



THE EXTRACELLULAR DNASE(S) OF VIBRIO CHOLERAE

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ABSTRACT

V.cholerae 01 produces a number of extracellular enzymes, several of which have been implicated in the virulence of the organism. These include the haemolysin(s), neuraminidase, cholera lectin (or soluble haemagglutinin) and cholera toxin. *V.cholerae* also secretes an extracellular deoxyribonuclease (DNase), but its role in the pathogenesis of the organism has not been determined. The gene encoding the extracellular DNase of *V.cholerae* has been cloned and expressed in *E.coli* K-12. This study has sought to characterize the DNase at the DNA level and determine the role it may play in the virulence and general physiology of *V.cholerae*. The DNase has also been used as a model for the extracellular secretion of proteins in Gram-negative bacteria.

The extracellular DNase of *V.cholerae* has been cloned by cosmid packaging and the gene localized to a 1.25kb DNA fragment. The gene product can not be visualized directly by Coomassie blue staining and has been detected indirectly via a DNA-hydrolysis assay after SDS-PAGE electrophoresis. The region of DNase activity migrates at a Mr24,000. The protein retains enzymatic activity in SDS, indicating that it is not readily denatured, but is inactivated with the addition of β -mercaptoethanol, implying disulphide bridging in this protein.

The normally extracellular DNase in *V.cholerae*, is localized in the periplasmic space when expressed in *E.coli*. This suggests that *E.coli* either lacks the necessary secretion machinery, or that it does not recognize the secretion signals on this protein. The DNase gene has been sequenced, and codes for a protein of Mr26,400. The ORF has a typical N-terminal signal sequence, and cleavage of this signal peptide results in a mature protein of Mr24,200. The presence of a signal sequence on this protein is consistent with other exported proteins, and although it is sufficient to export the DNase from the cytoplasm to the periplasm in *E.coli*, additional factors are required for its secretion into the extracellular environment.

Site-specific mutagenesis of the DNase gene (DNase-1) in the *V.cholerae* chromosome, has led to the identification of a second unrelated DNase (DNase2).

Inactivation of this second DNase (cloned by Newland et al., 1985), has resulted in a totally DNase negative phenotype of *V.cholerae* El Tor. While inactivation of these Dnases does not appear to attenuate *V.cholerae* as demonstrated with the infant mouse model of infection, these strains are now able to be transformed with plasmid DNA.

The construction of gene fusions with a series of carboxy-terminal deletions of the DNase to β -lactamase, have demonstrated that the DNase cannot supply the necessary export signals for their extracellular secretion, although these proteins are exported to the periplasm. The nature of the export signals have not been determined and may not simply require a primary sequence but also involve a specific conformation of the protein.

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

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Abbreviations

A adenine

aa amino acid

Ab antibody

ATP adenosine 5'-triphosphate

BHI brain heart infusion

bp base pair

BSA bovine serum albumin

CAT chloramphenicol acetyltransferase

C cytosine

cha chicken erythrocyte haemagglutinin

Cm chloramphenicol

cpm counts per minute

CT cholera toxin

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dNTP deoxyribonucleoside triphosphate

ddNTP dideoxyribonucleoside triphosphate

DDT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EM electron microscope

EMS ethyl methanesulphonate

Et Br ethidium bromide

G guanine

Gm₁ Galatosyl-N-acetyl-galactosaminy-Sialosyl-Lactosyl ceramide

Gm gentamycin

HA haemagglutinin

Hly haemolysin

Ig immunoglobulin

IM inner membrane
IPTG isopropyl- β -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
NAG non-agglutinable
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
pmx polymyxin
R^r resistant
RBC red blood cell
RF replicative form
Rif rifampicin
RNA ribonucleic acid
RNAP RNA Polymerase
rpm revolutions per minute
S^s sensitive
SD Shine-Dalgarno
SDS sodium dodecyl sulphate
SEM scanning electron microscope
SHA soluble haemagglutinin

Sm streptomycin

s/s single stranded

T thymine

Tc tetracycline

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

Tn transposon

Tris Tris (hydroxymethyl) aminomethane

ts temperature sensitive

U uracil

UV ultraviolet

v/v volume per volume

w/v weight per volume

X-gal N,N'-dimethyl formamide

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CHAPTER 1

INTRODUCTION

1.1 *Vibrio cholerae*

Vibrio cholerae of the O1 serotype is the causative agent of cholera, a severe diarrhoeal disease found only in man. The genus *Vibrio* belongs to the family *Vibrionaceae*; which also includes the genera *Aeromonas*, *Plesiomonas*, *Photobacterium* and *Lucibacterium* (Shewan and Veron, 1975). *Vibrios* are Gram-negative, motile rods and have a distinctive curved morphology with a single polar flagellum. *V. cholerae* is a facultative aerobe which grows well in media that are usually too alkaline (pH9.0 to pH9.6) for the growth of other bacteria. However, the organism is unusually sensitive to acid, and normal stomach acid is vibriocidal (Giannella *et al.*, 1973).

1.1.1 Biotype differentiation

Vibrios which are agglutinated by a cholera polyvalent O antiserum belong to the O-1 serotypic subgroup, the serotype as defined by Gardner and Venkatraman (1935). Within the O1 serotype are two biotypes, classical and El Tor (Feeley, 1965), both of which are capable of causing human cholera. The classical biotype has been responsible for all the major epidemics, until the onset of the seventh panademic, when El Tor *V. cholerae* were found to be the causative agent (Kamal, 1974). These two biotypes were initially differentiated by the ability of El Tor strains to produce a soluble haemolysin. However, since the early discovery of El Tor strains, considerable variation has been seen in their haemolytic nature, and a

number of other differentiating characteristics are now used. El Tor strains are resistant to the antibiotic polymyxin B, and can agglutinate chicken erythrocytes when grown on solid media, whereas classical strains cannot. (Barua and Mukherjee, 1963; Gan and Tjia, 1963). The most reliable test has been the sensitivity of classical *V. cholerae* to the group IV phages of Mukerjee, to which El Tor vibrios are resistant (Mukerjee and Roy, 1961; Monsur *et al.*, 1965).

1.1.2 Serotype differentiation

V. cholerae strains of either biotype can be further divided into three serotypes; Inaba, Ogawa and Hikojima, which are defined on the basis of possessing specific antigens (A,B and C) that are associated with the O-antigen of the lipopolysaccharide (Burrows *et al.*, 1946). The two major subclasses of O-antigen are termed Ogawa and Inaba. Strains of the Ogawa serotype have the antigens A and B and a small amount of C, whereas those of the Inaba serotype have the A and C antigens (Burrows *et al.*, 1946; Redmond *et al.*, 1973; Sakazaki and Tamura, 1971). The third less common and apparently unstable serotype, Hikojima, expresses high levels of all three antigenic factors A,B and C (Burrows *et al.*, 1946; Redmond *et al.*, 1973; Sakazaki and Tamura, 1971). It has been suggested that Hikojima strains may represent segregating diploids (Bhaskaran, 1971), or alternatively, that these strains could represent subclasses undergoing seroconversion as such serotype changes have been detected (Gangarosa *et al.*, 1967; Sack and Miller, 1969). Bacteriophage CP-T1 has been implicated in these serotype changes by means of a lysogenic conversion (Ogg *et al.*, 1978, 1979), but failure to detect any CP-T1 DNA in reported *V. cholerae* lysogens (Ogg *et al.*, 1979), does not support this conclusion (Guidolin and Manning, 1985).

1.1.3 History of Cholera

Cholera has been endemic in India for centuries, and from time to time has caused devastating epidemics in other parts of the world. Since 1817 seven

worldwide pandemics have occurred. The latest pandemic spread in the 1960's from Indonesia to the far East, India, the Middle East, Africa, and later to some parts of Europe (Cvjentanovic and Barua, 1972). In this pandemic the causative organism was identified as *V. cholerae* El Tor, and importantly, this was the first time that this biotype was shown to be responsible for a pandemic. The El Tor biotype was named after the quarantine station at which it was first isolated (Gotschlich, 1905 - cited by Pollitzer, 1959), and differed from the classical biotype which had been responsible for the six pandemics of this period, in that it was thought to lack the capacity to cause cholera in man. The El Tor vibrios were originally thought not to be true *V. cholerae*, and therefore were given the names *V. paracholerae* or *V. eltor*. The seventh pandemic which is still in progress, saw the El Tor biotype replacing the classical vibrios, and it was at this time the disease caused by the El Tor strains was defined as true cholera (Kamal, 1974). Biochemical studies conducted by Hugh (1965), demonstrated that both vibrios were nearly identical, leading to the acceptance of the biotype designation. It is now apparent that organisms of either biotype are capable of causing cholera in its severest form. In 1982 diarrhoea due to the classical biotype reappeared in epidemic form in Bangladesh (Samadi *et al.*, 1983).

Cholera has been a significant disease throughout the world in recent history, and this has resulted in an intensive research effort over the last twenty years. Major advances have been made in our understanding and treatment of the infective process, with a concerted push now being made towards the development of an effective vaccine against this disease.

1.1.4 Pathogenesis

Naturally acquired cholera has been described only in humans, and usually involves the ingestion of these organisms in contaminated drinking water or food (typically seafood). In a non-immune individual, there are a number of non-specific defence mechanisms. Gastric acid constitutes quite an effective barrier to infection, as has been demonstrated in a number of volunteer studies, where the minimum effective dose required to elicit choleraic diarrhoea by oral administration

can be reduced significantly, provided that sodium bicarbonate is given concomitantly to neutralise stomach acidity (Cash *et al.*, 1974a,b; Nalin *et al.*, 1978). *V. cholerae* organisms that successfully pass through this gastric acid barrier must also overcome small intestine peristalsis and a mucous layer coating the small intestine, which work together to prevent colonization.

Colonization of the small intestine by *V. cholerae* is an essential first step in the infectious process (Finkelstein, 1984). This process is multifactorial and involves motility (Guentzel and Berry, 1975; Yancey *et al.*, 1978), chemotaxis (Freter and O'Brien, 1981), protease production (Schneider and Parker, 1978), haemagglutinins (Finkelstein and Hanne, 1982), cholera enterotoxin (Pierce *et al.*, 1985) and a number of other extracellular and surface-associated factors (Manning, 1987). Adhesion to the intestinal epithelium facilitates the effective delivery of the toxin(s) to its site of action leading to the second phase of the disease process: the effect of the toxin(s) upon the host.

1.1.5 Adhesion

Vibrio cholerae O1 organisms must first penetrate the mucous layer of the small intestine to reach the enterocytes to which they attach. A potent mucinase is thought to assist the entrance into the mucous blanket covering the mucosa (Schneider and Parker, 1982). Motility is also regarded as a factor contributing to virulence, in terms of promoting adherence (Attridge and Rowley, 1983; Guentzel and Berry, 1975; Yancey *et al.*, 1978), facilitating penetration of the intestinal mucous blanket (Jones *et al.*, 1976). The locomotion of these bacteria is believed to be guided by chemotactic stimuli (Freter and O'Brien, 1981).

Although motility, chemotaxis and extracellular enzyme production are properties that enhance colonization by *V. cholerae*, the molecular components actually involved in adherence by these organisms to the intestinal mucous have been the source of considerable speculation. Among the surface antigens that have been implicated as being involved in adhesion are lipopolysaccharide (LPS) (Chitnis *et al.*, 1982), flagellar sheath protein and other flagellar antigens (Attridge and Rowley, 1983a, 1983b) major outer membrane proteins (Kabir, 1983; Kabir

and Showkat, 1983) and various haemagglutinins (Bhattacharjee and Srivastava, 1978; Hanne and Finkelstein, 1982; Jones *et al.*, 1976; Jones and Freter, 1976).

The haemagglutinins have received the most attention, because analagous studies with enterotoxigenic strains of *Escherichia coli* have shown that haemagglutination assays can serve as screening tests to establish the presence of adhesins that attach to receptors on small intestinal mucosa (Burrows *et al.*, 1976; Jones and Rutter, 1974; Moon *et al.*, 1979).

A variety of haemagglutinins produced by vibrios have been described, and studied as possible mediators of attachment (Bhattacharjee and Srivastava, 1978; Finkelstein and Hanne, 1982; Freter and Jones, 1976; Jones *et al.*, 1976; Jones and Freter, 1976). Hanne and Finkelstein (1982) have characterized the haemagglutinating patterns exhibited by classical and El Tor strains of *V. cholerae*. Four distinct haemagglutinins (HAs) have been described by these workers, of which three are cell-associated in *V. cholerae* O1. One is sensitive to L-fucose and resistant to D-mannose and requires Ca^{2+} for HA activity, and is found in classical strains (Hanne and Finkelstein, 1982; Holmgren *et al.*, 1983). The second type is resistant to L-fucose and sensitive to D-mannose and requires no Ca^{2+} for HA activity, and is distributed among El Tor biotype strains (Bhattacharjee and Srivastava, 1978; Hanne and Finkelstein, 1982). The third type is resistant to both L-fucose and D-mannose, and is detected in both classical and El Tor strains in liquid cultures by using chicken erythrocytes (Booth and Finkelstein, 1986; Hanne and Finkelstein, 1982). The fourth HA can be detected in cell-free supernatants and has been termed "cholera lectin" or soluble haemagglutinin (SHA). More recently, it has been referred to as the SHA/protease due to its proteolytic activity (Booth *et al.*, 1984; Finkelstein *et al.*, 1984), and is found in both classical and El Tor strains.

