.

Attridge, S.R., and Rowley, D. (1983b) The specificity of *Vibrio cholerae* adherence and the significance of the slime agglutinin as a second mediator of *in vitro* attachment. *J. Infect. Dis.* **147**: 873-881.

Bales, G.L., and Lankford, C.E. (1961) An adherence factor of *Vibrio cholerae*. *Bact. Proc.* p.118.

Barnes, W.M., Bevan, M., and Son, P.H. (1983) Kilo-sequencing: creation of an ordered nest of asymmetric deletion across a large target sequence carried on phage M13. *Meth. Enzymol.* **101:** 98-122.

Barrett, T.J., and Blake, P.A. (1981) Epidemiological usefulness of changes in hemolytic activity of *Vibrio cholerae* biotype El Tor during the seventh pandemic. *J. Clin. Microbiol.* **13**: 126-129.

Bauman, P., and Schubert, R.H.W. (1984) The family Vibrionaceae pp. 516-517. *In*: Bergey's Manual of Systemic Bacteriology. N.R. Krieg (ed.). Vol 1.

Beringer, J.E., Beynon, J.L., Buchanan-Wollaston, A.V., and Johnston, A.W.B. (1978) Transfer of the drug resistance transposon Tn*5* to *Rhizobium*. *Nature (London)* **276:** 633-634.

Berka, R.M., Gray, G.L., and Vasil, M.L. (1981) Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. *Infect. Immun.* **34:** 1071-1074.

Bernstein, A., Rolfe, B., and Onedera, K. (1972) Pleiotropic properties and genetic organization of the *tolAB* locus of *Escherichia coli* K-12. *J. Bacteriol.* **112**: 74-83.

Bhaskaran, K., and Sinha, V.B. (1971) Transmissible plasmid factors and fertility inhibition in *Vibrio cholerae*. *J. Gen. Microbiol.* **69:** 89-97.

Blake, P.A., Merson, M.H., Weaver, R.E., Hollis, D.G., and Heublein, P.C. (1979) Disease caused by a marine vibrio. Clinical characteristics and epidemiology. *N. Engl. J. Med.* **300:** 1-5.

Blake, P.A., Weaver, R.E., and Hollis, D.G. (1980) Diseases of humans (other than cholera) caused by vibrios. *Ann. Rev. Microbiol.* **34:** 341-364.

Bolivar, F., Rodiguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977) Construction and characterization of new vehicles II. A multipurpose cloning system. *Gene* **2**: 95-113.

Booth, B.A., Boesman-Finkelstein, M., and Finkelstein, R.A. (1983) *Vibrio cholerae* soluble hemagglutinin/protease is a metalloenzyme. *Infect. Immun.* **42:** 639-644.

Booth, B.A., Boesman-Finkelstein, M., and Finkelstein, R.A. (1984) *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. *Infect.Immun.* **45**: 558-560.

Braun, V. (1985) The iron-transport systems of *Escherichia coli*, p.617-652. *In* A.N.Martonosi (ed.), The enzymes of biological membranes, vol. 3. Plenum Publishing Corp., New York.

Braun, V., Günter, H., Neuss, B., and Tautz, C. (1985) Hemolytic activity of *Serratia marcescens*. *Arch. Microbiol.* **141:** 371-376.

Braun, V., Neuss, B., Ruan, Y., Schiebel, E., Schöffler, H., and Jander, G. (1987) Identification of the *Serratia marcescens* hemolysin determinant by cloning into *Escherichia coli*. *J. Bacteriol.* **169:** 2113-2120.

Brown, M.C.M., Weston, A., Saunders, J.R., and Humphreys, G.O. (1979) Transformation of *E.coli* C600 by plasmid DNA at different phases of growth. *FEMS Microbiol. Lett.* **5**: 219-222.

Brown, M.H., and Manning, P.A. (1985) Haemolysin genes of *Vibrio cholerae*: presence of homologous DNA in non-haemolytic O1 and haemolytic non-O1 strains. *FEMS Microbiol. Lett.* **30:** 197-201.

Brunner, K., Mauel, J., Cerottini, J.C., and Chapuis, B. (1968) Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogenic target cells *in vitro*; inhibition by isoantibody and by drugs. *Immunology* **14:** 181-196.

Burke, V., Robinson, J., Atkinson, H.M., and Gracey, M. (1982) Biochemical characteristics of enterotoxigenic *Aeromonas* spp. *J. Clin Microbiol.* **15:** 48-52.

Burrows, W., Mather, A.N., McGann, V.G., and Wagner, S.M. (1946) Studies on immunity to Asiatic cholera. II. The O and H antigenic structure of the cholera and related vibrios. *J. Infect. Dis.* **79**: 168-197.

Calderwood, S.B., and Mekalanos, J.J. (1987) Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. *J. Bacteriol.* **169:** 4759-4764.

Cash, R., Music, S., Libonati, J., Snyder, M., Wenzel, R., and Hornick, B. (1974a) Response of man to infection with *V. cholerae*. I. Clinical, serological and bacteriologic responses to a known inoculum. *J. Infect. Dis.* **129**: 45-52.

Cash, R.A., Music, S.I., Libonati, J.P., and Craig, J.P. (1974b) Response of man to infection with *Vibrio cholerae*. II. Protection from illness afforded by previous disease and vaccine. *J. Infect. Dis.* **130**: 325-333.

Cassel, D., and Pfeiffer, T. (1978) Mechanism of cholera toxin action: Covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **75:** 2669-2673.

Chakraborty, T., Huhle, B., Bergbauer, H., and Goebel, W. (1986) Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. *J. Bacteriol.* **167:** 368-374.

Chakraborty, T., Montenegro, M.A., Sanyal, S.C., Bulling, E., Helmuth, R., and Timmis, K.N. (1984) Cloning of enterotoxin gene from *Aeromonas hydrophila* provides conclusive evidence that this organism produces a cytotonic enterotoxin. *Infect. Immun.* **46:** 435-441.

Chitnis, D.S., Sharma, K.D., and Kamat, R.S. (1982) Role of somatic antigen of *Vibrio cholerae* in adhesion to intestinal mucosa. *J. Med. Microbiol.* **5**: 52-61.

Chou, P.Y., and Fasman, G.D. (1974a) Conformational parameters for amino acids in helical, β-sheet and random coil regions calculated from proteins. *Biochem.* **13**: 211-222.

Chou, P.Y., and Fasman, G.D. (1974b) Prediction of protein conformation. *Biochem.* **13**: 222-245.

Chou, P.Y., and Fasman, G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**: 45-148.

Churchward, G., Belin, D., and Nagamine, Y. (1984) A pSC101-derived plasmid which shows no sequence homology to other commonly used cloning vectors. *Gene* **31:** 165-171.

Clements, M. L., Levine, M.M., Young, C.R., Black, R.E., Lim, Y.L., Robins-Browne, R.M., and Craig, J.P. (1982) Magnitude, kinetics and duration of vibriocidal antibody responses in North Americans after ingestion of *Vibrio cholerae*. *J. Infect. Dis.* **145**: 465-473.

Clewell, D.B., and Helinski, D.R. (1969) Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. *Proc. Natl. Acad. Sci. USA* **62:** 1159-1166.

Clewell, D.B., and Helinski, D.R. (1970) Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. *Biochem.* **9:** 4428-4440.

Coleman, K., Dougan, G., and Arbuthnott, J.P. (1983) Cloning and expression in *Escherichia coli* K-12, of the chromosomal hemolysin (phospholipase C) determinant of *Pseudomonas aeruginosa*. *J. Bacteriol.* **153**: 909-915.

Craig, J.P., Yamamoto, K., Takeda, Y., and Minatani, T. (1981) Production of cholera-like enterotoxin by a *Vibrio cholerae* non-O1 strain isolated from the environment. *Infect. Immun.* **34**:90-97.

Curlin, G., Levine, R., Aziz, K.M.A., Mizanur Rahman, A.C.M., and Verway, W.F. (1975) Field trial of cholera toxoid. p 314-329. *In*: Proceedings of the 11th joint conference on cholera, U.S.-Japan Cooperative Medical Science Program.

Dallas, W.S., Moseley, S., and Falkow, S. (1979) The characterization of an *Escherichia coli* plasmid determinant that encodes for the production of a heat labile enterotoxin. p.113-122. *In* Plasmids of medical, environmental, and commercial importance. K.N. Timmis and A. Pühler (Eds.) Elsevier/North Holland Biomedical Press, New York.