Although the role of haemagglutinins as mediators of attachment to host epithelial cell surface receptors has been postulated in many reports (Bhattacharjee and Srivastava, 1978; Finkelstein and Hanne, 1982; Jones and Freter, 1976), the detection of structures such as fimbriae, correlating with these haemagglutinins, has been the source of considerable debate over the years. Evidence of fimbriae in *V. cholerae* was presented as early as 1968 (Tweedy and Park, 1968), but this isolated report could not be confirmed by subsequent

investigations (Nelson *et al.*, 1976; Nelson *et al.*, 1977). This led to the belief that adherence was the result of a more direct interaction(s) between the surface coat of the vibrios and the tips of the microvilli of the host intestinal epithelial cells. It was not until as recently as 1986 that there was definitive evidence of fimbrial production in *V. cholerae*. Ehara and coworkers (1986), detected peritrichously distributed fimbriae 5-7nm diameter on pathogenic El Tor isolates. Contradictory to this, Booth and Finkelstein (1986) could not detect fimbriae on vibrios, but this may reflect the importance of the appropriate growth conditions required for fimbriae production (Ehara *et al.*, 1986; Taylor *et al.*, 1987a). Importantly, a correlation between the colonization properties of *V. cholerae* and the presence of fimbriae could be shown. Pathogenic strains produced fimbriae and were able to colonize rabbits *in vivo*, while non-pathogenic strains unable to produce fimbriae did not colonize (Ehara *et al.*, 1986). Subsequently, Taylor and coworkers (1987a) isolated mutants of *V. cholerae* with a decreased capacity for intestinal colonization of suckling mice, by means of transposon Tn ϕ oA (Manoil and Beckwith, 1985) mutagenesis of the *V. cholerae* chromosome. The Tn ϕ oA mutants resulted in the loss of a 20.5kDa protein, the major subunit of a *V. cholerae* fimbrial structure. This colonization factor was designated TcpA (toxin co-regulated pilus) as it was shown to be co-ordinately regulated with cholera toxin (Taylor *et al.*, 1987a; Peterson *et al.*, 1988). *V. cholerae* cells expressing these pili were able to haemagglutinate mouse erythrocytes, and although the nature of the host receptors recognized by the pilus are unknown, haemagglutination was detected in the presence of L-fucose, a sugar that inhibits most of the haemagglutination mediated by *V. cholerae* strain 0395. This property was lost in tcpA::Tn ϕ oA mutants (Taylor *et al.*, 1987a). A defect in intestinal colonization concomitant with the loss of fimbriae in tcpA mutants has been observed, and it has been proposed that this pilus is an important colonization factor of *V. cholerae* O1 in man (Taylor *et al.*, 1987a; Herrington *et al.*, 1987; Shaw *et al.*, 1987). Finally, cholera toxin itself has been suggested to contribute to the mucosal adherence of *V. cholerae* (Pierce *et al.*, 1985), however, this notion has been disputed (Finn *et al.*, 1987).

1.1.6 Enterotoxin production

As *V. cholerae* organisms successfully associate with the mucosa of the proximal small intestine, they elaborate cholera enterotoxin. In contrast to the events leading to colonization, the effects of cholera enterotoxin are well understood. The protein itself is one of the best understood with respect to structure, function and biological activity (for reviews see Holmgren, 1981; Van Heynigan, 1977). Cholera toxin was first purified and characterized by Finkelstein and Lospalluto (1970), and this enabled the role of the toxin in pathogenesis of the disease to be studied (Finkelstein, 1972; Pierce *et al.*, 1971; Carpenter *et al.*, 1974). Cholera toxin is a M_r 84,000 protein, composed of one A subunit (M_r 27,000) and five B subunits (M_r 11,600). The A subunit, although synthesized as a single polypeptide chain, is usually proteolytically nicked to form two disulphide-linked polypeptides, A_1 (M_r 22,000) and A_2 (M_r 5,000) (Gill, 1976; Mekalanos *et al.*, 1979a). Each B subunit has a high binding affinity for its cell surface receptor, ganglioside-GM1. The A_1 polypeptide is an enzyme and promotes the activation of adenylate cyclase in target cells by catalyzing the ADP-ribosylation of a GTP-binding regulatory component of the cyclase complex (Gill *et al.*, 1976). The resulting accumulation of cyclic AMP in the intestinal mucosa leads to the severe fluid loss, characteristic of cholera. The secreted fluid is low in protein and rich in electrolytes, including Na^+ , K^+ , Cl^- , and HCO_3^- . It is this severe dehydration via fluid loss which if not treated promptly, can result in death.

The capacity to produce cholera toxin or a cholera-like enterotoxin is not limited to *V. cholerae* 01. Enterotoxins which are both biologically and immunologically similar to cholera toxin have been isolated from a number of non-01 *V. cholerae* (Yamamoto *et al.*, 1983a, 1983b; Craig *et al.*, 1981), and non-cholera vibrios (Kaper *et al.*, 1981; Zinnaka and Carpenter, Jr., 1972). The heat-labile (LT) enterotoxin of *E. coli* also belongs to this family of adenylate cyclase activating enzymes, and DNA sequence analysis has shown the LT genes to be 78% homologous to the cholera toxin genes (Mekalanos *et al.*, 1983). However, the LT operon *eltA,B* appears to be exclusively located on plasmids (Dallas *et al.*, 1979; Finkelstein, 1973), while the cholera toxin operon is located on the bacterial chromosome (Pearson and Mekalanos, 1982; Mekalanos *et al.*, 1983).

1.2 Vaccines

An initial clinical episode of cholera provides potent, long-lived protective immunity, and is the means of natural immunity acquired in endemic regions (Black *et al.*, 1981; Cash *et al.*, 1974). This immunity to re-infection can also be demonstrated experimentally in animals (Cray *et al.*, 1983; Tokunaga *et al.*, 1984). The ability to obtain such potent and lasting immunity, has resulted in a worldwide effort to obtain a suitable and effective vaccine.

The approaches taken towards vaccine development can be essentially divided into two categories:

(i) Killed whole cells and/or toxoids with the method of delivery of these antigens being either parenteral or oral.

(ii) Live oral cholera vaccines; i.e. either attenuated *V. cholerae* or heterologous organisms that express cholera antigens.

1.2.1 Killed vaccines

Antibacterial and antitoxic immunity both play roles in protection against cholera, but there has been much debate on which is more important (Holmgren and Svennerholm, 1977; Levine *et al.*, 1983a; Majumdar *et al.*, 1981). Killed vaccines can therefore be discussed in terms of killed whole cell vaccines and toxoids aimed at stimulating antibacterial and antitoxic immunity, respectively.

1.2.1.1 Toxoids

Stimulation of antitoxic immunity aimed at preventing cholera, has been largely carried out by the use of purified cholera toxin which has been inactivated *in vitro*. Treatment of purified cholera toxin with formaldehyde results in a toxoid that exhibits very little toxic activity, and when administered parenterally, stimulates antitoxic antibodies (Northrup and Chisari, 1972). Unfortunately the toxoid reverts to partial toxicity making it unacceptable for use (Northrup and Chisari, 1972).

While cholera toxin is highly immunogenic, nearly all of the antibodies are directed against the B subunit (Peterson *et al.*, 1979; Svennerholm, 1980; Holmgren, 1981). Svennerholm *et al.*, (1982a) used a B-subunit vaccine to stimulate antitoxic immunity. The major advantages to this, were its complete safety since it cannot revert to a biologically active form as with toxoids, and that it retained its capacity to adhere to toxin receptors on enterocytes. However, animal studies have shown the B subunit to be less potent than the complete toxoid in stimulating an antitoxin response (Pierce *et al.*, 1982). It is therefore likely that toxoid or B subunit vaccines would need to be used in conjunction with killed whole cells to stimulate both antibacterial and antitoxic antibodies for an efficient protective effect. Oral immunization also appears to be the more attractive route as it provides a longer lasting intestinal antibody response than parenteral administration (Cash *et al.*, 1974; Finkelstein, 1984).

1.2.1.2 Killed whole cells

Killed whole *V. cholerae* vaccines have been used as parenteral vaccines since the turn of the century and are still commercially available. They have been shown to stimulate high titres of serum vibriocidal antibodies (Feeley and Gangarosa, 1980), and do confer some protection against the homologous *V. cholerae* serotype (Feeley and Gangarosa, 1980), albeit for a short time. Killed vibrios have also been used as oral vaccines. Killed whole cells administered orally stimulate the appearance of antivibrio antibody (Freter, 1972), and challenge of vaccinees given killed whole cells orally has shown some protection (Cash *et al.*, 1974).

1.2.1.3 Combination vaccines

With the understanding that cholera stimulates the production of both antibacterial and antitoxic antibodies in the intestine, emphasis has been given to combining both elements in an oral vaccine to provoke an immune response against cholera toxin as well as the organism itself (Svennerholm and Holmgren, 1976; Svennerholm *et al.*, 1982a; Svennerholm *et al.*, 1984).

Vaccines consisting of killed whole *V. cholerae* organisms and the purified B

subunit of cholera toxin have been extensively evaluated. A single dose consisting of 2.5mg of the B subunit and 5×10^{10} killed vibrios stimulated a local secretory immunoglobulin A (sIgA) antitoxin response and, in most, a sIgA anti-LPS response comparable to responses induced by cholera (Svennerholm *et al.*, 1984). Three doses, each comprising 5mg of B subunit and 2×10^{11} killed vibrios or the killed vibrios alone, were studied for protective efficacy in North American volunteers (Black *et al.*, 1987). The combination oral vaccine provided 64% protection against cholera and the oral killed *V. cholerae* vaccine gave 56% protection. In a field trial subsequently performed in Bangladesh, an area where cholera is endemic, three doses of 1mg of B subunit and 10^{11} killed whole vibrios were found to provide 62% efficacy, while the killed-vibrio vaccine alone gave 53% protection in the year after vaccination (Clemens *et al.*, 1987). Results of studies with oral toxoid/killed whole-cell vaccine combinations demonstrate complete safety. However, the protective efficacy is only moderate, and multiple doses are required to induce the protection.

The molecular cloning and analysis of *V. cholerae* genes provides a means of modifying them by site-directed mutagenesis and the re-introduction of these modified genes back into *V. cholerae*. Taylor *et al.*, (1988b) have constructed several *V. cholerae* strains which are deleted encoding the gene for the A subunit of cholera toxin (CT), but carry a mutation which significantly increases the expression of the TCP pilus and the B subunit of CT. These two components have been estimated to represent up to 20% of the total bacterial cell protein in these strains. Killed cells which have produced increased amounts of B subunit and have a large number of pili on their surface, could greatly improve the protective efficacy of killed whole-cell cholera vaccines and such strains are currently being evaluated.

Although the major attraction of a non living cholera vaccine is its safety, parenterally or orally administered, killed whole cell and toxoid vaccines have been shown to be largely ineffective in producing long-lasting immunity, as they appear to lack the ability to induce local immune responses (sIgA) in the intestine (Levine, 1980; Cash *et al.*, 1974).

1.2.2 Live vaccines

Since the natural disease cholera is capable of inducing prolonged immunity (Cash *et al.*, 1974; Levine *et al.*, 1983b), the use of attenuated, non-toxinogenic mutants of *V. cholerae* as live oral cholera vaccines has become increasingly popular. The observation that live *V. cholerae* are more immunogenic than killed vibrios when administered orally (Cash *et al.*, 1974; Levine *et al.*, 1983a), is consistent with the view that live bacteria are especially efficient at delivering protective antigens to responsive mucosal lymphoid tissue (Pierce *et al.*, 1988). Live *V. cholerae* are readily translocated from the bowel lumen to submucosal lymphoid tissue by epithelial M cells that overlie Peyer's patches. This, however, is not the case with killed *V. cholerae*, where the uptake of killed vibrios by M cells is not detected (Owen *et al.*, 1986). Long-term protection against the disease is therefore probably mediated by intestinal secretory IgA antibodies and directed against a variety of bacterial antigens (Fubara and Freter, 1973).

Non-toxinogenic *V. cholerae* 01 strains from environmental sources have been isolated and evaluated as potential live oral vaccine candidates (Cash *et al.*, 1974; Levine *et al.*, 1982). These strains failed to colonize or did so minimally, and failed to provide protection in experimental challenge studies (Cash *et al.*, 1974; Levine *et al.*, 1982). The first laboratory attenuated *V. cholerae* strain was a chemically mutagenized El Tor Ogawa (Honda and Finkelstein, 1979). This strain, designated Texas Star-SR, was able to produce the immunogenic B subunit while failing to produce detectable A subunit or the holotoxin. This strain was tested in human volunteers, and although significant protection was afforded against re-infection, 24% of the volunteers experienced a mild diarrhoea (Levine *et al.*, 1984). The Texas Star strain therefore suffered a significant drawback in that it still gave rise to unacceptable side-effects. The method of attenuation, nitrosoguanidine mutagenesis, induces multiple mutations which may have remained unidentified in this strain. Importantly, the nature of the mutation in the A subunit was not identified, and the possibility of reversion to a biologically active toxin was also a consideration. Although such a strain was clearly not acceptable, its use in trials (Levine *et al.*, 1983b) did support the concept of using attenuated live oral strains to mimic infection derived immunity to cholera.

The need to construct attenuated *V. cholerae* strains without the possibility of reversion to virulence, has led to the application of genetic engineering to overcome these problems. The molecular cloning of the cholera toxin genes, has allowed specific deletions in the toxin genes to be introduced into certain pathogenic strains of *V. cholerae* (Kaper *et al.*, 1984a; Kaper *et al.*, 1984b; Mekalanos *et al.*, 1983). These strains have been fed to volunteers, and although the volunteers are highly protected to subsequent challenge, a mild diarrhoea is induced by the immunization (Kaper *et al.*, 1985). The precise reason for this reactogenicity is unknown, but may be related to the heavy colonization of the small intestine by these vibrios. Another possible factor is that these strains produce another toxin(s), one of which is related to Shiga toxin (Madden *et al.*, 1984; O'Brien *et al.*, 1984).