Datta, A.R., Wentz, B.A., and Hill, W.E. (1987) Detection of hemolytic *Listeria monocytogenes* by using DNA colony hybridization. *Appl. Environ. Microbiol.* **53:** 2256-2259.

Datta, A.R., Wentz, B.A., Shook, D., and Trucksess, M.W. (1988) Synthetic oligodeoxynucleotide probes for detection of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **54:** 2933-2937.

Davis, B.D., Dulbecco, R., Eisen, H.N., and Ginsberg, H.S. (1980) Microbiology. 3rd ed. Harper and Row, Publishers, Inc.

De la Cruz, F., Müller, D., Ortiz, J.M., and Goebel, W. (1980) Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. *J. Bacteriol.* **143:** 825-833.

De la Cruz, F., Zabala, J.C., and Ortiz, J.M. (1983) Hemolysis determinant common to *Escherichia coli* strains of different O serotypes and origins. *Infect. Immun.* **41:** 881-887.

de Moor, C.E. (1963) A non-haemolytic vibrio. *Trop. Geogr. Med.* **15:** 97-107.

De, S.N. (1959) Enterotoxicity of bacteria-free culture filtrate of *Vibrio cholerae. Nature* **183:** 1533-1534.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980) Broad host range DNA cloning system for Gram-negative bacteria: constuction of a gene bank of *Rhizobium meliloti. Proc. Natl. Acad. Sci. USA* **77**: 7347-7351.

Dixon, J.M.S. (1960) The fate of bacteria in the small intestine. *J. Pathol. Bacteriol.* **79:** 131-140.

Donta, S.T., and Haddow, A.P. (1978) Cytotoxic activity of *Aeromonas hydrophila. Infect. Immun.* **21:** 989-993.

Easmon, C.S.F., and Adlam, C. (1984) Staphylococci and staphylococcal infections. Academic Press, Inc., London.

Ehara, M., Ishibashi, M., Watanabe, S., Iwanaga, M., Shimotori, S., and Naito, T. (1986) Fimbriae of *Vibrio cholerae* 01: observation of fimbriae on the organisms adherent to the intestinal epithelium and development of a new medium to enhance fimbriae production. *Trop. Med.* **28**: 21-33.

Ehara, M., Ishibashi, M., Ichinose, Y., Iwanaga, M., Shimotori, S., and Naito, T. (1987) Purification and partial characterization of fimbriae of *Vibrio cholerae* 01. *Vaccine* **5:** 283-288.

Evans, D.G., Evans Jr., D.J., and Tjoa, W. (1977) Haemagglutination of human group A erythrocytes by enterotoxigenic and enteropathogenic *Escherichia coli* isolated from adults with diarrhoea: correlation with colonization factor. *Infect. Immun.* **18:** 330-337.

Faast, R., Ogierman, M.A., Stroeher, U.H., and Manning, P.A. (1989) Nucleotide sequence of the structural gene, *tcpA*, for a major pilin subunit of *Vibrio cholerae. Gene* in press.

Fairweather, N., Kennedy, S., Foster, T.J., Kehoe, M., and Dougan, G. (1983) Expression of a cloned *Staphylococcus aureus* α-hemolysin determinant in *Bacillus subtilus* and *Staphylococcus aureus. Infect. Immun.* **41:** 1112-1117.

Feeley, J.C. (1965) Classification of *Vibrio cholerae* (*Vibrio comma*), including El Tor vibrios, by intrasubspecific characteristics. *J. Bacteriol.* **89**:665-678.

Felmlee, T., Pellet, S., Lee, E.Y., and Welch, R. (1985a) *Escherichia coli* haemolysin is released extracellularly without cleavage of a signal peptide. *J. Bacteriol.* **163**: 88-93.

Felmlee, T., Pellet, S., and Welch, R. (1985b) Nucleotide sequence of an *Escherichia coli* chromosomal haemolysin. *J. Bacteriol.* **163**: 94-105.

Ferencz, A., Orskov, I., Orskov, F., and Klemm, P. (1980) Deoxyribonucleic acid is a significant component of the small intestinal mucus. *Acta. Path. Microbiol. Scand.* **80**: 347-348

Finkelstein, R.A. (1969) The role of choleragen in the pathogenesis and immunology of cholera. *Tex. Rep. Biol. Med.* **27** S1: 181-201.

Finkelstein, R.A. (1973) Cholera. *Crit. Rev. Microbiol.* **2**: 553-623.

Finkelstein, R.A., and Hanne, L.F. (1982) Purification and characterization of the soluble hemagglutinin (cholera lectin) produced by *Vibrio cholerae*. *Infect. Immun.* **36**: 119-1208.

Finkelstein, R.A., Arita, M., Clements, J.D., and Nelson, E.T. (1978) Isolation and purification of an adhesive factor ("cholera lectin") from *Vibrio cholerae*. p.137-151. *In*: Proceedings of the 13th Joint Conference on Cholera. U.S.-Japan Cooperative Medical Science Program. J.P. Craig and H. Fukumi (eds). NIH publication no. 78-1590. U.S. Government Printing Office,

Finkelstein, R.A., Boesman-Finkelstein, M., and Holt, P. (1983) *Vibrio cholerae* hemagglutinin-lectin-protease hydrolyzes fibronectin and ovomucin: F.M. Burnet revisited. *Proc. Natl. Acad. Sci. USA* **80:** 1092-1095.

Focareta, T. (1989) The extracellular Dnase(s) of *Vibrio cholerae*. PhD. Thesis, University of Adelaide.

Focareta, T., and Manning, P.A. (1987) Extracellular proteins of *Vibrio cholerae*: molecular cloning, nucleotide sequence and characterization of the deoxyribonuclease (DNase) together with its periplasmic localization in *Escherichia coli* K-12. *Gene* **53**: 31-40.

Forrest, B.D., LaBrooy, J.T., Attridge, S.R., Boehm, G., Beyer, L., Morona, R., Shearman, D.J.C., and Rowley, D. (1989) Immunogenecity of a candidate live oral typhoid/cholera hybrid vaccine in humans. *J. Infect. Dis.* **159:** 145-146.

Franzon, V.L., Clark, C.A., Williams, S.G., and Manning, P.A. (1989) Nucleotide sequence and construction of a mutant in a mannose-fucose resistant haemagglutinin (MFRHA) of *Vibrio cholera* 01. *In*: (Eds. Y.Takeda and R.B. Sack). Advances in Research on Cholera and Related Diarrheas Vol. 8. KTK Publications, Tokyo, in press.

Fraser, T.H., and Bruce, B.J. (1978) Chicken ovalbumin is synthesized and secreted by *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **75:** 5936-5940.

Freer, J.H., and Arbuthnott, J.P. (1983) Toxins of *Staphylococcus aureus*. *Pharmacol. Ther.* **19:** 55-106.

Freter, R. (1974) Gut-associated immunity to cholera. p.315-331. *In*: Cholera. D. Barua and W. Burrows (eds). W.B. Saunders Co.

Freter, R., and O'Brien, P.C.M. (1981) Role of chemotaxis in the association of motile bacteria with intestinal mucosa: fitness and virulence of nonchemotactic *Vibrio cholerae* mutants in infant mice. *Infect. Immun.* **34**: 222-233.

Gaillard, J.-L., Berche, P., and Sansonetti, P. (1986) Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. *Infect. Immun*. **52:** 50-55.

Gaillard, J.-L., Berche, P., Mounier, J., Richard, S., and Sansonetti, P. (1987) In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect. Immun*. **55:** 2822-2829.

Galen, J.E., Vimr, E.R. Lawrisuk, L., and Kaper, J. (1987) Cloning, sequencing, mutation and expression of the gene, *nanH*, for *Vibrio cholerae* neuraminidase. p.41-42. *In*: Abstracts of the 23rd Joint Conference on Cholera. U.S.-Japan Cooperative Medical Science Program.

Gan, K.H., and Tjia, S.K. (1963) A new method for the differentiation of *Vibrio comma* and Vibrio El Tor. *Am. J. Hyg.* **77:** 184-186.

Gangarosa, E.J., Sonati, A., Saghari, H., and Feeley, J.C. (1967) Multiple serotypes of *Vibrio cholerae* from a case of cholera. *Lancet* **i:** 646-648.

Gardner, A.D., and Venkatraman, K.V. (1935) The antigens of the cholera group of vibrios. *J. Hyg. (Camb).* **35**: 262-282.

Garger, S.J., Griffith, O.M., and Grill, L.K. (1983) Rapid purification of plasmid DNA by a single centrifugation in a two step cesium chloride-ethidium bromide gradient. *Biochem. Biophys. Res. Commun.* **117**: 835-842.

Gariépy, J., Judd, A.K., and Schoolnik, G.K. (1987) Importance of disulfide bridges in the structure and activity of *Escherichia coli* enterotoxin ST1b. *Proc. Natl. Acad. Sci. USA.* **84:** 8907-8911.

Gentz, R., and Bujard, H.G. (1985) Promoters recognized by *Escherichia coli* RNA polymerase selected by function: highly efficient promoters from bacteriophage T5. *J. Bacteriol.* **164**: 70-77.

Germanier, R., and Fürer, E. (1975) Isolation and characterization of a *galE* mutant Ty21a of *Salmonella typhi*: a candidate strain for live oral typhoid vaccine. *J. Infect. Dis.* **131**: 553-558.

Gill, D.M., and Meren, R. (1978) ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA.* **75:** 3040-3054.

Goebel, W., Royer-Pokora, B., Lindenmaier, W., and Bujard, H. (1974) Plasmids controlling synthesis of hemolysin in *Escherichia coli*: molecular properties. *J. Bacteriol.* **118**: 964-973.

Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer, B.W., and Stormo, G. (1981) Translational initiation in prokaryotes. *Ann. Rev. Microbiol.* **35:** 365-403.

Goldberg, S.L., and Murphy, J.R. (1984) Molecular cloning of the hemolysin determinant from *Vibrio cholerae* El Tor. *J. Bacteriol.* **160**: 239-244.

Goldberg, S.L., and Murphy, J.R. (1985) Cloning and characterization of the hemolysin determinants from *Vibrio cholerae* RV79(Hly$^+$), RV79(Hly$^-$) and 569B *J. Bacteriol.* **162:** 35-41.

Gough, J.A., and Murray, N.E. (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* **166:** 1-19.

Gray, L., Mackman, N., Nicaud, J.M., and Holland, I.B. (1986) The carboxy-terminal region of haemolysin 2001 is required for secretion of the toxin from *Eschericha coli. Mol. Gen. Genet.* **205**: 127-133.

Groves, R.D., and Welshimer, H.J. (1977) Separation of pathogenic from apathogenic *Listeria monocytogenes* by three in vitro reactions. *J. Clin. Microbiol.* **5:** 559-563.

Guidolin, A., and Manning, P.A. (1985) *Vibrio cholerae* bacteriophage CP-T1: characterization of the receptor. *Eur. J. Biochem.* **153**: 89-94.

Guidolin, A., and Manning, P.A. (1988) Molecular analysis of the packaging signal in bacteriophage CP-T1 of *Vibrio cholerae. Mol. Gen. Genet.* **212:** 514-521.

Halegoua, S., Sekizawa, J., and Inouye, M. (1976) A new form of structural lipoprotein of outer membrane of Escherichia coli. *J. Biol. Chem.* **252:** 2324-2330.

Halter, R., Pohlner, J., and Meyer, T.F. (1989) Mosaic-like organization of IgA protease genes in *Neisseria gonorrhoeae* generated by horizontal genetic exchange *in vivo. EMBO J.* **8:** 2737-2744.

Hanne, L.F., and Finkelstein, R.A. (1982) Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae. Infect. Immun.* **36**: 209-214.

Hantke, K. (1982) Negative control of iron uptake systems in *Escherichia coli. FEMS Microbiol. Lett.* **15:** 83-86.

Hawkes, R., Niday, E., and Gordon, J. (1982) A dot-immuno binding assay for monoclonal and other antibodies. *Anal. Biochem.* **119**: 142-147.

Hawley, D.K., and McClure, W.R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucl. Acids Res.* **11**: 2237-2255.

Higgins, C., Hiles, I., Whalley, K., and Jamieson, D. (1985) Nucleotide binding by membrane components of bacterial periplasmic binding protein-dependent transport systems. *EMBO J.* 4: 1033-1040.

Holmes, D.S., and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**: 193-195.

Holmgren, J. (1981) Actions of cholera toxin and the prevention and treatment of cholera. *Nature* **292**: 413-417.

Honda, T., Goshima, K., Takeda, Y., Sugino, Y., and Miwatani, T. (1976a) Demonstration of the cardiotoxicity of the thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus*. *Infect. Immun.* **13:** 163-171.

Honda, T., Taga, S., Takeda, T., Hasibuan, M.A., Takeda, Y., and Miwatani, T. (1976b) Identification of lethal toxin with the thermostable direct hemolysin produced by *Vibrio parahaemolyticus*, and some physicochemical properties of the purified toxin. *Infect. Immun.* **13**: 133-139.

Honda, T., Shimizu, M., Takeda, Y., and Miwatani, T. (1976c) Isolation of a factor causing morphological changes of CHO cells from the culture filtrate of *Vibrio parahaemolyticus*. *Infect. Immun.* **14:** 1028-1033.

Honda, T., and Finkelstein, R.A. (1979a) Selection and characterization of a novel *Vibrio cholerae* mutant lacking the A (ADP-ribosylating) portion of the cholera enterotoxin. *Proc. Natl. Acad. Sci. USA*. **76**: 2052-2056.

Honda, T., and Finkelstein, R.A. (1979b) Purification and characterization of a hemolysin produced by *Vibrio cholerae* biotype El Tor; another toxic substance produced by cholera vibrios. *Infect. Immun.* **26:** 1020-1027.

Honda, T., Ni, Y., and Miwatani, T. (1988) Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infect. Immun.* **56:** 961-965.

Howard, S.P., and Buckley, J.T. (1982) Membrane glycoprotein receptor and hole forming properties of a cytolytic protein toxin. *Biochemistry* **21:** 1662-1667.

Howard, S.P., and Buckley, J.T. (1985) Protein export by a Gram-negative bacterium: Production of aerolysin by *Aeromonas hydrophila.* **161**: 1118-1124.

Howard, S.P., and Buckley, J.T. (1986) Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila. Mol. Gen. Genet.* **204**: 289-295.

Hugh, R. (1964) The proposed conservation of the generic name Vibrio Pacini 1854 and designation of the neotype strain of *Vibrio cholerae* Pacini 1854. *Int. Bull. Bacteriol. Nomen. Tax.* **14:** 87-101.

Hughes, C., Müller, D., Hacker, J., and Goebel, W. (1985) Genetics and pathogenic role of *Escherichia coli* hemolysin. *Toxicon.* **20:** 247-252.

Ichinose, Y., Yamamoto, K., Nakasone, N., Tanabe, M.J., Takeda, T., Miwatani, T., and Iwanaga, M. (1987) Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae. Infect. Immun.* **55:** 1090-1093.

Inouye, M., and Halegoua, S. (1980) Secretion and membrane localization of proteins in *Escherichia coli. CRC Crit. Rev. Biochem.* **7:** 339-371.

Inouye, S., Viasnk, G.P., Hsuing, H., and Inonye, M. (1984) Structural flexibility in the hydrophobic region of the *Escherichia coli* prolipoprotein signal peptide. *J. Biol. Chem.* **259**: 3729-3733.

Inouye, S., Wang, S., Sekizama, J., Halegona, S., and Inonye, M. (1977) Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. *Proc. Natl. Acad. Sci. USA*. **74**: 1004-1008.

Johnson, M.K. (1977) Cellular location of pneumolysin. *FEMS Microbiol. Lett.* **2:** 243-245.

Jones, G.W., and Freter, R. (1976) Adhesive properties of *Vibrio cholerae*: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. *Infect. Immun.* **14**: 240-245.

Jones, G.W., Abrams, G.D., and Freter, R. (1976) Adhesive properties of *Vibrio cholerae*: adhesion to isolated rabbit brush border membranes and haemagglutinating activity. *Infect. Immun.* **14**: 232-239.

Jonsson, P., Lindberg, M., Haraldsson, I., and Wadström, T. (1985) Virulence of *Staphylococcus aureus* in a mouse mastitis model: studies of alpha hemolysin, coagulase, and protein A as possible virulence determinants with protoplast fusion and gene cloning. *Infect. Immun.* **49:** 765-769.

Kabir, S. (1983) Immunochemical properties of the major outer membrane protein of *Vibrio cholerae*. *Infect. Immun.* **39**: 452-455.