The ability of live attenuated strains to colonize efficiently is an important factor for immunizing efficacy (Owen *et al.*, 1986; Pierce *et al.*, 1988). Early studies have emphasized the role of mucosal colonization for bacterial immunogenicity and resistance to re-colonization as a defence against re-infection (Holmgren and Svennerholm, 1977). This protection to re-infection can be explained in two ways. Protection may be due to secretory antibodies preventing colonization by binding to and blocking specific colonization factors on the organism. Therefore the best colonizers produce these factors abundantly and are the most efficient at evoking specific immune responses to these antigens. Secondly, efficient mucosal colonization could aid in the delivery of a number of antigens (not necessarily colonization factors) to mucosal lymphoid tissue and therefore a more vigorous immune response to these antigens.

It has been proposed that the act of colonization to the small intestine by adherent vibrios, could itself be responsible for the mild diarrhoea seen in attenuated *V. cholerae* vaccines (Pierce *et al.*, 1988; Taylor *et al.*, 1988b). High numbers of bacteria multiplying in the usually sterile ileal environment could disrupt the local balance to induce a fluid outpouring. Therefore, with the process of colonization being essential for a protective immune response, it may not be possible to construct a non-reactive live attenuated oral *V. cholerae* vaccine. However, there may be a combination of factors which contribute to the reactogenicity of these strains, such as a haemolysin/cytotoxin or a Shiga-like

toxin (Levine *et al.*, 1988). If this were the case, then the deletion of the genes for such cytotoxins could result in an efficient and protective live oral vaccine without the adverse side effects.

1.2.2.1 Heterologous vaccines

Due to the problems of reactogenicity that are associated with attenuated live oral *V. cholerae* vaccines, the potential for heterologous carrier strains carrying *V. cholerae* protective antigens has also received attention in recent years. This strategy, however, necessitates the identification of the important protective antigens, with the components of the cell envelope involved in adhesion and colonization being the main targets. Recombinant DNA technology and the recent advances in our understanding of the genetics of *V. cholerae* has made such a task feasible.

Of prime importance in such a strategy is the need for a well suited carrier host. Attenuated Salmonellae seem well suited to this task (Stevenson and Manning, 1985; Germanier and Furer, 1975). The major advantage of these strains is their ability to efficiently penetrate the gut lymphoid tissue (Peyer's patches), while attenuated *V. cholerae* organisms are thought to only enter by the normal sampling mechanism of the M cells (Owen *et al.*, 1986). Therefore an improved response could be expected from immunization with an attenuated Salmonella with the added advantage of stimulating an immune response to the Salmonella host.

The major problem confronted by using a heterologous organism for a carrier strain, is the identification of the antigens in *V. cholerae* which are not only immunogenic, but also protective. To date, a number of such antigens have been defined, and these include LPS (Clements *et al.*, 1982), cholera toxin (Curlin *et al.*, 1975; Svennerholm *et al.*, 1982a) and the TCP pilus (Taylor *et al.*, 1988b; Sharma *et al.*, 1988). The live oral typhoid vaccine, *S. typhi* Ty21a (Germanier and Furer, 1975), has been used as a carrier for the *V. cholerae* LPS O-Antigen (Manning, 1987). The oral immunization of volunteers with such a strain has led to encouraging levels of specific IgA in the gut (Manning, 1987). The incorporation of a number of antigens into an attenuated Salmonella carrier could further

improve the protective capacity in such an oral immunization programme.

1.3 Genetics of *V. cholerae*

The mechanism of disease of *V. cholerae* 01 has come under considerable study over the years, with the resulting advance in our knowledge of the disease process of this organism. The genetics of *V. cholerae*, however, has remained less developed by comparison with other bacterial systems (e.g. *E. coli* and *S. typhimurium*). The past decade has seen a major advance in our understanding of *V. cholerae* at the molecular level with the application of genetic techniques, and the recognition of the contribution that these studies can make in determining the virulence factors elicited by this organism.

1.3.1 Genetic Mapping

Conjugal gene transfer in *V. cholerae* is mediated by the naturally occurring conjugative P plasmid or P factor (Bhaskaran, 1960). The *V. cholerae* conjugation system resembles the F-mediated system of *E. coli*, except that recombination frequencies are much lower, and non-selected markers are only poorly linked to selected markers (Bhaskaran, 1960; Parker *et al.*, 1979). Unlike F, P does not appear to stably integrate into the bacterial chromosome to form Hfr donors (Datta *et al.*, 1973; Sublett and Romig, 1981; Wohlhieter *et al.*, 1975). Parker and co-workers (1979) constructed a relatively coherent linear chromosomal map of the classical strain 162. However, this mapping procedure was inefficient because transfer frequencies were so low, and the resulting map could not be circularized because linkage was not detected between the *his-1* and *pro-2* markers that comprised its termini.

Johnson and Romig (1979) developed a transposon-facilitated recombination (Tfr) system in *V. cholerae* that increased the efficiency of chromosomal gene transfer. Their Tfr donor strains contained copies of the ampicillin resistance transposon Tn1 inserted into both the P plasmid and the bacterial chromosome

(Johnson and Romig, 1979; Sublett and Romig, 1981). The identical copies of the transposon Tn1 in the chromosome and the P factor, provided homology for P factor integration. The improved donors initiated high-frequency transfer from origins specified by the chromosomal Tn1 insertion site (Johnson and Romig, 1979). By using classical Tfr donors the order of most of the genes on the classical strain 162 map were confirmed, and significant linkage was detected between the previously unlinked *pro-2* and *his-1* markers (Sublett and Romig, 1981). Using a modification of this Tfr system, Green *et al.*, (1983) found the El Tor biotype markers for haemolysin production (*hly*), chicken erythrocyte haemagglutination (*cha*) and polymyxin B resistance (*pmx*) to be closely linked to each other, and were located between the *pyrA* and *his* loci on the genetic map of *V. cholerae*. They also found that the segment of the El Tor genetic map including the loci *pro-pyrA-leu-ura* was inverted with respect to the map of the classical *V. cholerae* strain 162, and that the three El Tor biotype markers (*hly*, *cha* and *pmx*) were located in this region (Newland *et al.*, 1984). The resulting genetic map from these and other studies, has shown that the classical and El Tor strains are similar in most respects, with perhaps the inversion of the region containing the El Tor biotype locus being the most significant.

1.3.2 Cholera toxin (CT)

CT is probably the best studied molecule produced by *V. cholerae*, in terms of its structure and its biological function. The genes encoding this enterotoxin have also come under considerable examination.

Phenotypically non-toxinogenic mutants of *V. cholerae* were first isolated from previously toxinogenic strains by the use of the mutagenic vibriophages VcA1 and VcActs1 (Mekalanos *et al.*, 1982). These mutants were shown to be deleted for part or all of the toxin genes, by probing with DNA corresponding to the genes for the heat-labile (LT) enterotoxin of *E. coli*. LT had previously been found to be very closely related to CT in both its structure, antigenicity and mode of action (Clements and Finkelstein, 1979; Gill *et al.*, 1976). Furthermore, Southern blot analysis utilizing probes from the LT genes, demonstrated that toxigenic strains of

V. cholerae possessed DNA sequences homologous to the LT genes (Moseley and Falkow, 1980). This sequence homology was made use of to clone the enterotoxin genes from the classical *V. cholerae* strain 569B (Pearson and Mekalanos, 1982; Kaper and Levine, 1981). DNA sequence data of the cloned CT and LT genes, revealed extensive homology between the heat-labile toxin genes (*eltAB*) of *E. coli* and the cholera toxin genes (*ctxAB*). Both the A and B cistrons showed approximately 75% DNA homology, and this was reflected at the amino acid level with the A and B polypeptides showing 78% and 80% homology, respectively (Mekalanos *et al.*, 1983). These workers also showed the *V. cholerae* CT genes were present as a single transcriptional unit, similar to that of the *E. coli* LT genes, with the gene for the A subunit (*ctxA*) being transcribed first. The A subunit is expressed as a single polypeptide which is subsequently proteolytically nicked to give rise to the A1 and A2 subunits. The gene for the B subunit (*ctxB*) has a more efficient ribosome binding site, giving rise to the one to five stoichiometry of A to B subunits (Mekalanos *et al.*, 1983; Dallas and Falkow, 1979). The genes for LT have been found to exist mainly on plasmids while those of cholera toxin are chromosomally located (Dallas and Falkow, 1980; Spicer *et al.*, 1981). Furthermore, although sequence data show a striking homology between the LT and CT genes, this homology is immediately lost upstream of the structural gene within the promoter regions (Mekalanos *et al.*, 1983), and suggests that these genes may have had a common origin but have evolved separately to suit their hosts.

The structural genes which constitute the cholera toxin operon, *ctxAB*, have been genetically mapped and shown to lie between the *nal* and *his* genes on the *V. cholerae* El Tor chromosome (Sporecke *et al.*, 1984). The classical strains of *V. cholerae* have two widely separated copies of *ctxAB*, one of which maps at the same position on the chromosome as in El Tor strains, while the location of the second copy is at present unknown (Sporecke *et al.*, 1984; Mekalanos, 1983). *V. cholerae* strains of the El Tor biotype were originally reported to contain only a single copy of the cholera toxin operon (Moseley and Falkow, 1980; Kaper *et al.*, 1981). However, a number of El Tor strains have now been shown to contain multiple copies of *ctxAB* arranged on large tandem repeats (Mekalanos, 1983). The size of these repeats is variable, and has been shown to be due to a

difference in the copy number of a smaller 2.7kb tandemly repeated sequence, designated RS1 (Mekalanos, 1983). The RS1 sequence is located at the novel joint of these duplications, as well as upstream and downstream of *ctxAB* (Mekalanos, 1983; Mekalanos, 1985). The cholera toxin operon therefore appears to be located on a variable genetic element composed of a central core region containing *ctxAB*, flanked by direct repeats of RS1.

This genetic element is capable of increasing its copy number within a strain, and can be selected for *in vivo* by infection with *V. cholerae* in animal models, and thus has been implicated in the enhancement of its pathogenicity (Mekalanos, 1983). This gene amplification relies on recombination occurring within copies of RS1 that flank the *ctxAB* genes, and has been shown to be dependent on the *recA* gene product of *V. cholerae* (Goldberg and Mekalanos, 1985a, 1985b).

The cloning of the *ctxAB* operon of *V. cholerae* into *E. coli*, resulted in a significant decrease in toxin production when compared to *V. cholerae* (Pearson and Mekalanos, 1982). While it was thought possible that *ctxAB* transcriptional and translational signals were not recognized efficiently by the *E. coli* synthetic machinery, an alternative explanation for this difference was that *E. coli* lacked the *V. cholerae* regulatory genes. Mutations at a locus called *tox* caused a sharp decrease in toxin production in the classical strain 569B (Baine *et al.*, 1978). The chromosomal locations of these *tox* mutations have been mapped and lie between *cys* and *fmt*, while *ctxAB* lies between *his* and *nal* (Mekalanos *et al.*, 1979). These mutations were therefore clearly at a locus distinct from the CT structural genes. Consistent with the idea of a positive regulatory protein(s) acting to increase expression of the *ctxAB* operon, was a study of the promoter regions from a variety of *ctxAB* copies cloned from both El Tor and classical strains (Mekalanos *et al.*, 1983). The sequences of these promoters were found to be identical, except for the number of tandem repetitions of the sequence TTTTGAT located about 80 base pairs upstream of the start of *ctxA* (Mekalanos *et al.*, 1983).

Miller and Mekalanos (1984) reported genetic evidence supporting a role for these repeats in toxin expression in *V. cholerae*. These investigators cloned a positive regulatory gene from *V. cholerae* that regulated synthesis of cholera toxin at the transcriptional level, and termed it *toxR* (Miller and Mekalanos, 1984). The *toxR* gene has subsequently been identified as coding for a 32,527 dalton protein

and the nucleotide sequence predicts a transmembrane structure, with the DNA binding domains of the ToxR protein in the cytoplasmically located N-terminal portion (Miller *et al.*, 1987). ToxR is thought to exert its effect at the transcriptional level by binding to the tandemly repeated copies of the 7bp sequence upstream of the transcriptional start point (Miller *et al.*, 1987). Genetic evidence has also shown that the cloned *toxR* gene corresponds to the previously described *tox* locus (Miller and Mekalanos, 1985).

Recent studies have identified a second regulatory gene called *toxS*, which enhances the activity of *toxR* (Peterson *et al.*, 1988). The *toxS* gene is in the same transcriptional unit as *toxR*, and its properties are consistent with it encoding a periplasmic protein. These authors have suggested a working model for the *toxR* regulon, in which the membrane inserted ToxR must dimerize to bind to the specific repetitive DNA sequences that define its binding sites near the promoters of the *toxR*-regulated genes. Environmental, physical or nutritional parameters influence the periplasmically located ToxS. The ToxR,S system has now been shown to regulate not only the *ctx* operon, but also a number of other virulence factors and outer membrane proteins of *V. cholerae*, in either a positive or negative fashion (Taylor *et al.*, 1987a; Peterson *et al.*, 1988; Miller and Mekalanos, 1988; Miller *et al.*, 1989).

1.3.3 Outer Membrane Proteins

The cell envelope of *V. cholerae* is typical for Gram-negative bacteria in its structure and composition. The outer membrane contains lipopolysaccharide (LPS) and a small number of major outer membrane proteins (Kabir, 1980; Kelley and Parker, 1981; Manning *et al.*, 1982). A group of proteins with sizes in the range of 43 to 47 kDa probably represent the major cell porins, with the 45 kDa protein being the most abundant species (Kelley and Parker, 1981; Manning *et al.*, 1982). These porins are significantly larger than those found in enteric bacteria such as *E. coli* and *S. typhimurium* (DiRienzo *et al.*, 1978). In addition to these proteins, there is an OmpA-like heat-modifiable 35 kDa protein (Alm *et al.*, 1988) and the OmpV protein of 25 kDa (Stevenson *et al.*, 1985), which probably

represent the major structural proteins of the *V. cholerae* outer membrane.

The gene for the 25 kDa outer membrane protein of *V. cholerae* has been cloned, and termed *ompV* (Stevenson *et al.*, 1985). Although the OmpV protein is a major outer membrane protein in *V. cholerae* (Manning and Haynes, 1984), the gene is poorly expressed in *E. coli* (Stevenson *et al.*, 1985). This is in marked contrast to the cloned 22 kDa dalton minor protein of *V. cholerae*, which when present in *E. coli* is expressed in large amounts (Manning *et al.*, 1985). A number of possibilities exist for the low expression of *ompV*, but analysis of the nucleotide sequence of this gene points towards the role of a positive regulatory element being required for proper gene expression (Pohlner *et al.*, 1986a). Pohlner *et al.*, (1986a), however, postulate that this positive regulator acts at the level of translation of the *ompV* mRNA compared with ToxR which functions at the level of transcription (Miller and Mekalanos, 1984). The initiation codon and ribosome binding site of the *ompV* gene fall within a region capable of forming a stem-loop structure in the RNA. This is thought to be responsible for the low level of expression, by inhibiting the binding of the mRNA to the 16S ribosomal subunit. It has been proposed that a positive regulatory element is involved which is present in *V. cholerae* but absent from *E. coli* (Pohlner *et al.*, 1986a).

The immunogenicity of OmpV has also been investigated. The derived amino acid sequence of this protein has marked hydrophilic regions, several of which have been shown to correspond to the antigenic determinants on either the native or denatured forms of the protein (Pohlner *et al.*, 1986a, 1986b). OmpV is present in all *V. cholerae* strains of both biotypes and serotypes (Manning and Haynes, 1984). The importance of OmpV in the virulence of *V. cholerae* is uncertain as a transposon insertion in the *ompV* gene does not affect colonizing ability of the organism or reduce its pathogenicity in the *in vivo* models tested (Taylor *et al.*, 1987b).

1.3.4 Lipopolysaccharide (LPS)

The LPS of *V. cholerae* has a similar overall structure to that of other Gram-negative organisms. It consists of a lipid A region which is the hydrophobic portion and forms part of the lipid bilayer of the outer membrane, the core oligosaccharide, and the O-antigen (Luderitz *et al.*, 1966; Gmeiner *et al.*, 1971). The chemical composition of the core and O-antigen regions have been determined, but neither the structure of the LPS molecules nor the chemical nature of the antigens (A,B and C) have been defined (Redmond, 1978, 1979; Sen *et al.*, 1979, 1980; Hisatsune and Kondo, 1980). Ogawa but not Inaba strains possess the sugar 4-amino-4-deoxy-L-arabinose and so it is thought that this sugar could be associated with the B antigen (Redmond, 1978).

The genes determining an O-antigen have been well defined in Salmonella (Levinthal and Nikaido, 1969; Nikaido *et al.*, 1967), but very little is known about the genetics of O-antigen biosynthesis in *V. cholerae*, although the genes coding for the O-antigen of the Inaba and Ogawa serotypes have been cloned and expressed in *E. coli* K-12 (Manning *et al.*, 1986). These cosmid clones are capable of expressing their serotype specificity and therefore the information required for determining the Ogawa or Inaba serotype must be present on the cloned DNA. Analysis of the DNA coding for both these serotypes has defined the O-antigen region to be contained within a 19kb SstI fragment, with no differences being detected in the restriction patterns of the Inaba and Ogawa regions (Ward *et al.*, 1987). This suggests that only minor changes are involved in serotype conversion.

The *oag* locus, responsible for serotype specificity, has been mapped between the *ilv* and *arg* loci of *V. cholerae* (Bhaskaran, 1959; Parker *et al.*, 1979). Transposon insertion mutants have been isolated and mapped by Southern hybridization analysis to the region of the *V. cholerae* chromosome determining O-antigen biosynthesis. These chromosomal mutations have also been shown to map between *ilv* and *arg* (Ward and Manning, 1989), confirming it to be the same locus as that described by Bhaskaran (1959) and Parker *et al.*, (1979). Ward *et*

al., (1987) have suggested that the locus encoding O-antigen biosynthesis, *oag*, be redesignated *rfb*, in accordance with the established convention (Hitchcock *et al.*, 1986).

1.3.5 Fimbriae

One of the first reports of a haemagglutinin associated with a pilus-like structure in *V. cholerae* was by Taylor *et al.*, (1987). This pilus has been termed TCP for toxin co-regulated pilus, as its expression is coordinately regulated with cholera toxin by *toxR* (Peterson *et al.*, 1988). This pilus shares the same mannose- fucose resistant haemagglutination pattern (MFRHA) as that described by Hanne and Finkelstein (1982). However, at least two distinct MFRHA's are inferred from results of Southern hybridization analysis (V. Franzon - personal communication). *V. cholerae* mutants defective in the TCP pilus do not colonize in both the infant mouse model and humans (Taylor *et al.*, 1987a, 1988b; Herrington *et al.*, 1987).

Genetic analysis of this region by transposon *TnphoA* (Manoil and Beckwith, 1985) insertion mutagenesis, has led to the tentative organization of the *tcp* operon (Taylor *et al.*, 1988b). The authors have based their model of operon organization on that for the pyelonephritis-associated pili (PAP) operon of uropathogenic *E. coli* (Lindberg *et al.*, 1986), where the major structural subunit, *tcpA*, makes up the pilus structure, while the actual adhesin is located at the tip of the pilus (Taylor *et al.*, 1988b; Lindberg *et al.*, 1987). The *tcpA* gene coding for the major structural subunit has been cloned and sequenced (R.Faast-personal communication). This gene codes for a 20.5 kDa protein, and the deduced amino acid sequence shows strong homology to the pilins of other bacteria such as *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and *Bacteroides nodosus* (Taylor *et al.*, 1988b). The proposed tip adhesin of the *tcp* operon, *TcpG* has not as yet been identified, but Franzon *et al.*, (1988) have detected amino acid homology of their MFRHA with *PapG*, the tip adhesin in the PAP pilus system (Lindberg *et al.*, 1987). This suggests that their MFRHA may be a tip adhesin, although it probably belongs to another pilus system in *V. cholerae* distinct from TCP.

The genes for TCP have been shown by Southern hybridization to be present in both classical and El Tor biotypes of *V. cholerae*, but not in non-toxicogenic environmental El Tor isolates (Taylor *et al.*, 1988b). However, the *tcp* genes are only expressed in classical strains (Taylor *et al.*, 1988b). Sharma *et al.*, (1988) have shown that the functional *tcp* genes cloned from a classical strain can be introduced into an El Tor strain with the subsequent expression of the TCP pilus on the cell surface. This points towards the idea that El Tor strains do not express the TCP pilus due to a defect in one of the *tcp* operon genes. Whether this defect is in a *tcp* structural gene, or one of the regulatory or assembly genes remains to be seen.

Hall *et al.*, (1988) have shown that *V. cholerae* strains express at least two other types of fimbriae. TcpA fimbriae are 5-6nm in diameter and form bundles of parallel undulating filaments up to 15 μ m long; Type B are 3nm wide and of wavy morphology, and Type C are rigid isolated filaments 5-6nm wide and 180-800nm long. A fourth, morphologically distinct fimbrial type has been found on pathogenic El Tor organisms, but further studies are required to confirm these observations (Hall *et al.*, 1988). These fimbriae, or at least their adhesins, could in fact correspond to the other haemagglutinins of *V. cholerae* described by Hanne and Finkelstein (1982). The role of these other fimbriae has not as yet been established, although it appears that Type B and Type C are not as important as TCP in adherence (Hall *et al.*, (1988). The fourth type of fimbriae which has been seen only on El Tor strains (Hall *et al.*, 1988), may functionally replace the non-expressed TCP pilus in this biotype (Sharma *et al.*, 1988).

1.4 Extracellular proteins in Gram negative bacteria

The export and secretion of proteins are fundamental properties of living cells. Gram-negative cells export polypeptides to the periplasmic and outer membrane subcompartments of their cell envelopes, but few secrete proteins into the extracellular environment (Pugsley and Schwartz, 1985; Davis and Tai,1980). Gram-negative bacteria are not widely recognized for their ability to secrete proteins into the growth medium, but a number of species are able to do so,

particularly those belonging to the Vibrionaceae and Pseudomonaceae.

Proteins released into the extracellular environment by these organisms, must cross both the inner and outer membranes during their export. Very little is known of how this occurs, despite an increasingly detailed understanding of the mechanism of secretion of proteins across the cytoplasmic membranes of eukaryotes and prokaryotes (for reviews see Hall and Silhavy, 1981; Randall and Hardy, 1984; Oliver, 1985).

V. cholerae releases a variety of extracellular proteins. These include proteases (Schneider *et al.*, 1981; Young and Broadbent, 1982), haemagglutinins (Hanne and Finkelstein, 1982), haemolysins (Manning *et al.*, 1984; Goldberg and Murphy, 1984; Richardson *et al.*, 1986), DNases (Tsan, 1978), neuraminidase (Ada *et al.*, 1961; Vimr *et al.*, 1988), mucinase (Schneider and Parker, 1982) and cholera toxin (Finkelstein, 1969). The study of the extracellular proteins of *V. cholerae* and other Gram-negative organisms, has focused mainly on their involvement in the virulence of the organism. The use of these proteins as models for extracellular export, and the study of export defective mutants should help in understanding this process.

Interestingly, when the genes for extracellular proteins are cloned into *E. coli*, they are usually localized in the periplasmic space. This phenomenon can be demonstrated with a number of extracellular proteins of *V. cholerae*, including cholera toxin (Pearson and Mekalanos, 1982), haemolysin (Mercurio and Manning, 1985) and neuraminidase (Vimr *et al.*, 1988). This has also been observed in *E. coli* with the extracellular enzymes of *Aeromonas hydrophila* (Chakraborty *et al.*, 1986; Howard and Buckley, 1986; Gobius and Pemberton, 1988) as well as *Pseudomonas aeruginosa* (Lory *et al.*, 1983; Lory and Tai, 1983; Coleman *et al.*, 1983), suggesting that *E. coli* may have a block in the extracellular export of proteins which is normally present in the natural host. The nature of this block is unknown, but could be due to *E. coli* not recognizing the appropriate export signals on these extracellular proteins, or simply a lack of the necessary export machinery required for this process. The inability of *E. coli* to secrete these extracellular proteins does not come as a complete surprise, since as a rule it does not usually secrete any of its own proteins outside of the cell.

Most extracellularly located proteins are initially synthesized with amino-terminal

signal sequences and are thought to initiate their secretion in a manner analogous to the export of periplasmic and outer membrane proteins (Randall and Hardy, 1984). However, the pathway taken by secreted proteins as they traverse the cell envelopes of Gram-negative bacteria and the role of additional extragenic factors in the secretion of specific proteins remain to be elucidated. Clearly, a typical signal sequence would not be sufficient to cause selective export of a protein across the double membrane of a Gram-negative bacterium. A mechanism must exist to differentiate soluble proteins destined for the periplasm from those destined for the extracellular environment.

The simplest possible pathway for extracellular export would involve recognition of the protein by a receptor at zones of adhesion and co- or post-translational movement across inner and outer membranes simultaneously. The results obtained by Lory *et al.*, (1983) appear to support such a model for the secretion of exotoxin A by *P. aeruginosa*. Lory *et al.*, (1983) observed that the export of toxin occurred as rapidly as the toxin was synthesized. Furthermore, they found that when processing of this protein was inhibited by ethanol, the precursor was found on the surface of the cell, with no detectable levels in the periplasm. These data were used to devise a model in which exotoxin A is exported directly to the outer membrane through areas of contact between inner and outer membranes, with proteolysis being required for its immediate release into the medium. The proteolytic step involves the removal of the signal peptide, but removal of a peptide anchor subsequent to signal peptidase action might also be involved (Davis and Tai, 1980; Lory *et al.*, 1983). Alternatively, proteins could cross one membrane and then the other enroute to the extracellular medium. This less direct pathway for protein export would require transfer of the molecule across the inner membrane, sorting either on the outside surface of the inner membrane or in the periplasm, and transfer across the outer membrane. Such a multistage pathway appears to be followed by the extracellular toxin, aerolysin, of *A. hydrophila* (Howard and Buckley, 1985).

A number of extracellular proteins of *Serratia marcescens* seem to be an exception to the rule of being localized in the periplasm when cloned in *E. coli*. The extracellular serine protease and nucleases of *S. marcescens* when cloned into *E. coli*, are secreted into the extracellular medium, with no evidence of cell

lysis or concurrent release of cytoplasmic or periplasmic proteins, thereby establishing it as a true form of extracellular secretion (Yanagida *et al.*, 1986; Ball *et al.*, 1987; Clegg and Allen, 1985). No accessory genes are required for the extracellular release of these enzymes in *E. coli*, and therefore it appears that *E. coli* is capable of secreting certain proteins extracellularly. This suggests that at least in some cases, the information required for the secretion of extracellular proteins resides within the protein sequence itself.

Yanagida *et al.*, (1986) purified the serine protease of *S. marcescens* and compared the nucleotide sequence of the cloned DNA and the N- and C-terminal amino acid sequences of the secreted protease. They found the proenzyme was processed not only at the N-terminal signal sequence but also the C-terminal part of the proenzyme. These investigators have proposed that the N-terminal signal sequence of the proenzyme and its processing are involved in secretion through the inner cytoplasmic membrane, while processing of the large C-terminal part of the proenzyme is involved in its excretion through the outer membrane. The involvement of the C-terminal region in excretion of an extracellular protein has also been seen with the IgA protease of *Neisseria gonorrhoeae*. This protein is also exported to the external medium when cloned into *E. coli* (Pohlner *et al.*, 1987). Thus, one cannot limit the study of extracellular secretion to the identification of an additional secretory apparatus required for this process, as it appears that in some instances at least, the information required for secretion resides within the protein itself.