Kabir, S., and Showkat, A. (1983) Characterization of surface properties of *Vibrio cholerae*. *Infect Immun.* **39**: 1048-1058.

Kabir, S., Ahmad, N., and Ali, S. (1984) Neuraminidase production by *Vibrio cholerae* O1 and other diarrheagenic bacteria. *Infect. Immun.* **44:** 747-749.

Kaper, J.B., Moseley, S.L., and Falkow, S. (1981a) Molecular characterization of environmental and nontoxinogenic strains of *Vibrio cholerae*. *Infect. Immun.* **32**: 661-667.

Kaper, J.B., Lockman, H., and Colwell, R.R. (1981b) *Aeromonas hydrophila*: ecology and toxigenicity of isolates from an estuary. *J. Appl. Bacteriol.* **50:** 359-377.

Kaper, J.B., Lockman, H.A., Baldini, M.M., and Levine, M.M. (1984a) A recombinant live oral cholera vaccine. *Biotechnology* **2**: 345-349.

Kaper, J.B., Lockman, H.A., Baldini, M.M., and Levine, M.M. (1984b) Recombinant nontoxinogenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. *Nature* **308**: 655-658.

Kaper, J.B., Campen, R.K., Seidler, R.J., Baldini, M.M., and Falkow, S. (1984c) Cloning of the thermostable direct or Kanagawa phenomenon associated hemolysin of *Vibrio parahaemolyticus*. *Infect. Immun.* **45:** 290-292.

Kaper, J.B., Mobley, H.L.T., Michalski, J.M., Herrington, D.A., and Levine, M.M. (1986) Recent advances in developing a safe and effective live oral attenuated *Vibrio cholerae* vaccine. *In* Advances in Research on Cholera and Related Diarrheas. Vol. 6. KTK Scientific Publications, Tokyo.

Kartner, N., and Ling, V. (1989) Multidrug resistance in cancer. *Sci. Am.* **260:** 26-33.

Kathariou, S., Metz, P., Hof, H., and Goebel, W. (1987) Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *J. Bacteriol.* **169:** 1291-1297.

Kehoe, M., and Timmis, K.N. (1984) Cloning and expression in *Escherichia coli* of the streptolysin O determinant from *Streptococcus pyogenes*: characterization of the cloned streptolysin O determinant and demonstration of the absence of substantial homology with determinants of other thiol-activated toxins. *Infect. Immun.* **43:** 804-810.

Kehoe, M., Duncan, J., Foster, T.J., Fairweather, N., and Dougan, G. (1983) Cloning, expression and mapping of the *Staphylococcus aureus* alpha-hemolysin determinant in *Escherichia coli* K-12. *Infect. Immun.* **41:** 1105-1111.

Kehoe, M.A., Miller, L., Walker, J.A., and Boulnois, G.J. (1987) Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging thiol-activated toxins. *Infect. Immun.* **55:** 3228-3232.

Kelly, M.T., and Avery, D.M. (1980) Lactose-positive *Vibrio* in seawater: a cause of pneumoniae and septicemia in a drowning victim. *J. Clin. Microbiol.* **11:** 278-280.

Kennedy, N., Beutin, L., Achtman, M., Skurray, R., Rahmsdorf, U., and Herrlich, P. (1977) Conjugation proteins of the F sex factor. *Nature* **270**: 580-585.

König, B., König, W., Scheffer, J., Hacker, J., and Goebel, W. (1986) Role of *Escherichia coli* alpha-hemolysin and bacterial adherence in infection: requirement for release of inflammatory mediators from granulocytes and mast cells. *Infect. Immun.* **54:** 886-892.

König, W., Faltin, Y., Scheffer, J., Schöffler, H., and Braun, V. (1987) Role of cell-bound hemolysin as a pathogenicity factor for *Serratia* infections. *Infect. Immun.* **55:** 2554-2561.

Kozak, M. (1983) Comparison of initiation of protein synthesis in procaryotes, eucaryotes and organelles. *Microbiol. Rev.* **47**: 1-45.

Kreger, A., and Lockwood, D. (1981) Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. *Infect. Immun.* **33:** 585-590.

Kreger, A.S. (1984) Cytolytic activity and virulence of *Vibrio damsela*. *Infect. Immun.* **44:** 326-331.

Kurioka, S., and Liu, P.V. (1967) Effect of the hemolysin of *Pseudomonas aeruginosa* on phosphatides and on phospholipase C activity. *J. Bacteriol.* **93:** 670-674.

Kyte, J., and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**: 105-132.

Lebek, G., and Greunig, H-M. (1985) Relation between the hemolytic property and iron metabolism in *Escherichia coli*. *Infect. Immun.* **50:** 682-686.

Lee, J.V., Donovan, T.J., and Furniss, A.L. (1978) Characterization, taxonomy, and amended description of *Vibrio metschnikovii*. *Int. J. Syst. Bacteriol.* **28:** 99-111.

Leimeister-Wächter, M., and Chakraborty, T. (1989) Detection of Listeriolysin, the thiol-dependant hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Literia seeligeri*. *Infect. Immun* **57:** 2350-2357.

Lennette, E.H., Balows, A., Hausler Jr., W.J., and Truant, J.P. (1980) Manual of Clinical Microbiology. American Society for Microbiology.

Levine, M.M., Black, R.E., Clements, M.L., Cisneros, L., Saah, A., Nalin, D.R., Gill, D.M., Craig, J.P., Young, C.R., and Ristaino, P. (1982) The pathogenicity of non-enterotoxigenic *Vibrio cholerae* serotype 01 biotype El Tor isolated from sewage water in Brazil. *J. Infect. Dis.* **145**: 296-299.

Levine, M.M., Black, R.E., Clements, M.L., Young, C.R., Lanata, C., Sears, S., Honda, T., and Finkelstein, R.A. (1983) Texas Star-SR: Attenuated *Vibrio cholerae* oral vaccine candidate. *Develop. Biol. Standard.* **53:** 59-65.

Levine, M.M., Black, R.F., Clements, M.L., Lanata, C., Sears, S., Honda, T., Young, C.R., and Finkelstein, R.A. (1984) Evaluation in humans of attenuated *Vibrio cholerae* El Tor Ogawa strain Texas Star-SR as a live oral vaccine. *Infect. Immun.* **43**: 515-522.

Levine, M.M., Kaper, J.B., Herrington, D., Losonsky, G., Morris, J.G., Clements, M.L., Black, R.E., Tall, B., and Hall, R. (1988) Volunteer studies of deletion mutants of *Vibrio cholerae* 01 prepared by recombinant techniques. *Infect. Immun.* **56**: 161-167.

Liu, P.V. (1979) Toxin of *Pseudomonas aeruginosa*, p. 63-68. *In* Doggett, R.G. (ed.), *Pseudomonas aeruginosa*. Academic Press, Inc., New York.

Ljungh, A., and Wadstrom, T. (1983) Toxins of *Vibrio parahaemolyticus* and *Aeromonas hydrophila*. *J. Toxicol. Toxin. Rev.* **1:** 257-307.

Lockman, H., and Kaper, J.B. (1983) Nucleotide sequence analysis of the A2 and B subunits of *Vibrio cholerae* enterotoxin. *J. Biol. Chem.* **258**: 13722-13726.

Lockman, H.A., Galen, J.E., and Kaper, J.B. (1984) *Vibrio cholerae* enterotoxin genes: Nucleotide sequence analysis of DNA encoding ADP-ribosyltransferase. *J. Bacteriol.* **159:** 1086-1089.

Lockwood, D.E., Kreger, A.S., and Richardson, S.H. (1982) Detection of toxins produced by *Vibrio fluvialis*. *Infect. Immun.* **35:** 702-708.

Lospalluto, T.J., and Finkelstein, R.A. (1972) Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid). *Biochem. Biophys. Acta.* **257:** 158-166.

Love, M., Teebken-Fisher, D., Hose, J.E., Farmer III, J.J., Hickman, F.W., and Fanning, G.R. (1981) *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damsel fish *Chromis punctipinnis*. *Science* **214:** 1139-1140.

Ludwig, D.S., Ribi, H.O., Schoolnik, G.K., and Kornberg, R.D. (1986) Two-dimensional crystals of cholera toxin B-subunit receptor complexes: Projected structure at 17- AA resolution. *Proc. Natl. Acad. Sci. USA* **83:** 8585-8588.

Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P., and van Alphen, L. (1975) Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K-12 into four bands. *FEBS Lett.* **58**: 254-258.

Mackman, N., Nicaud, J.M., Gray, L., and Holland, I.B. (1986) Secretion of haemolysin by *Escherichia coli*. *Curr. Top. Microbiol. Immunol.* **125**: 159-181.

Madden, J.M., McCardell, B.A., and Shah, D.B. (1984) Cytotoxin production by members of genus Vibrio. *Lancet* **i**: 1217-1218.

Maki, D.G., Hennekens, C.G., Philips, C.W., Shaw, W.V., and Bennet, J.V. (1973) Nosocomial urinary tract infection with *Serratia marcescens*. *J. Infect. Dis.* **128:** 579-587.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Maniatis, T., Jeffrey, A., and Kleid, G. (1975) Nucleotide sequence of the rightward operator of phage lambda. *Proc. Natl. Acad. Sci. USA* **72:** 1184-1188.

Manning, P.A. (1987) Involvement of cell envelope components in the pathogenesis of *Vibrio cholerae*: targets for cholera vaccine development. *Vaccine* **5**: 83-87.

Manning, P.A. (1988) Molecular genetic approaches to the study of *Vibrio cholerae*. *Microbiol. Sci.* **5**: 196-201.

Manning, P.A. (1989a) A molecular approach to cholera vaccines. *Today's Life Sciences* **1:** 38-47.

Manning, P.A. (1989b) New and improved vaccines against cholera: avirulent Salmonellae expressing cloned *Vibrio* genes as potential oral immunogens. *In* (Eds. G.C. Woodrow and M.M. Levine) The Molecular Route to New and Improved Vaccines. Marcel Dekker Inc., New York.

Manning, P.A., Brown, M.H., and Heuzenroeder, M.W. (1984) Cloning of the structural gene (hly) for the hemolysin of *Vibrio cholerae* El Tor strain O17. *Gene* **31**: 225-231.

Manning, P.A., Heuzenroeder, M.W., Yeadon, J., Leavesley, D.I., Reeves, P.R., and Rowley. D. (1986) Molecular cloning and expression in *Escherichia coli* K-12 of the O antigens of the Ogawa and Inaba serotypes of the lipopolysccharide of *Vibrio cholerae* O1 and their potential for vaccine development. *Infect. Immun.* **53**: 272-277.

McCardell, B.A., Madden, J.M., and Shah, D.B. (1985) Isolation and characterization of a cytolysin produced by *Vibrio cholerae* serogroup non-O1. *Can. J. Microbiol.* **31:** 711-720.

McIntosh, M.A., and Earhart, C.F. (1976) Effect of iron on the relative abundance of two large polypeptides of the *Escherichia coli* outer membrane. *Biochem. Biophys. Res. Commun.* **70:** 315-322.

McIntyre, O.R., and Feeley, J.C. (1965) Characteristics of non-cholera vibrios isolated from cases of human disrrhoea. *Bull. Wld. Hlth. Org.* **32:** 627-632.

Mekalanos, J.J. (1983) Duplication and amplification of toxin genes in *Vibrio cholerae. Cell* **35**: 253-263.

Mekalanos, J.J. (1985) Cholera toxin: genetic analysis, regulation and role in pathogenesis. *Curr. Top. Microbiol. Immunol.* **118**: 97-118.

Mekalanos, J.J., Collier, R.J., and Romig, W.R. (1979) Enzymatic activity of cholera toxin. II. Relationships to proteolytic processing, disulfide bond reduction, and subunit composition. *J. Biol. Chem.* **254**: 5855-5861.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D.N., Harford, N., Groyne, F., and de Wilde, M. (1983) Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. *Nature* **306**: 551-557.

Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J., and Cossart, P. (1987) Isolation of the structural gene encoding the SH-activated hemolysin of *Listeria monocytogenes*: listeriolysin O is homologous to streptolysin O and pneumolysin. *Infect. Immun.* **55:** 3225-3227.

Mengaud, J., Vicente, M.-F., Chenevert, J., Pereira, J.M., Geoffrey, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.-C., and Cossart, P. (1988) Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. *Infect. Immun.* **56:** 766-772.

Mercurio, A., and Manning, P.A. (1985) Cellular localization and export of the soluble haemolysin of *Vibrio cholerae* El Tor. *Mol. Gen. Genet.* **200:** 472-475.

Messing, J., and Vieira, J. (1982) A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene* **19**: 269-276.

Miller, J. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, New York.

Miller, V.L., and Mekalanos, J.J. (1984) Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. *Proc. Natl.Acad. Sci. USA* **81**: 3471-3475.

Miller, V.L., Taylor, R.K., and Mekalanos, J.J. (1987) Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. *Cell* **48**: 271-279.

Miller, V.L., DiRita, V.J., and Mekalanos, J.J. (1989) Identification of *toxS*, a regulatory gene whose product enhances ToxR-mediated activation of the cholera toxin promoter. *J. Bacteriol.* **171**: 1288-1293.

Minshew, B.H., Jorgenson, J., Counts, G.W., and Falkow, S. (1978) Association of hemolysin production and hemagglutination of human erythrocytes and virulence for chicken embryos of extraintestinal *Escherichia coli* isolates. *Infect. Immun.* **20:** 50-54.

Miyake, M., Honda, T., and Miwatani, T. (1988) Purification and characterization of *Vibrio metschnikovii* cytolysin. *Infect. Immun.* **56:** 954-960.

Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K., and Yamai, S. (1969) In vitro hemolytic characteristics of *Vibrio parahaemolyticus*: its close correlation with human pathogenecity. *J. Bacteriol.* **100:** 1147-1149.

Miyamoto, Y., Obana, Y., Nikkawa, T., Yamai, S., Kato, T., Yamada, Y. and Ottashi, M. (1980) Simplified purification and biophysiochemical characteristics of Kanagawa phenomenon associated hemolysin of *Vibrio parahemolyticus. Infect. Immun.* 28: 567-576.

Monsur, K.A., Rizvi, S.S.H., Huq, M.I., and Beneson, A.S. (1965) Effect of Mukerjee's Group IV phage on El Tor vibrios. *Bull. Wld. Hlth. Org.* **32:** 211-216.

Morris, J.G. Jr., Wilson, R., Hollis, D.G., Weaver, R.E., Miller, H.G., Tacket, C.O., Hickman, F.W., and Blake, P.A. (1982) Illness caused by *Vibrio damsela* and *Vibrio hollisae. Lancet* **i:** 1294-1297.

Moseley, S.L., and Falkow, S. (1980) Nucleotide sequence homology between the heat labile enterotoxin gene of *Escherichia coli* and *Vibrio cholerae* DNA. *J. Bacteriol.* **144:** 441-446.

Mukerjee, S., and Roy, G. (1961) Evaluation of tests for differentiating *Vibrio cholerae* and El Tor vibrios. *Ann. Biochem. Exp. Med.* **21:** 129-132.

Müller, D., Hughes, C., and Goebel, W. (1983) Relationship between plasmid and chromosomal determinants of *Escherichia coli. J. Bacteriol.* **153:** 846-851.

Nalin, D.R., Levine, R.J., Levine, M.M., Hoover, D., Bergquist, E., McLaughlin, J., Libonati, J., Alam, J., and Hornick, R.B. (1978) Cholera, non-vibrio cholera and stomach acid. *Lancet* **ii:** 856-859.

Neilands, J.B. (1982) Microbial envelope proteins related to iron. *Ann. Rev. Microbiol.* **36:** 285-309.

Nelson, E.T., Clements, J.D., and Finkelstein, R.A. (1976) *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. *Infect. Immun.* **14**: 527-547.

Nelson, E.T., Hochli, M., Hackenbrock, C.R., and Finkelstein, R.A. (1977) Electron microscopic observations on intestinal colonization by *Vibrio cholerae*: freeze-etching studies. p.81-87. *In*: Symposium on cholera: Sapporo 1976 (Proceedings of the 12th Joint Conference on Cholera, U.S.-Japan Cooperative Medical Science Program). H. Fukumi and Y. Zinnaka (eds.)

Newland, J. W., Green, B.A., Foulds, J., and Holmes, R.K. (1985) Cloning of extracellular DNase and construction of a DNase-negative strain of *Vibrio cholerae. Infect. Immun.* **47**: 691-696.