1.4.1 *E. coli* haemolysin

The extracellular haemolysin, produced by some strains of *E. coli*, is probably the best studied example of an extracellular protein in *E. coli*. The haemolysin is present in certain pathogenic strains of *E. coli*, particularly those that are isolated from extra-intestinal infections in humans (Hughes *et al.*, 1982; Minshew *et al.*, 1980) or from animal intestinal origin (Smith, 1963). The alpha-haemolytic phenotype, i.e. the synthesis of active haemolysin and its secretion into the surrounding medium, is controlled by determinants which are located either on

transmissible plasmids or the chromosome (de la Cruz *et al.*, 1979; Goebel *et al.*, 1974). This determinant is a highly conserved 7.5kb sequence, and has some characteristics of a transposon-like element (Zabala *et al.*, 1984). Four genes *hlyA*, *hlyB*, *hlyC* and *hlyD* are required for the synthesis and release of haemolysin into the culture medium (Mackman *et al.*, 1986; Wagner *et al.*, 1983). The *E. coli* haemolysin is a 107 kDa polypeptide encoded by the *hlyA* gene (Mackman and Holland, 1984). The product of *hlyC*, a 20kDa cytoplasmic protein, is required to promote the formation of a haemolytically active 107kDa polypeptide by some form of post-translational modification. However, *hlyC* is not required for the export of HlyA (Nicaud *et al.*, 1985). The secretion of HlyA is dependent on the products of two additional genes, *hlyB* and *hlyD* (Mackman *et al.*, 1986). HlyB encodes two distinct polypeptides: a 66kDa protein and a 46kDa protein that appears to derive from an internal translational start, and these are both membrane bound (Felmlee *et al.*, 1985b). A 53kDa protein is encoded by *hlyD*, and it is located primarily in the inner membrane (Mackman *et al.*, 1986). Secretion of the haemolysin, which involves translocation through both membranes, appears to involve a novel mechanism, as the haemolysin does not carry an N-terminal signal sequence (Felmlee *et al.*, 1985a). Mackman *et al.*, (1986) have proposed a model in which the Hly proteins B and D form a specific complex, spanning the inner and outer membranes, that initially binds the 107kDa protein and then promotes its direct translocation into the medium. The binding of HlyA to HlyB and HlyD located in the inner membrane, and its excretion into the medium, is probably the result of a direct interaction of HlyA with the secretion machinery. Although the haemolysin does not possess an N-terminal signal sequence, the C-terminal region appears to play a role in the secretion of this polypeptide (Gray *et al.*, 1986; Felmlee and Welch, 1988). Removal of the terminal 27 amino acids of HlyA does not destroy haemolytic activity, but is sufficient to prevent the haemolysin from reaching the external medium (Gray *et al.*, 1986). This raises the possibility that the C-terminal region of the haemolysin includes a specific topogenic sequence, and that this sequence is necessary for recognition by the secretion machinery.

A possible role for HlyB in the secretion process has also been suggested (Gray *et al.*, 1986). Nucleotide sequence data of HlyB show two blocks of highly

conserved sequences close to the C-terminus (Felmlee *et al.*, 1985b), which correspond to an ATP binding site similar to a number of *E. coli* and *S. typhimurium* polypeptides that are involved in cytoplasmic membrane transport systems (Higgins *et al.*, 1985). One role for the HlyB peptide in HlyA secretion may therefore be to provide energy for translocation through a proteinaceous pore provided by HlyD (Gray *et al.*, 1986). The failure of *E. coli* to secrete haemolysins encoded by other bacteria (Lory and Tai, 1983; Goldberg and Murphy, 1984; Mercurio and Manning, 1985) may therefore be due to the absence of additional *hly* determinants which are concerned with haemolysin release in these organisms.

1.4.2 *V. cholerae* haemolysin

Many strains of *V. cholerae* are able to produce and secrete a haemolysin into the culture medium (Honda and Finkelstein, 1979). Haemolytic activity is commonly associated with the El Tor biotype but not the classical biotype of *V. cholerae*, and this has been one of the means of distinguishing biotypes. However, non-haemolytic El Tor strains have been isolated (Barrett and Blake, 1981; Finkelstein, 1966). The haemolysin determinant from *V. cholerae* El Tor has been cloned by several groups, and although the protein is secreted to the external medium in *V. cholerae*, it is found localized in the periplasmic space of *E. coli* (Goldberg and Murphy, 1984; Manning *et al.*, 1984; Mercurio and Manning, 1985).

Two proteins, and their corresponding genes, *hlyA* and *hlyB*, have been identified (Manning *et al.*, 1984; Alm and Manning, manuscript in preparation). Transposon mutagenesis studies by these workers, have shown that the *hlyA* gene codes for an 82 kDa protein which corresponds to the haemolysin structural gene. This protein size is in good agreement with Goldberg and Murphy, (1984) who have also cloned the El Tor haemolysin. Recently, mutations in the *hlyB* gene of the *V. cholerae* chromosome have been constructed in *hlyB* which inhibit the secretion of the haemolysin from *V. cholerae* resulting in an accumulation of the active haemolysin in the periplasm (R. Alm - personal communication). Therefore, it appears that the *V. cholerae* haemolysin requires at least one other

gene product for its secretion, although there may be other genes involved, as the cloned *V. cholerae hlyA,B* determinant in *E. coli* is not sufficient to export the haemolysin beyond the periplasmic space (Mercurio and Manning, 1985).

It is tempting to find similarities between the mode of secretion of the *E. coli* haemolysin and that of the *V. cholerae* haemolysin. Although these proteins are operationally similar, and both require a number of other genes for their secretion, there are some essential differences in their mechanism of export. While the haemolysin of *E. coli* has no N-terminal signal sequence (Felmlee *et al.*, 1985b), the haemolysin of *V. cholerae* El Tor has been shown to possess an 18 amino acid typical signal sequence which is cleaved to give the mature 80 kDa protein (Alm *et al.*, 1988). Activation of the *V. cholerae* haemolysin does not appear to be necessary, as the *hlyA* gene cloned alone exhibits haemolytic activity, in contrast to that of the *E. coli* haemolysin (Alm *et al.*, 1988; Nicaud *et al.*, 1985). A mutation in *hlyB* in *V. cholerae* results in a periplasmic intermediate of HlyA, while there is strong evidence that there is no periplasmic intermediate in the secretion of the HlyA protein of *E. coli* (Gray *et al.*, 1986; Felmlee and Welch, 1988). Finally, the *hly* determinant of *E. coli* carrying a deletion of *hlyA*, is unable to complement the *hlyA* gene of *V. cholerae* for secretion into the external medium (unpublished results).

Secretion of at least some extracellular proteins in Gram-negative bacteria appears to be a two-step process: initial translocation, facilitated by an N-terminal signal sequence across the inner membrane, and mediated by specific outer membrane proteins to the exterior of the cell (Pugsley and Schwartz, 1985). The *hly* determinant of *V. cholerae* comprises the essential elements of this process, and although secretion of the haemolysin to the outside of the cell can not be demonstrated in *E. coli*, the cloning of additional genes (for specific outer membrane proteins) may facilitate this. On the other hand, the *E. coli hly* determinant seems to follow a more novel secretion process which is independent of an N-terminal signal sequence and devoid of a periplasmic intermediate. This could reflect the fact that the *E. coli hly* determinant is on a transposon-like element (Zabala *et al.*, 1984), and has evolved its own export pathway which is self-contained and thereby making it functional in any bacterium in which it may reside, in particular *E. coli*.

1.4.3 Cholera toxin (CT) and Heat-labile enterotoxin (LT)

CT of *V. cholerae* and LT of enterotoxigenic *E. coli*, are structurally and functionally similar oligomeric proteins comprised of six subunits, an A subunit that activates adenylate cyclase and five B subunits which bind to GM1-ganglioside receptors (Gill *et al.*, 1981; Lonnroth and Holmgren, 1973). Extensive studies on the export of heat-labile enterotoxins (LT) from *E. coli* have shown that the toxin subunits are initially synthesized as precursors which are rapidly processed and translocated across the inner membrane into the periplasm (Hirst *et al.*, 1983; Hirst *et al.*, 1984a; Hofstra and Wilholt, 1984). Surprisingly, the assembled holotoxin remains within the periplasm of *E. coli* and is not secreted through the outer membrane (Clements *et al.*, 1985; Hofstra and Wilholt, 1984). This contrasts with *V. cholerae* which efficiently secretes cholera toxin into the surrounding medium (Mekalanos *et al.*, 1983). However, LT is secreted into the medium when plasmids encoding it are transferred into *V. cholerae* (Hirst *et al.*, 1984b; Neill *et al.*, 1983).

Many of the steps in the export and secretion of CT and LT are presumably common in both *V. cholerae* and *E. coli*. This includes synthesis of subunit precursors, translocation across the cytoplasmic membrane, maturation and subunit assembly (Palva *et al.*, 1981; Mekalanos *et al.*, 1983; Hirst *et al.*, 1983). The capacity of *V. cholerae* to secrete cholera toxin into the extracellular medium is, however, an additional step not found in the export of LT by *E. coli*. This is unlikely to be due solely to differences between cholera toxin and LT, because the cloning of the *ctxAB* genes in *E. coli* results in the accumulation of cell-associated CT (Pearson and Mekalanos, 1982). Studies on the synthesis and location of CT and LT in *V. cholerae* imply that these toxins are secreted via a periplasmic pool. Treatment of *V. cholerae* with lincomycin causes the accumulation of CT in the periplasm (Levner *et al.*, 1980), while pulse chase experiments have detected newly synthesized (radiolabelled) toxin subunits entering the periplasmic compartment very rapidly after synthesis. The efflux of these radiolabelled subunits from the periplasm coincides with the appearance of radiolabelled subunits in the medium (Hirst and Holmgren, 1986).

The site of assembly of A and B subunits during their export is also relevant to the mechanism of toxin secretion, because this governs whether *V. cholerae* secretes the subunits separately or as an assembled holotoxin. The A subunits of LT are only secreted when they are synthesized in a strain that also produces B subunits (Hirst *et al.*, 1984b). This implies that the two subunits must assemble prior to their secretion in order to facilitate an extracellular location for the A subunit. However, in strains that produce only B subunits, it was found that these were efficiently secreted into the medium even though no A subunits were synthesized (Hirst *et al.*, 1984b). Therefore, an association between A and B subunits is not a prerequisite for the B subunits to enter the secretory step of the export pathway.

Although the nature of the secretory step in *V. cholerae* is unknown, several physiological features could be responsible for this event. These include: (i) a "leaky" outer membrane that allows passive diffusion into the medium, (ii) modification enzymes that alter the structure of secreted proteins and cause them to adopt a secretory proficient conformation, or (iii) a secretory apparatus that recognizes and mediates the secretion of proteins through the outer membrane.

While a leaky outer membrane seems highly unlikely, as periplasmic enzymes of *V. cholerae* are not generally found in the external medium (Neill *et al.*, 1983), the modification of the protein may be relevant. CT has been shown to have nicked A subunits when secreted from *V. cholerae* (Lonnroth and Holmgren, 1973), but un-nicked A subunits when cell-associated in *E. coli* (Pearson and Mekalanos, 1982). However, the holotoxins of both porcine and human LT can be completely secreted from *V. cholerae* without their subunits being nicked (Hirst *et al.*, 1984b). *V. cholerae* may contain a "secretory apparatus" which mediates the secretion of a protein by some specific interaction. The B subunits of LT and CT may contain important domains necessary for this interaction with the secretory apparatus, with the A subunit only being secreted by virtue of its association with the B subunit pentamer. The nature of the interaction between the B subunits and the secretory apparatus, and the identity of the latter remain to be elucidated.

Although Gram-negative bacteria appear to display more than one type of mechanism for the secretion of extracellular proteins to the external environment (Davis and Tai, 1980; Lory *et al.*, 1983; Gray *et al.*, 1986; Pugsley and Schwartz, 1985), the Vibrionaceae appear to generally utilize a two stage process for the secretion of their proteins, where the proteins are channelled via the periplasm to the external environment. This notion is supported by the isolation of secretion defective mutants of *Aeromonas hydrophila* which accumulate extracellular proteins in the periplasmic space (Howard and Buckley, 1983). The Vibrionaceae, however, may possess more than one type of secretion pathway, as mutants defective in the export of extracellular proteins, do not necessarily affect the secretion of others (Holmes *et al.*, 1975; Howard and Buckley, 1983).

1.5 Nucleases

Nucleases are phosphodiesterases which either require a terminus for hydrolysis (exonuclease) or do not require a terminus (endonuclease). Exonucleases do not act on circular DNA, either single-stranded or double-stranded, but require a chain terminus, either a 3' end, a 5' end, or both. These nucleases can be further distinguished by their preference for a single-stranded or a duplex DNA and by the

production of mono- or oligonucleotides. Examples of this type of exonuclease are the exonucleases I - VI of *E. coli* (Kornberg, 1974), bacteriophage T4 encoded exonuclease, (Lehman, 1971) snake venom phosphodiesterase (Khorana, 1961) and Mammalian DNase IV (Lehman, 1971). The DNA polymerase I of *E. coli*, also known as exonuclease I and IV, has both 3' → 5' and 5' → 3' exonuclease activity, required for its proofreading and excision-repair function (Brutlag and Kornberg, 1972). Exonuclease V or the RecBC nuclease of *E. coli* which functions in recombination, acts on both single-stranded and duplex DNA and also has both 3' → 5' and 5' → 3' exonuclease function. Snake venom phosphodiesterase on the other hand completely degrades the DNA, and also acts on RNA (Khorana, 1961).

The endonucleases, which do not require a terminus, often show a strong preference for either single-stranded DNA or duplex DNA. A second characteristic of endonucleases is recognition of base sequences. This was once thought to be a property of only restriction endonucleases, which recognize a sequence of usually four to eight nucleotides of a duplex. While recognition of base sequences is most strictly defined for the restriction endonucleases, sequence preference appears to characterize all endonucleases. For example, bacteriophage T4-encoded endonuclease IV has a cytosine-specific cleavage site, while pancreatic DNase I and spleen DNase II produce digests with characteristic frequencies of oligonucleotides. The sequences of the 3' and 5' termini of the products form a pattern distinctive for each enzyme (Afinsen *et al.*, 1971).

1.5.1 Extracellular Nucleases

A number of bacteria secrete extracellular nucleases. These include the streptococcal extracellular deoxyribonucleases A,B,C and D (Wannamaker, 1958), the micrococcal nuclease of *Staphylococcus aureus* (Davis *et al.*, 1977), the exonuclease of *Bacillus subtilis* (Kerr *et al.*, 1967), the nucleases of *Serratia marcescens* (Eaves and Jeffries, 1963) and the deoxyribonuclease(s) of *V. cholerae* (Tsan, 1978).

A group of four immunologically and electrophoretically distinct nucleases designated DNase A,B,C and D are known to be secreted into the growth medium by many strains of group A streptococci (Wannamaker, 1958). DNase B is generally produced in the greatest amount, with lesser levels of DNases A,C and

D. DNases B and D endonucleolytically degrade both single-stranded and double-stranded DNA, as well as RNA. These enzymes have an absolute requirement for divalent cations (Wannamaker *et al.*, 1967; Citak and Grey, 1980). In the case of staphylococcal nuclease, three types are known to exist, although they probably represent precursor forms of the micrococcal nuclease (Davis *et al.*, 1977). The specificity of this enzyme is not known, although it has a marked preference for denatured DNA (Davis *et al.*, 1977). *S. marcescens* produces a number of nucleases (Eaves and Jeffries, 1963) and two of these have been cloned (Clegg and Allen, 1985; Ball *et al.*, 1987). One of the extracellular nucleases of *S. marcescens* has been purified and characterized extensively (Nestle and Roberts, 1969a). The purified nuclease is able to attack both single-stranded and double-stranded DNA and RNA (Nestle and Roberts, 1969b). Digestion of RNA and DNA by the *S. marcescens* nuclease results in the production of oligonucleotide fragments of definite length; essentially only di-, tri- and tetranucleotides terminating in a 5'-phosphate. When the *S. marcescens* nuclease is compared with other nucleases, it is found to most closely resemble two nucleases from organs of higher animals (Nestle and Roberts, 1969b). These enzymes, isolated from chicken pancreas and rat liver, are both Mg^{2+} and Mn^{2+} activated endonucleases which cleave both RNA and DNA (Eley and Roth, 1966; Curtis *et al.*, 1966). *V. cholerae* produces a number of nucleases (Tsan, 1978) but these enzymes have not been purified, and their mode of action and substrate specificity is not known.

1.5.2 The Deoxyribonucleases of *V. cholerae*

The DNases of *V. cholerae* are true extracellular proteins (Tsan, 1978), and there appears to be a periplasmic pool of active enzyme as well as them being found in the extracellular medium (Young and Broadbent, 1985). Lincomycin, a potent inhibitor of protein synthesis, increases the synthetic rate and the periplasmic pool size of CT (Levner *et al.*, 1980) and a similar effect can be seen with DNase production (Young and Broadbent, 1985). This suggests that the DNase(s) belong to a group of extracellular proteins of *V. cholerae* that are secreted into the medium via a periplasmic pathway.

The role of the DNase(s) in the pathogenesis of cholera has not been

determined. While the role of CT in the disease process has been clearly established (Finkelstein, 1969, 1972;), the effect of DNase may be more subtle. A possible clue as to the role of DNase(s) in the infectious process has come from the findings of Ferencz *et al.*, (1980). These investigators found that DNA was a significant component of the small-intestinal mucous. Although the origin of DNA at this site is unknown, it was speculated that the DNA originates from the intestinal mucous cells which show a rapid turnover. The importance of the small-intestinal mucous for colonization has been examined (Freter and Jones, 1976), and the role of colonization in the disease process discussed previously (see section 1.1.4). The DNase(s) of *V. cholerae* could facilitate the degradation of the DNA-rich, viscous mucous covering of the small intestine and along with motility and other colonization factors aid in the efficient colonization of this surface by these organisms. Mutants of *V. cholerae* deficient in DNase(s), would help in evaluating the extent to which these enzymes contribute to the virulence of *V. cholerae*.

The DNase also may have a role in nutrition. DNA is a good carbon and nitrogen source, and also a source of bases. Isolates of *V. cholerae* are usually prototrophic, but when auxotrophs are isolated, they usually have a *pur* or *pyr* requirement which would be compensated for by the presence of the DNase (Bhaskaran, 1955).

1.5.3 Transformation in *V. cholerae*

Another less direct consequence of the extracellular DNases of *V. cholerae*, is the role they may play in preventing the uptake of foreign DNA into the cell. When compared with other enteric organisms, *V. cholerae* has a low incidence of R plasmids (Hedges *et al.*, 1977; Prescott *et al.*, 1968). The limited ability of *V. cholerae* to accept and maintain foreign plasmids may be a reflection of the DNases produced by this organism. To date, no suitable and reproducible transformation or transfection system has been developed in *V. cholerae*.

The production of an extracellular nuclease in *S. marcescens* has also been associated with obtaining low yields of plasmid DNA and poor transformability

(Timmis and Winkler, 1973; Winkler, 1968). The isolation of nuclease negative mutants of *S. marcescens* termed *nuc* enabled the preparation of plasmid DNA from this organism in high and reproducible yields, supporting the view that DNA degradation was due to the extracellular nuclease present in lysates of cell suspensions (Timmis and Winkler, 1973). Reid *et al.*, (1982) showed that with inactivation of this nuclease, six of eight *S. marcescens* strains tested could be transformed with the plasmid pBR322. Based on the properties of the *S. marcescens* endonuclease (Eaves and Jeffries, 1963; Nestle and Roberts, 1969b), various heat treatments were incorporated into the transformation protocols in an attempt to inactivate this enzyme (Reid *et al.*, 1982). The optimal conditions for transformation were 65½C for one minute pre-treatment and 6 hours incubation with DNA; longer periods of incubation severely reduced the viability of the cells. It should also be noted that the high transformability of certain *E. coli* strains coincides with the loss of the *E. coli* periplasmic DNase encoded by *endA* (Hanahan, 1983).

The importance of transformation and transfection systems in any bacterial species is seen with the ever-increasing use of recombinant DNA technology to genetically manipulate bacteria. The main interest in *V. cholerae* has of course resided in its potential for vaccine development, either as a modified and attenuated strain, or for the cloning of its protective antigens into heterologous carriers.

The introduction of recombinant DNA molecules into *V. cholerae* has proved to be a tedious process, and this has usually involved the indirect and often inefficient method of bacterial conjugation. With the advent of mobilizable cloning vectors, the process of conjugation has become more efficient in transferring recombinant DNA from one bacterial species to another. The broad host range IncQ/P-4 group plasmids RSF1010 and R300B, isolated from *Salmonella* (Guerry *et al.*, 1974) and the R1162 plasmid of *P. aeruginosa* (Holloway and Krishnapillai, 1975) have been modified to provide additional endonuclease cleavage sites and effective markers such as antibiotic resistance genes, that can be used for the selection of plasmid-carrying transformant bacteria (Bagdasarian *et al.*, 1979; Bagdasarian *et al.*, 1981). These small plasmids of 8-9kb can be transferred among different bacterial strains by mobilization (i.e. are Mob⁺) if conjugal transfer

functions are provided by a coexisting transfer-proficient (Tra⁺) plasmid. This property of the vectors has enabled them to be used for the cloning of DNA from and into almost any Gram-negative bacterial strain (Bagdasarian and Timmis, 1982).

The second group of broad host range plasmids that have been studied extensively are the IncP-1 conjugative plasmids RP1, RP4, RK2 and R68 (Burkardt *et al.*, 1979). A Tra⁻, tetracycline resistant deletion derivative of the RK2 plasmid, pRK290 of 20kb in length has been constructed for use as a cloning vector (Ditta *et al.*, 1980). This vector can be mobilized into a range of Gram-negative bacteria by plasmids that specify the RK2-type plasmid transfer system. The disadvantage of this vector, however, is the low number of suitable cloning sites. More recently, mobilizable cloning vectors utilizing the Mob region of the RP4 plasmids have been constructed (Simon *et al.*, 1983; Simon, 1984). These plasmids are pBR322 and pACYC184 based, with the RP4 mobilization origin cloned into them, and can be efficiently mobilized by plasmids carrying the RP4 transfer (Tra) genes. This Tra region has also been integrated into the *E. coli* chromosome to produce hosts to serve as donors in mobilization (Simon *et al.*, 1983). This latter group of mobilizable plasmids utilize the pMB1 (Bolivar *et al.*, 1977) and P15A replicons (Chang and Cohen, 1978), respectively, and are therefore not as broad in their host range as the previously described mobilizable cloning vectors.

The strategy of using mobilizable cloning vectors, however, does have its disadvantages. The cloning of recombinant DNA, and subsequent manipulation of this DNA is limited to using these vectors, most of which lack the usual large array of unique restriction endonuclease cloning sites present in other cloning vectors. This system also requires the additional conjugation step, and usually requires another plasmid present encoding the necessary transfer functions.

The relatively new technique of transformation by electroporation, whereby living cells are subjected to a rapidly changing high-strength electric field to produce transient pores in their outer membranes to facilitate diffusion of DNA into the cell, has proved successful with larger cells such as plant and mammalian cells (Fromm *et al.*, 1985). Attempts to permeabilize small cells such as bacteria, however, have until recently, met with only limited success (Calvin and Hanawalt, 1988).

The ability to demonstrate transformation in *V. cholerae*, by inactivation of the extracellular DNase(s), would be an important step to facilitate genetic manipulation in this organism.

The importance of the DNase to all these processes can best be established by the construction of *V. cholerae* mutants specifically deficient in extracellular DNase(s).

1.6 Aims of this Study

The study of *V. cholerae* at the molecular level, has led to a dramatic increase in our knowledge of this organism. Attention has focussed mainly on the virulence factors elaborated by *V. cholerae* and their contribution to the infectious process. It is now apparent that this organism depends on a variety of virulence properties rather than any single trait. *V. cholerae* also belongs to a select group of Gram-negative organisms that are capable of secreting a number of proteins into the extracellular environment. While the process of secretion through the cytoplasmic membrane in both prokaryotic and eukaryotic cells is now well understood, the molecular mechanisms for the subsequent correct localization of proteins outside the cell remain largely to be elucidated.

The objectives of this study are to characterize the extracellular DNase(s) at the DNA level, with the aim of developing a transformation system to facilitate genetic manipulation of *V. cholerae*; and to determine the role of the DNase in the pathogenicity of this organism. Finally, the *V. cholerae* DNase provides a model system for the study of secretion of extracellular proteins from a Gram-negative bacterial cell.

CHAPTER 2

MATERIALS AND METHODS

2.1 Growth media

The following nutrient media were used for bacterial cultivation. Nutrient broth (NB) (Difco), prepared at double strength (16 g/litre) with added sodium chloride (NaCl) (5 g/litre) was the general growth medium for *E.coli* K-12 strains. *V.cholerae* strains were grown in Brain Heart Infusion (BHI) (Difco) prepared as directed by the manufacturers. Luria broth (LB) and 2x 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 MgSO₄, 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. DNA-agar consisted of 2% Herring Sperm DNA (Sigma) dissolved in nutrient agar by boiling for 10min. Soft agar contains equal volumes of NB and NA. H agar consisted of bacto-tryptone (16 g/litre) (Difco), NaCl (8 g/litre) and bacto-agar (12 g/litre) (Difco). H top agar was like H agar but also contained 8 g/litre bacto-agar.

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

Incubations were at 37°C unless otherwise specified. Normally, liquid 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. The chemical mutagen, ethyl-methane-sulfonate, was also purchased from Sigma. Caesium chloride (Cabot) was technical grade. Ethylenediamine-tetra-acetic-acid, disodium salt (EDTA) was Analar analytical grade.

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

The following electrophoresis grade reagents were obtained from the sources indicated: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N'-methylene bisacrylamide and urea (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. The substrate 5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal), 5-Bromo-4-chloro-3-indolyl-phosphate (X-pho) and isopropyl- β -D-thiogalactopyranoside (IPTG) were purchased from Boehringer-Mannheim.

M13 sequencing primer and [³²P]-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. [³⁵S]-Methionine (1,270 Ci/mMole) was purchased from Amersham. Phosphorylated *Bam*HI, *Eco*RI, *Hind*III and *Pst*I linkers (8-mer) were purchased from New England Biolabs, Inc., Beverley, Mass. Linkers were obtained in a lyophilized form and resuspended in 0.1 ml of TE buffer, pH 8.0 and stored frozen at -20°C.

2.3 Enzymes

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

Restriction endonucleases *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hind*III, *Kpn*I, *Mlu*I, *Nde*I, *Nru*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sau*3A, *Sma*I, *Taq*I, *Xba*I and *Xho*I were purchased from either Boehringer-Mannheim, New England Biolabs, Pharmacia or Amersham. Nuclease *Bal*31 nuclease was purchased from New England Biolabs.

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

2.4 Maintenance of bacterial strains

All strains were maintained as lyophilized cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate sterile broth. Half the contents were then transferred to a 10 ml bottle of NB and grown with shaking at 37°C for 16 h. The other half was streaked onto two nutrient agar plates and incubated at 37°C for 16 h. Antibiotics were added to the media when appropriate. If the colony form

was uniform, single colonies were selected and picked off plates for subsequent storage or use. Short-term storage of strains in routine use was achieved by 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) followed by incubation at 37°C for 16h just prior to use.