Nicaud, J.M., Mackman, N., Gray, L., and Holland, I.B. (1985) Characterization of HlyC and mechanism of activation and secretion of haemolysin 2001 from *E.coli. FEBS Lett.* **187**: 339-344.

Nicaud, J-M., Mackman, N., Gray, L., and Holland, I.B. (1986) The terminal 23 kDa peptide of *E.coli* haemolysin 2001 contains all the information necessary for its secretion by the haemolysin (Hly) export machinery. *FEBS Lett.* **204:** 331-335.

Nishibuchi, M., and Kaper, J.B. (1985) Nucleotide sequence of the thermostable direct haemolysin gene of *Vibrio parahaemolyticus. J. Bacteriol.* **162:** 558-564.

Nishibuchi, M., Seidler, R.J., Rollins, D.M., and Joseph, S.W. (1983) Vibrio factors cause rapid fluid accumulation in suckling mice. *Infect. Immun.* **40:** 1083-1091.

Nishibuchi, M., Ishibashi, M., Takeda, Y., and Kaper, J.B. (1985) Detection of the thermostable direct haemolysin gene and related DNA sequences in *Vibrio parahaemolyticus* and other Vibrio species by the DNA colony hybridization test. *Infect. Immun.* **49:** 481-486.

Norqvist, A., Davies, J., Norlander, L., and Normark, S. (1978) The effect of iron starvation on the outer membrane protein composition of *Neisseria gonorrhoeae. FEMS Microbiol. Lett.* **4:** 71-75.

O'Brien, A.D., Chen, M.E., Holmes, R.K., Kaper, J.B., and Levine, M.M. (1984) Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* (Shiga-like) cytotoxin. *Lancet* **i**: 77-78.

Ofek, I., and Beachey, E.H. (1980) Bacterial adherence. *Ad. Intern. Med.* **25:** 503-532.

Ogg, J.E., Shrestha, M.B., and Poudaryl, L. (1978) Phage-induced changes in *Vibrio cholerae*: serotype and biotype conversions. *Infect. Immun.* **19**: 231-238.

Ogg, J.E., Ogg, B.J., Shrestha, M.B., and Poudayl, L. (1979) Antigenic changes in *Vibrio cholerae* biotype El Tor serotype Ogawa after bacteriophage infection. *Infect. Immun.* **24**: 974-978.

Okada, K., Mitsuyama, M., Miake, S., and Amako, K. (1987) Monoclonal antibodies against the haemolysin of *Vibrio vulnificus. J. Gen. Microbiol.* **133:** 2279-2284.

Oropeza-Wekerle, R.L., Müller, E., Kern, P., Meyerman, R., and Goebel, W. (1989) Synthesis, inactivation, and localization of extracellular and intracellular *Escherichia coli* hemolysins. *J. Bacteriol.* **171:** 2783-2788.

Osborn, M.J., Gander, J.E., Parisi, E., and Carson, J. (1972) Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* **247:** 3962-3972.

Palmiter, R.D., Gagnon, J., and Walsh, K.A. (1978) Ovalbumin: a secreted protein without a transient hydrophobic leader sequence. *Proc. Natl. Acad. Sci. USA.* **75:** 94-98.

Parrisius, J., Bhakdi, S., Roth, M., Tranum-Jensen, J., Goebel, W., and Seeliger, H.P.R. (1986) Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. *Infect. Immun.* **51:** 314-319.

Paton, J.C., and Ferrante, A. (1983) Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infect. Immun.* **41:** 1212-1216.

Paton, J.C., Berry, A.M., Lock, R.A., Hansman, D., and Manning, P.A. (1986) Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. *Infect. Immun.* **54:** 50-55.

Paton, J.C., Lock, R.A., and Hansman, D.J. (1983) Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect. Immun.* **40:** 1085-1087.

Pearson, G.D.N., and Mekalanos, J.J. (1982) Molecular cloning of the *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* **79:** 2976-2980.

Pesigan, T.P., Gomez, C.Z., and Gaetos, D. (1967) Variants of agglutinable vibrios in the Philippines. *Bull. Wld. Hlth. Org.* **37:** 795-797.

Peterson, J.W., Lospalluto, J.J., and Finkelstein, R.A. (1972) Localization of cholera toxin in vivo. *J. Infect. Dis.* **126:** 617-628.

Peterson, K.M., Taylor, R.K., Miller, V.L., Di Rita, V.J., and Mekalanos, J.J. (1988) Coordinate regulation of virulence determinants in *Vibrio cholerae*. *In*: Molecular Biology of Infectious Diseases. Elsevier, Paris. p.165-172.

Pierce, N.F., Cray, W.R., Kaper, J.B., and Mekalanos, J.J. (1988) Determinants of immunogenicity and mechanisms of protection by virulent and mutant *Vibrio cholerae* 01 in rabbits. *Infect. Immun.* **56:** 142-148.

Pierce, N.F., Kaper, J.B., Mekalanos, J.J., and Cray, W.C. (1985) Role of cholera toxin in enteric colonization by *Vibrio cholerae* 01 in rabbits. *Infect. Immun.* **50**: 813-816.

Pinkney, M., Beachey, E., and Kehoe, M. (1989) The thiol-activated toxin Streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun.* **57:** 2553-2558.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T.F. (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* **325**: 458-462.

Pohlner, J., Meyer, T.F., Jalajakamari, M.B., and Manning, P.A. (1986) Nucleotide sequence of ompV, structural gene for a major *Vibrio cholerae* outer membrane protein. *Mol. Gen. Genet.* **205**: 494-500.

Poole, K., and Braun, V. (1988) Influence of growth temperature and lipopolysaccharide on hemolytic activity of *Serratia marcescens*. *J. Bacteriol.* **170:** 5146-5152.

Poole, K., Schiebel, E., and Braun, V. (1988) Molecular characterization of the hemolysin determinant of *Serratia marcescens*. *J. Bacteriol.* **170:** 3177-3188.

Poole, M.D., and Oliver, J.D. (1978) Experimental pathogenecity and mortality in ligated ileal loop studies of the newly reported halophilic lactose-positive *Vibrio* spp. *Infect. Immun.* **20:** 126-129.

Portnoy, D.A., Jacks, P.S., and Hinrichs, D.J. (1988) Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J. Exp. Med.* **167:** 1459-1471.

Prentki, P, Karch, F., Iida, S., and Meyer, J. (1981) The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. *Gene* **14:** 289-299.

Pribnow, D. (1975a) Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. *Proc. Natl. Acad. Sci. USA* **72:** 784-789.

Pribnow, D. (1975b) Bacteriophage T7 early promoters: nucleotide sequences of two RNA polymerase binding sites. *J. Mol. Biol.* **99:** 419-443.

Pribnow, D. (1979) Genetic controls signals in DNA. *In*: Biological regulation and development I: Gene expression. p.219-277. R.F. Goldberger (ed.). Plenum press, New York.

Pritchard, A.E., and Vasil, M.L. (1986) Nucleotide sequence and expression of a phosphate-regulated gene encoding a secreted hemolysin of *Pseudomonas aeruginosa*. *J. Bacteriol.* **167:** 291-298.

Pugsley, A.P., and Schwartz, M. (1985) Export and secretion of proteins by bacteria. *FEMS Microbiol. Rev.* **32:** 3-38.

Ratcliff, S.W., Luh, J., Ganesan, A.T., Behrens, B., Thompson, R., Montenegro, M., Morelli, G., and Trautner, T.A. (1979) The genome of *B. subtilis* phage SPP1: the arrangement of restriction endonuclease generated fragments. *Mol. Gen. Genet.* **168:** 165-172.

Rader, A.E., and Murphy, J.R. (1988) Nucleotide sequences and comparison of the hemolysin determinants of *Vibrio cholerae* El Tor RV79(Hly^+) and RV79(Hly^-) and Classical 569B(Hly^-). *Infect. Immun.* **56:** 1414-1419.

Rahim, Z., Sanyal, S.C., Aziz, K.M.S., Huq, M.Z., and Chaudhury, A.A. (1984) Isolation of enterotoxigenic, hemolytic and antibiotic-resistant *Aeromonas hydrophila* strains from infected fish in Bangladesh. *Appl. Environ. Microbiol.* **48:** 865-867.

Randall, L.L., and Hardy, S.J.S. (1984) Export of proteins in bacteria. *Microbiol. Rev.* **48:** 290-298.