Bacterial strains were prepared for long-term storage by suspension of several colonies in a small volume of sterile skimmed milk. Approximately 0.2 ml aliquots of this thick bacterial suspension were dispensed into sterile 0.25x4 in. freeze drying ampoules and the end of each ampoule was plugged with cotton wool. The samples were then lyophilised in a freeze drier. After the vacuum was released, the cotton wool plugs were pushed well down the ampoule and a constriction 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

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

2.6 Plasmids

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

2.7 Antisera

2.7.1 Antiserum production

Antiserum against the denatured form of the β -lactamase was prepared as follows:

Antiserum to the denatured form of the protein was raised to bands extracted from polyacrylamide gels. This was accomplished by electrophoresing cell periplasmic preparations (10 mg/ml) on a SDS polyacrylamide gel, staining a strip with Coomassie Brilliant Blue to identify the location of the desired band, then aligning this strip with the gel and excising the corresponding location. The strip was washed in distilled water to remove SDS and then homogenized and injected with 1 part to 5 of Span85:Paraffin oil. Rabbits were immunized subcutaneously, by three repeated injections at fortnightly intervals.

2.7.2 Selective absorption of antiserum by intact cells

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

2.8 Transformation procedure

Transformation was performed essentially by the method described by Brown *et al.* (1979). *E.coli* K-12 strains were made competent for transformation with plasmid DNA as follows: an overnight shaking culture (in NB) was diluted 1:20 into BHI and incubated with shaking until the culture reached an OD of 0.6 (4×10^8 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 100mM MgCl₂, centrifuged again and resuspended in a tenth volume of cold 100 mM CaCl₂. 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: 10mM Tris-HCl, 1mM EDTA, pH 8.0) and left on ice for a further 30 min. The cell/DNA mixture was heated at 42°C for 2 min and then 3 ml BHI was added followed by incubation with shaking at 37°C for 1-2 hours. The culture was plated onto selection plates directly or concentrated by centrifugation and plated. Cells with sterile buffer were included as a control.

When transforming the *V.cholerae* DNase negative mutants, *V.cholerae* strains and *E.coli* DH1 were all grown in Luria broth to an OD₆₅₀ of 0.6, washed with 10mls of cold 50mM MgCl₂ and resuspended in 2mls of cold 50mM CaCl₂, and left overnight at 4°C. Dilutions of each culture were plated onto nutrient agar plates as well. The following day, before use, the competent cells were all standardized to a concentration of 1x10⁹ cells/ml (using the 10⁻⁶ dilution of each of the cultures plated onto nutrient agar the night before).

2.9 DNA extraction procedures

2.9.1 Plasmid DNA isolation

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

Method 1: Rapid plasmid preparation by the boiling method of Holmes and Quigley (1981) was performed as follows: Cells from 1 ml of a 10 ml shaking overnight culture were pelleted (this yielded about 10⁹ cells) in an Eppendorf 5414 centrifuge for 30 secs, resuspended in 50 µl STET buffer (5%(w/v) sucrose, 5%(v/v) Triton X-100, 50mM EDTA, 50mM Tris-HCl, pH 8.0). Lysozyme (5mg/ml; 5 µl) was added and the suspension left at room temperature for several min. Samples were then placed in boiling water for 35 secs and immediately spun for 10 min in an Eppendorf centrifuge. The chromosomal pellet was removed and plasmid DNA in the supernatant precipitated with 0.6 volumes of propan-2-ol at -20°C for 10 min. DNA was pelleted by centrifugation in an Eppendorf centrifuge

for 10 min, washed once with 1 ml 70%(w/v) ethanol, dried *in vacuo* and dissolved in 20 μ l 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 50mM Tris-HCl, pH 8.0. Lysozyme (50 μ l, 10 mg/ml freshly prepared in H₂O) and 50 μ l 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 μ l 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 (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM 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 (0.2M 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 CsCl saturated isopropanol. CsCl was then removed by dialysis overnight against three changes of 2 litres TE at 4°C. DNA was stored at 4°C.

2.9.2 Preparation of *V.cholerae* genomic DNA

V.cholerae genomic DNA was prepared according to Manning *et al.* (1986). Cells from a 20 ml shaking overnight culture were pelleted in a bench centrifuge for 10 min and washed once with TES buffer (50mM Tris-HCl, pH 8.0, 5mM EDTA, 50mM NaCl). The pellet was then resuspended in 2 ml 25% sucrose, 50mM Tris-HCl, pH 8.0 and 1 ml lysozyme (10 mg/ml in 0.25M 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, 50mM 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 two extractions each with TE saturated phenol and diethyl ether. The genomic DNA was then precipitated from the solution by the addition of two volumes of cold 95%(v/v) ethanol. The precipitate was washed twice with 70%(v/v) ethanol, dried *in vacuo* for 60 min and dissolved in 1 ml TE buffer. This was achieved by heating to 56°C for several min. This generally yielded high molecular weight DNA at concentrations ranging from 0.5-1.0 mg/ml.

2.10 Analysis and manipulation of DNA

2.10.1 DNA quantitation

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

2.10.2 Restriction endonuclease digestion of DNA

Cleavage reactions of the restriction enzymes *Hind*III, *Bam*HI, *Eco*RI, *Pst*I, *Cl*AI and *Xba*I were performed using SPK buffer (10x: 200mM Tris-HCl, pH 8.0, 50mM MgCl₂, 5mM dithioerythritol, 1mM EDTA, 500mM KCl and 50% glycerol). The remaining restriction digests were carried out as described by Davis *et al.* (1980). 0.1-0.5 μ g of DNA or purified restriction fragments were incubated with 2 units of each restriction enzyme in a final volume of 20 μ l, at 37°C, for 1-2 hours. The reactions were terminated by heating at 65°C for 10 min. Prior to loading onto a gel, a one tenth volume of tracking dye (15%(w/v) Ficoll, 0.1%(w/v) bromophenol blue) was added.

2.10.3 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 hours in TBE buffer (67mM Tris base, 22mM boric acid and 2 mM EDTA, final pH 8.8). After electrophoresis the gels were stained in distilled water containing 2 μ g/ml ethidium bromide. DNA bands were visualized by trans-illumination with UV light and photographed on either Polaroid 667 positive film or 665 negative film.

For preparative gels Sea Plaque (Seakem) low-gelling-temperature agarose at a concentration of 0.6%(w/v) was used for separation of restriction fragments, which were recovered by one of the following methods.

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

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

2.10.4 Isolation of DNA fragments less than 1,000bp

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

2.10.5 Calculation of restriction fragment size

The size of restriction enzyme fragments were calculated by comparing their relative mobility with that of *EcoRI* digested *Bacillus subtilis* bacteriophage SPP1 DNA. The calculated sizes of the SPP1 *EcoRI* 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: 8.0; 7.1; 6.0; 4.78; 3.44; 2.77; 1.93; 1.88; 1.55; 1.43; 1.2; 1.03; 0.7; 0.48.

2.10.6 *In vitro* cloning

DNA to be subcloned (3 μ g) was cleaved in either single or double restriction enzyme digests. This was combined with 1 μ g of similarly cleaved vector DNA, then ligated with 2 units of T4 DNA ligase in a volume of 50 μ l in a final buffer concentration of 20mM Tris-HCl, pH 7.5, 10mM MgCl₂, 10mM dithiothreitol (DTT), 0.6mM ATP for 16 hours at 4°C. The reaction was stopped by heat inactivation of the T4 DNA ligase at 65°C for 10 min. The ligated DNA was then used directly for transformation of strain DH1. Transformants were screened for insertional inactivation of the appropriate drug resistance (Ap or Tc), wherever possible, prior to plasmid DNA isolation.

2.10.7 Generation of deletions using nuclease *BaI*31

DNA (200 μ l) was digested with the appropriate restriction endonuclease and heat inactivated at 65°C for 10 min. The linearized DNA was then digested with *BaI*31 (the digest contained 225 μ l linearized DNA, 30mM Tris-HCl, pH 8.0, 2mM MgCl₂, 12mM CaCl₂, 0.25mM NaCl, 3 units *BaI*31) and samples were taken from the digest at various times (1 min intervals) and the reaction stopped by the addition of 10 μ l GEBS (GEBS: 20%(v/v) glycerol, 50mM EDTA, 0.05%(w/v) bromophenol blue, 0.5%(w/v) sarkosyl). The samples were desalted in 1 ml Sepharose CL-6B

columns, dried *in vacuo* and resuspended in 20 μ l of water. The ends were filled in with Klenow by adding 2.5 μ l of 10x nick translation buffer [10x :0.5M Tris-HCl, pH 7.2, 0.1 M MgSO₄, 1 mM DTT, 500 μ g/ml BSA (Pentax Fraction V), and 1 μ l each of dNTP's (2mM)] and 5 units/ μ l of Klenow and incubating at room temperature for 30 min. The reaction was stopped with 5 μ l GEBS and heated at 65°C for 10 min. The DNA was desalted and dried down as before and the linkers were ligated to the DNA. Linker ligation was performed by adding 10 μ l of 1x linker kinase buffer (10x :0.66M Tris-HCl, pH 7.6, 10mM ATP, 10mM spermidine, 0.1M MgCl₂, 150mM DTT, 2 mg/ml BSA), 3 μ l of phosphorylated linkers and 2 units of T4 DNA ligase and incubated overnight at 4°C. The reaction was stopped with 30 μ l of 1/4 GEBS and heated at 65°C for 10 min. The DNA was desalted and digested with the appropriate restriction endonuclease to cleave the linkers, heated at 65°C for 10 min and then ligated overnight at 4°C. The ligated DNA was transformed into *E.coli* K-12 strain DH1 and the sizes of the deletions were determined by isolating plasmid DNA, digesting with appropriate restriction endonucleases and analyzing the digests on a 0.8%(w/v) agarose gel.

2.10.8 Dephosphorylation of DNA using alkaline phosphatase

0.1-0.5 μ g of digested plasmid DNA was incubated with 1 unit of molecular biology grade alkaline phosphatase for 30 min at 37°C. The reaction was terminated by the addition of EDTA, pH 8.0 to give a final concentration of 3mM followed by heating at 65°C for 10 min. The reaction mix was then extracted twice with hot TE saturated phenol and twice with diethyl ether. DNA was precipitated overnight at -20°C with two volumes of absolute ethanol and 1/10 volume of 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.

2.10.9 End-filling with Klenow fragment

Protruding ends created by cleavage with restriction endonucleases were filled in using the Klenow fragment of *E.coli* DNA polymerase I. Typically, 1 μ g of digested DNA, 2 μ l of 10x nick-translation buffer (Maniatis *et al.*, 1982), 1 μ l of each dNTP (2mM) 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.

2.10.10 End-filling with T4 DNA polymerase

Plasmid DNA was cleaved and cohesive ends converted to blunt ends with T4 DNA polymerase in a final volume of 25 μ l containing 2 μ g DNA, 2 units T4 DNA polymerase, 1 μ l of each dNTP (2mM) and 1 μ l of 10x T4 DNA polymerase buffer (Maniatis *et al.*, 1982). After a 5 min incubation at 37°C, the reaction was stopped by heating at 65°C for 10 min. Salt, unincorporated nucleotides and enzyme were removed by passage through a Sepharose CL-6B column.

2.10.11 Ligation of Linkers to blunt DNA ends

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

2.10.12 Construction of gene banks

In cosmid vector pHC79

Genomic fragments of approximately 40 kilobases (kb) were obtained by controlled partial digestion with the restriction endonuclease *Sau3A* (Maniatis *et al.*, 1982). The cosmid vector used was pHC79 (Hohn and Collins, 1980) which was restricted with *Bam*HI and treated with alkaline phosphatase to prevent self-ligation. The two DNAs were mixed, ligated overnight and packaged *in vitro* into bacteriophage lambda using a Promega Packagene kit. The packaged phage were then used to infect *E.coli* K-12 strain DH1. Cells harbouring cosmid clones were detected by plating onto nutrient media containing Ap. At least 90% of colonies were Tc^S.

2.10.13 Nick translation method

Nick translation reactions with DNA polymerase I were modified from Maniatis *et al.* (1982) and carried out as follows: 25 μ Ci α -[³²P]-dCTP (1,700 Ci/mmol in ethanol) was dried *in vacuo* in an Eppendorf tube, resuspended with 80 μ l water, 10 μ l of 10x nick translation buffer (500mM Tris-HCl, pH 7.2, 100mM MgCl₂, 1mM DTT, 500 μ g/ml BSA) 1 μ l each of 2mM dATP, dGTP, dTTP. DNA (1 μ g) was added and incubated at 37°C for 10 min. DNA polymerase I (5 units) was added to the mix and allowed to incubate at 16°C for 2 hours. [³²P]-labelled DNA was separated from unincorporated label by centrifugation through a mini-column of Sepharose CL-6B.

2.10.14 Southern transfer and hybridization

Bidirectional transfers of DNA from agarose gels to nitrocellulose paper (Schleicher and Schll) were performed as described by Southern (1975) and modified by Maniatis *et al.* (1982).

Prior to hybridization with radio-labelled probe, filters were incubated for 4 hours at 44°C in a pre-hybridization solution containing 50%(v/v) formamide, 50 mM sodium phosphate buffer, pH 6.4, 5 x SSC (0.34M NaCl, 75mM sodium citrate, pH 7.0), 5x Denhardt's reagent and 83 µg/ml single stranded Herring Sperm DNA (Sigma) (Maniatis *et al.*, 1982). Pre-hybridization fluid was discarded and replaced with fresh hybridization buffer (as for pre-hybridization solution, with the exclusion of Herring sperm DNA). Denatured probe (approximately 10⁶ cpm/µg) was added and hybridization allowed to occur for 16-24 hours at 44°C.