Redmond, J.W. (1979) The structure of the O-antigenic side chain of the lipopolysaccharide of *Vibrio cholerae* 569B (Inaba). *Biochem. Biophys. Acta.* **584:** 346-352.

Redmond, J.W., Korsch, M.J., and Jackson, G.D.F. (1973) Immunochemical studies of the O-antigens of *Vibrio cholerae*. Partial characterization of an acid-labile antigenic determinant. *Aust. J. Exp. Biol. Med. Sci.* **51:** 229-235.

Rella, M., Mercenier, A., and Haas, D. (1985) Transposon insertion mutagenesis of *Pseudomonas aeruginosa* with a Tn*5* derivative: application to physical mapping of the *arc* gene cluster. *Gene* **33:** 293-303.

Richardson, K., Michalski, J., and Kaper, J.B. (1986) Hemolysin production and cloning of two hemolysin determinants from classical *Vibrio cholerae*. *Infect. Immun.* **54:** 415-420.

Rizvi, S., Huq, M.I., and Benenson, A.S. (1965) Isolation of haemagglutinating non-El Tor vibrios. *J. Bacteriol.* **89:** 910-912.

Rood, J.I., and Gawthorne, J.M. (1984) Apple software for analysis of the size of restriction fragments. *Nucl. Acids Res.* **12:** 689-694.

Rosenberg, M., and Court, D. (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. *Ann. Rev. Genet.* **13:** 319-353.

Roy, C., Mridha, K., and Mukerjee, S. (1965) Action of polymyxin on cholera vibrios. Techniques of determination of polymyxin-sensitivity. *Proc. Soc. Exp. Biol. Med.* **119:** 893-896.

Sack, R.B., and Miller, C.E. (1969) Progressive changes of vibrio serotypes in germ-free mice infected with *Vibrio cholerae*. *J. Bacteriol.* **99**: 688-695.

Sakazaki, R., and Tamura, K. (1971) Somatic antigen variation in *Vibrio cholerae*. *Jpn. J. Med. Sci. Biol.* **24**: 93-100.

Sakurai, Y., Matsuzaki, A., and Miwatani, T. (1973) Purification and characterisation of the thermostable direct haemolysin of *Vibrio parahaemolyticus*. *Infect. Immun.* **8:** 775-780.

Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H., and Roe, B.A. (1980) Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* **143**: 161-178.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.

Sanyal, S.C., Alam, K., Neogi, P.D.B., Huq, M.I., and Al-Mahmud, K.A. (1983) A new cholera toxin. *Lancet* **i:** 1337.

Sanyal, S.C., Neogi, P.K.B., Alam, K., Huq, M.I., and Al-Mahmud, K.A. (1984) A new enterotoxin produced by *Vibrio cholerae* O1. *J. Diarrhoeal Dis.* **2:** 3-12.

Saunders, F.K., Mitchell, T.J., Walker, J.A., Andrew, P.W., and Boulnois, G.J. (1989) Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for in vitro activity. *Infect. Immun.* **57:** 2547-2552.

Schaller, H., Gray, C., and Herrmann, K. (1975) Nucleotide sequence of an RNA polymerase binding site from the DNA of bacteriophage fd. *Proc. Natl. Acad. Sci. USA* **72**: 737-741.

Scheffer, J., König, W., Hacker, J., and Goebel, W. (1985) Bacterial adherence and hemolysin production from *Escherichia coli* induces histamine and leukotriene release from various cells. *Infect. Immun.* **50:** 271-278.

Schiebel, E., and Braun, V. (1989) Integration of the *Serratia marcescens* haemolysin into human erythrocyte membranes. *Molec. Microbiol.* **3:** 445-453.

Schneider, D.R., and Parker, C.D. (1978) Isolation and characterization of protease-deficient mutants of *Vibrio cholerae*. *J. Infect. Dis.* **138:** 143-151.

Schneider, D.R., Sigel, S.P., and Parker, C.D. (1981) Characterization of *Vibrio cholerae* protease activities with peptide digest analysis. *J. Clin. Microbiol.* **13:** 80-84.

Sciortino, C.V., and Finkelstein, R.A. (1983) *Vibrio cholerae* expresses iron-regulated outer membrane proteins in vivo. *Infect. Immun.* **42:** 990-996.

Seeburg, P.H., Nusslein, C., and Schaller, H. (1977) Interaction of RNA polymerase with promoters from bacteriophage fd. *Eur. J. Biochem.* **74:** 107-113.

Sen, R. (1969) A classification of cholera vibrios into biotypes and their value for epidemiological purposes. *Ind. J. Med. Res.* **57:** 856-863.

Sharma, D.P., Stroeher, U.H., Thomas, C.J., Attridge, S.R., and Manning, P.A. (1988) The characterization of a major non-LPS protective antigen on *Vibrio cholerae* strain Z17561 and molecular cloning of the Tcp pilus. *In*: Abstracts of the US-Japan Cholera Conference.

Shewan, J.M., and Veron. M. (1975) The genus Vibrio. p.340-345. *In*: Bergey's Manual of Determinative Bacteriology. T.M. Buchanan (ed.) 8th edition, Williams and Wilkins, Baltimore.

Shine, J., and Dalgarno, L. (1974) The 3'terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. *Proc. Natl. Acad. Sci. USA* **71**: 1342-1346.

Shinoda, S., Miyoshi, S., Yamanaka, H., and Miyoshi-Nakahara, N. (1985) Some properties of *Vibrio vulnificus* hemolysin. *Microbiol. Immunol.* **29:** 583-590.

Siebenlist, S., Simpson, R.B., and Glbert, W. (1980) *E. coli* RNA polymerase interacts homologously with two different promoters. *Cell* **20**: 269-281.

Sigel, S.P., and Payne, S.M. (1982) Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio cholerae*. *J. Bacteriol.* **150:** 148-155.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**: 784-791.

Sinha, V.B., and Srivastava, B. (1978) Suppression of pathogenicity by P and V plasmids in *Vibrio cholerae*. *J. Gen. Microbiol.* **104:** 251-255.

Smith, H.W. (1963) The haemolysins of *Escherichia coli*. *J. Pathol. Bacteriol.* **85:** 197-207.

Smith, H.W., and Linggood, M.A. (1971) Observation on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. *J. Med. Microbiol.* **4:** 467-485.

Sokatch, J.R. (1979) Roles of appendages and surface layers in adaptation of bacteria to their environment. p.229-289. *In*: The Bacteria. J.R. Sokatch and L.N. Ornsten (eds.) Vol. 7. Academic Press, London.

Southern, E.M. (1975) Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517.

Spicer, E.K., and Noble, J.A. (1982) *Escherichia coli* heat-labile enterotoxin. Nucleotide sequence of the A subunit gene. *J. Biol. Chem.* **257:** 5716-5721.

Spira, W.M., Sack, D.A, Fedorka-Cray, P.J., Sanyal, S., Madden, J., and McCardell, B. (1986) Description of a possible new extracellular virulence factor in nontoxigenic *Vibrio cholerae* O1. *In*: Advances in Research on Cholera and Related Diarrheas. Vol. 3. S. Kuwahara and N.F. Pierce (eds.). p.267-270. KTK Scientific Publishers, Tokyo.

Studnicka, G.M. (1987) Nucleotide sequence homologies in control regions of prokaryotic genomes. *Gene* **58:** 45-57.

Suzuki, J., Kobayashi, S., Kagaya, K., and Fukazawa, Y. (1988) Heterogeneity of hemolytic efficiency and isoelectric point of Streptolysin O. *Infect. Immun.* **56:** 2474-2478.

Svennerholm, A.-M., Sack, D.A., Holmgren, J., and Bardhan, P.K. (1982) Intestinal antibody responses after immunization with cholera B subunit. *Lancet* **i:** 305-307.

Tabor, S., and Richardson, C.C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA* **84:** 4767-4771.

Takanami, M., Sagimoto, K., Sugisaki, H., and Okamoto, T. (1976) Sequence of promoter for coat protein of bacteriophage fd. *Nature* **260**: 297-302.

Takeda, Y. (1983) Thermostable direct hemolysin of *Vibrio parahaemolyticus. Pharmacol. Ther.* **19:** 123-146.

Taylor, R.K., Miller, V.L., Furlong, D.B., and Mekalanos, J.J. (1987a) Use of *pho*A gene fusions to identify a pilus colonization factor co-ordinately regulated with cholera toxin. *Proc. Natl. Acad. Sci. USA* **84**: 2833-2837.

Taylor, R., Spears, P., and Mekalanos, J.J. (1987b) Applications of the Tcp pilus in cholera vaccine development. p.105-106. *In*: Abstracts of the 23rd Joint Conference on Cholera. U.S.-Japan Cooperative Medical Science Program.

Taylor, R., Shaw, C., Peterson, K., Spears, P., and Mekalanos, J.J. (1988) Safe, live *Vibrio cholerae* vaccines? *Vaccine* **6**: 151-154.

Thorn, R.M., Palmer, J.C., and Manson, L.A. (1974) A simplified ^{51}Cr-release assay for killer cells. *J. Immunol. Methods* **4:** 301-315.

Timmis, K.N., and Winkler, U. (1973) Isolation of covalently closed circular deoxyribonucleic acid from bacteria which produce extracellular nuclease. *J. Bacteriol.* **113**: 508-509.

Tinoco Jr., J., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Gothers, D.M., and Gralla, J. (1973) Improved estimation of secondary structure in ribonucleic acids. *Nature* **246**: 40-41.

Towbin, H., Staehlin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**: 4350-4354.

Tsan, Y.C. (1978) Nucleic acids hydrolysis by *Vibrio cholerae*. *Chin. J. Microbiol.* **11**: 114-115.

Tweedy, J.M., Park, R.W.A., and Hodgkiss, W. (1968) Evidence for the presence of fimbriae (pili) on vibrio species. *J. Gen. Microbiol.* **51:** 235-244.

Tweten, R.K. (1988) Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin and pneumolysin. *Infect. Immun.* **56:** 3235-3240.

Ujiiye, A., Nakatomi, M., Utsunomiya, A., Mitsui, K., Sogame, S., Iwanaga, M., and Kobari, K. (1968) Experimental cholera in mice. I. First report on the oral infection. *Trop. Med.* **10**: 65-71.

van Dongen, W.M.A.M., van Vlerken, M.M.A., and de Graaf, F.K. (1987) Nucleotide sequence of a DNA fragment encoding a *Vibrio cholerae* haemagglutinin. *Mol. Gen. (Life Sci. Adv.)* **6:** 85-91.

van Epps, D.F., and Andersen, B.R. (1974) Streptolysin O inhibition of neutrophil chemotaxis and mobility: nonimmune phenomenon with species specificity. *Infect. Immun.* **9:** 27-33.

van Heyningen, S. (1977) Cholera toxin. *Biol. Rev.* **52**: 509-549.

Vasil, M.L., Berka. R.M., Gray, G.L., and Nakai, H. (1982) Cloning of a phosphate-regulated hemolysin gene (Phospholipase C) from *Pseudomonas aeruginosa*. *J. Bacteriol.* **152:** 431-440.

Vasil, M.L., Holmes, R.K., and Finkelstein, R.A. (1974) Studies on the toxinogenesis in *Vibrio cholerae*. II. An in vitro test for toxin production. *Infect. Immun.* **9:** 195-197.

Vieira, J., and Messing, J. (1982) The pUC plasmids, a M13mp-7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259-268.

Vicente, M.F., Baquero, F., and Perez-Diaz, J.C. (1985) Cloning and expression of the *Listeria monocytogenes* haemolysin in *E.coli*. *FEMS Microbiol. Lett.* **30:** 77-79.

Vimr, E.R., Lawrisuk, L., Galen, J., and Kaper, J.B. (1988) Cloning and expression of the *Vibrio cholerae* neuraminidase gene *nanH* in *Escherichia coli*. *J. Bacteriol.* **170**: 1495-1504.

von Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites. *Eur. J. Biochem.* **133**: 17-21.

von Heijne, G. (1984) How signal sequences maintain cleavage specificity. *J. Mol. Biol.* **173:** 243-251.

von Heijne, G. (1985) Signal sequences. The limits of variation. *J. Mol. Biol.* **184:** 99-105.

von Mechow, S., Vaidya, A.B., and Bramucci, M.B. (1985) Mapping of a gene that regulates hemolysin production in *Vibrio cholerae*. *J. Bacteriol.* **163:** 799-802.

Waalwijk, C., van der Bosch, J.F., MacLaren, D.M., and de Graaf, J. (1982) Hemolysin plasmid coding for the virulence of a nephropathogenic *Escherichia coli* strain. *Infect. Immun.* **35:** 32-37.

Wagner, W., Vogel, M., and Goebel, W. (1983) Transport of haemolysin across the outer membrane of *Escherichia coli* requires two functions. *J. Bacteriol.* **154**: 200-210.

Walker, J.A., Allen, R.L., Falmange, P., Johnson, M.K., and Boulnois, G.J. (1987) Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. *Infect. Immun.* **55:** 1184-1189.

Wall, V.W., Kreger, A.S., and Richardson, S.H. (1984) Production and partial characterization of a *Vibrio fluvialis* cytotoxin. *Infect. Immun.* **46:** 773-777.

Ward, H.M., Morelli, G., Kamke, M., Morona, R., Yeadon, J., Hackett, J.A., and Manning, P.A. (1987) A physical map of the chromosomal region determining O-antigen biosynthesis in *Vibrio cholerae* 01. *Gene* **55**: 197-204.

Wong, K.R., Green, M.J., and Buckley, J.T. (1989) Extracellular secretion of cloned aerolysin and phospholipase by *Aeromonas salmonicida. J. Bacteriol.* **171:** 2523-2527.

Wright, A.C., Morris Jr., G., Maneval Jr., D.R., Richardson, K., and Kaper, J.B. (1985) Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus. Infect. Immun.* **50:** 922-924.

Yamamoto, K., Takeda, Y., Minatani, T., and Craig, J.P. (1983a) Purification and some properties of a non-O1 *Vibrio cholerae* enterotoxin that is identical to cholera enterotoxin. *Infect. Immun.* **39**: 1128-1135.

Yamamoto, K., Takeda, Y., Minatani, T. and Craig, J.P. (1983b) Evidence that a non-O1 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. *Infect Immun.* **41**: 896-901.

Yamamoto, K., Alo-omani, M., Honda, T., Takeda, Y., and Miwatani, T. (1984) Non-01 *Vibrio cholerae* hemolysin: purification, partial characterization and immunological relatedness to El Tor hemolysin. *Infect. Immun.* **45:** 192-196.

Yamamoto, K., Ichinose, Y., Nakasone, N., Tanabe, M., Nagahama, M., Sakurai, J., and Iwanaga, M. (1986) Identity of hemolysins produced by *Vibrio cholerae* non-O1 and *Vibrio cholerae* O1, biotype El Tor. *Infect. Immun.* **51:** 927-931

Yamanaka, H., Satoh, T., and Shinoda, S. (1987) Preparation of specific antiserum against *Vibrio vulnificus* hemolysin by immunization with hemolysin-bound liposomes. *FEMS Microbiol. Lett.* **41:** 313-316.

Yancey, R.J., Willis, D.L., and Berry, L.J. (1978) Role of motility in experimental cholera in adult rabbits. *Infect. Immun.* **22**: 387-392.

Young, D.B., and Broadbent, D.A. (1985) The effect of lincomycin on exoprotein production by *Vibrio cholerae. J. Clin. Microbiol.* **146**: 13-17.

Zabala, J., Garcia-Lobo, J., Diaz-Aroca, E., de la Cruz, F., and Ortitz, J. (1984) *Escherichia coli* α-haemolysin synthesis and export genes are flanked by a direct repetition of IS*91*-like elements. *Mol. Gen. Genet.* **197**: 90-97.

Zabala, J.C., De la Cruz, F., and Ortiz, J.N. (1982) Several copies of the same insertional sequence are present in α-hemolytic plasmids belonging to four different incompatibility groups. *J. Bacteriol.* **151:** 472-476.

Zen-Yoji, H., Kudoh, Y., Igarashi, H., Ohta, K., and Fukai, K. (1974) Purification and identification of enteropathogenic toxins "a" and "a'" produced by *Vibrio parahaemolyticus* and their biological and pathological activities. p.237-243. *In*: T.Fujino, G.Sakaguchi, R.Sakazaki and Y.Takeda (eds.) International symposium on *Vibrio parahaemolyticus.* Saikon Publishing Co., Tokyo.

Zinnaka, Y., and Carpenter, C.C.J.Jr. (1972) An enterotoxin produced by noncholera vibrios. *John Hopkins Med. J.* **131**: 403-411.