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

2.10.15 Kinasing single stranded DNA

Single stranded DNA (primers) were kinased using gamma-[³²P]-dATP. The reaction mix consisted of 100mM DTT, 1µl 10X kinase buffer, 3 units of Polynucleotide kinase, 10µl of gamma-[³²P] and 60µg of primer. This reaction mix was made up to 10µl in water and incubated at 37°C for 30min. The 10X kinase buffer consisted of 500mM Tris pH7.4 and 100mM MgCl₂.

2.11 Isolation and analysis of RNA

2.11.1 Isolation of cellular RNA

Total cellular RNA, from *V. cholerae*, or *E. coli* was isolated by a modification of the methods of Aiba *et al.* (1981) and Glisin *et al.* (1974). An overnight shaking culture was diluted 20-fold (in nutrient media, as indicated), and incubated with shaking, until the culture reached an O.D.₆₅₀ = 0.8. Sodium azide (NaN₃) was

added to a final concentration of 15mM, and the cells were chilled on ice for 10 min. Cells were harvested by centrifugation (7000 RPM, 10 min., 4°C, SS34, Sorvall) and resuspended in 3 ml 0.02M Sodium acetate pH 5.5, 0.5% SDS, 1mMEDTA, 10 mMNaN₃. 3 ml. phenol (equilibrated in 0.02M sodium acetate, pH 5.5) was added and the mixture incubated at 60°C for 5 min. with gentle shaking. After briefly spinning in a bench centrifuge, to separate the phases, the aqueous phase was re-extracted with phenol. Nucleic acid was precipitated from the aqueous phase by the addition of 3 volumes ethanol and chilling at -70°C for 30 min., followed by centrifugation (15000 RPM), 30 min., 4°C, SS34, Sorvall). The pellet was resuspended in 3 ml. 0.02M sodium acetate pH 5.5, 0.5% SDS, 1mM EDTA, reprecipitated, and recentrifuged, as above. The final precipitate was redissolved in 4.5 ml. 0.1 M EDTA pH8.0, with an equal volume of 7.5M Caesium chloride in 0.1mM EDTA pH 8.0, layered onto 2.5 ml. of 5.2M Caesium chloride in a Beckman 9/16" x 3 1/2" polyallomer tubes and centrifuged at 3000 RPM, for 18 hours, at 20°C in an SW-41 rotor in a Beckman L8-80 ultracentrifuge. The resulting pellet of RNA was dissolved in 200µl 0.1M EDTA pH 8.0, precipitated twice using one tenth volume of 3M sodium acetate pH 4.5 and two and a half volumes of ethanol, washed with 70% ethanol, and resuspended in 200 µl 0.1M EDTA pH 8.0. Samples were stored at -70°C.

2.11.2 Electrophoresis of RNA fragments

Polyacrylamide gel electrophoresis

In vitro produced RNA was separated from unincorporated label by electrophoresis through 6% acrylamide/9M urea. Gels were prepared using glass plates 16.5 cm x 22 cm and spacers and combs of high density polystyrene 0.5 mm thick. Gels were pre-electrophoresed for 30 min at 500 V constant voltage for 2 1/2 hours, by which time the bromophenol blue dye front had reached the bottom of the gel. The electrophoresis buffer contained 89mM Tris base, 89mM boric acid and 2.5mM EDTA pH8.3, and samples were loaded in a formamide/dye mixture as described by Sancar *et al.* (1982).

After electrophoresis, the glass plates were separated by heating one side of the

gel with hot running water, the gel generally binding to the cold plate. The gel was covered in plastic wrap and placed on X-ray film for autoradiography, for 16-24 hours at room temperature, without the use of intensifying screens.

2.11.3 Primer Extension Analysis of RNA

The RNA to be extended (20 μ g) was mixed with 10ng of kinased primer, and made up to 100 μ l by the addition of 5 μ l 4M NaCl and water. This was precipitated by the addition of 300 μ l of ethanol and dried *in vacuo*. The pellets were resuspended in 10 μ l hybridization buffer, spun down and heated to 75°C for 3min. This mix was then spun down and incubated at 42°C for 60min. The hybridization mix was then spun down and 24 μ l of extension mix added. 10 units of AMV reverse transcriptase was added and incubated at 42°C for 60 min. The reaction mix was ethanol precipitated by the addition of 3.0 μ l of 3M NaAc pH5.5 and 100 μ l ethanol. The pellets were then resuspended in 2 μ l of water and 3 μ l of formamide loading buffer, and boiled at 100°C before loading. The extension product was run on a 6% polyacrylamide gel with a sequencing reaction used as a marker. The hybridization mix consisted of 10mM Tris pH 8.3 and 200mM KCl made in water. The extension mix consisted of 10mM Tris pH8.3, 14mM MgCl₂, 14mM DTT and 700 μ M of each deoxynucleotide. Formamide loading buffer was made up as 95% deionized formamide and 100mM NaOH with 0.1% bromophenol blue.

2.12 Mutagenesis

2.12.1 Chemical mutagenesis

The chemical mutagen ethyl methanesulfonate (EMS) was used in an attempt to mutagenize the *V.cholerae* chromosome. A culture of *V.cholerae* was grown to late exponential phase in Luria broth, and one volume of this culture was mixed with an equal volume of freshly prepared stock solution of EMS (0.1ml of EMS in

2.5ml of minimal medium warmed to 37°C). This mixture was aerated on a shaking incubator at 37°C for 2 hours. The culture was then diluted 10-fold into fresh minimal medium, and allowed to grow out for several hours followed by plating for single colonies onto nutrient agar. *V.cholerae* cells were patched onto DNA-agar and duplicated onto nutrient agar plates. Mutants defective for DNase production were screened by looking for colonies without a zone of clearing on DNA-agar plates after the addition of 1N HCl.

2.12.2 Transposition with Tn1725

Tn1725 (Cm^R) transposition to plasmid DNA was performed in the following manner: Plasmid pRU669 (R_{ts}1::Tn1725) (Ubben and Schmitt, 1986) was transferred by conjugation, into an *E.coli* K-12 derivative harbouring the target plasmid. This was achieved by mating for 3 hours at 30°C in a standing culture which consisted of 0.1 ml of an overnight culture of C600 [R_{ts}1::Tn1725] with 0.9 ml of the culture to be mutagenized and 1 ml BHI broth. Following plating of 0.1 ml of mating mix on NA containing Cm and Ap, independent exconjugants were purified and used for growing up an overnight cultures at 37°C in NB containing both antibiotics to select for the transposon (Cm) and the plasmid (Ap). Triton X-100 lysates (10 ml) prepared from these cultures were used to transform C600, again selecting for both the plasmid and the transposon. Following overnight incubation at 37°C, transformants were randomly chosen for analysis of their plasmid DNA.

2.12.3 Transposition with TnphoA

The plasmid pRT291 was used which carried TnphoA on pRK290. The mobilizing strain SM10 (Simon *et al.*, 1983) was used to transfer pRT291 by conjugation into *V.cholerae* selecting for exconjugants by means of the Sm^R of *V.cholerae*. The strain was then superinfected with pH1JI, selecting for Sm^R, Gm^R and Km^R. Colonies that came up were Tc^S because of the loss of pRT291 due to its incompatibility with pH1JI, yet retained Km^R because TnphoA had transposed from pRT291. X-pho (40µg/ml) was incorporated into the selection plates at the

last step, with colonies producing an active, exported hybrid protein coming up as blue colonies. Blue colonies were only detected after 48 hours incubation at 37°C.

2.12.4 Oligonucleotide site-directed mutagenesis

Oligonucleotide mutagenesis was carried out using the pMa/c phasmid vector by the gapped duplex DNA method (Stanssens *et al.*, 1986). The desired gene (DNase) to be mutagenized was cloned into the *EcoRI* site of the pMc-type phasmid and s/s DNA was isolated in a similar manner to that described for M13 s/s DNA. A derivative of M13 was used which preferentially packaged this phasmid DNA. This derivative, M13K07, was obtained from Pharmacia. The phasmid pMa5-14 was digested with *EcoRI* and 0.1 pmole of this DNA was mixed with 0.5 pmole of the s/s DNA. To this mix 5 μ l of 1.5M KCl/100mM Tris-HCl, pH7.5 and the volume adjusted to 40 μ l with H₂O. The reaction was incubated at 100°C for 4min., and then placed at 65°C for 5min. and allowed to cool to RT.

For the gap-filling and sealing reactions, 8 μ l of the hybridization mixture and 8 pmoles of the phosphorylated mutagenic oligonucleotide were placed in an Eppendorf tube, The mixture was heated to 65°C for 5min. and then allowed to cool to RT. To this, 24 μ l H₂O and 4 μ l 10X fill-in buffer (625mM KCl, 275mM Tris-HCl, 150mM MgCl₂, 20mM DTT, 0.5mM ATP and 0.25mM of the four dNTP's, pH7.5), 1 μ l T4-DNA ligase and 1 μ l DNA polymerase (Klenow) was added, mixed and incubated for 45min. at RT. This mixture was stored on ice until it was used for transformation.

The filled-in gdDNA was transformed into the *E.coli* strain WK6mutS. For selection of transformants, the antibiotic (Ap) to which resistance was encoded by the gapped strand was used. A 100 μ l aliquot of the transformation mixture was used to inoculate a 10ml broth supplemented with Ap. The following day, phasmid DNA was prepared from this culture. The mixed phasmid DNA population obtained was allowed to segregate by transforming the su⁻ strain WK6 and selecting for Ap. Phasmid DNA was isolated from this strain and screened for the mutation.

2.13 Protein analysis

2.13.1 Minicell procedures

Minicells were purified and the plasmid-encoded proteins labelled with [³⁵S]-methionine as described by Kennedy *et al.*, (1977) and modified by Achtman *et al.* (1979). This involved separation of minicells from whole cells (500 ml overnight culture in LB medium) by centrifugation through two successive sucrose gradients, pre-incubating to degrade long lived mRNAs, then pulse labelling with [³⁵S]-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).

2.13.2 Bacterial cell-free coupled transcription-translation

The prokaryotic DNA-directed translation kit was obtained from Amersham, and this system was essentially as described by DeVries and Zubay (1967), and modified by Collins (1979). Reactions were carried out according to manufacturers specifications.

2.13.3 SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 11-20% gradients for proteins using a modification of the procedure of Lugtenberg *et al.* (1975) as described previously by Achtman *et al.* (1978). Samples were heated at 100°C for 3 min prior to loading. Gels were generally electrophoresed at 100 V for 5 hours (11-20% gradient gels). Protein staining was achieved by incubation, with gentle agitation overnight at room temperature in 0.06%(w/v) Coomassie Brilliant Blue G250 (dissolved in 5%(v/v) perchloric acid). Destaining was accomplished with several changes of 5%(v/v) acetic acid, with gentle agitation for 24 hours.

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

2.13.4 Silver Staining

The modified procedure of Morrissey, (1981), was used to detect proteins in polyacrylamide gels. Following electrophoresis, the gel was pre-fixed in 50% methanol, 10% acetic acid for 30min., and 5% methanol, 7% acetic acid for a further 30min. The gel was fixed for 30min. in 10% glutaraldehyde (Univar) and rinsed with several changes of deionized H₂O for 2 hours. The gel was soaked for 30min. in 5 μ g/ml DTT and 0.1% silver nitrate was added after rinsing. After 30min. the gel was rinsed with deionized H₂O and developer added. The developer (50 μ l 37% formaldehyde in 100ml 3% sodium carbonate) was changed twice and left until the desired level of staining was attained. Staining was stopped by adding 5ml 2.3M citric acid directly to the developer and agitating for 10min.

2.13.5 Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60°C for 2 hours on a Bio-Rad gel drier. [³⁵S]-methionine autoradiography was performed at room temperature for 1-7 days without intensifying screens using Kodak XR-100 film. For autoradiography with [³²P]-phosphate labelled DNA, the gels were exposed to film for 6-72 hours at -70°C, using intensifying screens.

2.13.6 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 (50ml, OD₆₅₀ of 0.6). Cells were pelleted in a Sorvall SS-34 rotor, (10,000rpm for 10 min. at 4°C) and resuspended in 1ml of 20% sucrose, 30mM Tris-HCl pH8.1, transferred to SM-24 tubes and chilled on ice. Cells were converted to sphaeroplasts with 0.1ml of 1mg/ml lysozyme in 0.1M EDTA pH7.3 for 30min. 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 3ml 3mM EDTA pH7.3. Cells were lysed by 60, 1s bursts with a Branson ultrasonifier. Unlysed cells and large cell debris were removed by low spin centrifugation (5,000rpm, 5min. at 4°C). The supernatant containing the membranes and the cytoplasm was centrifuged at 35,000rpm 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, 10mM Tris-HCl pH7.8, 1mMEDTA. 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 0.1ml of pH6.8, 10mM sodium phosphate buffer. Proteins present in the culture supernatant (extracellular fraction) were precipitated by the addition of ammonium sulphate to a concentration of either 40% or 80% saturation at 4°C. After centrifugation, the protein pellet was dissolved in 40mM Tris-HCl pH7.5, 2mM MgCl₂ and then dialysed against multiple changes of the same buffer.

2.13.7 Whole cell preparation

1 ml of an overnight culture (1×10^9 cells) was placed in a microfuge tube and the cells were collected by centrifugation (30 secs, Eppendorf 5414). The cell pellet was resuspended in 100 μ l 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.

2.13.8 Western transfer and protein blotting

The procedure used was a modification of that described by Towbin *et al.* (1979). Samples were subjected to SDS-PAGE (11-20% gradient gels) and transferred to nitrocellulose (Schleicher and Schill) at 200 mA for 2 hours in a Trans-Blot Cell (Biorad). The transfer buffer used was 25mM Tris-HCl, pH 8.3, 192mM 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, 20mM Tris-HCl, 0.9%(w/v) NaCl) to block non-specific protein binding sites. Antiserum was diluted 1/1000 in TTBS, 0.02%(w/v) skim milk powder (unless stated otherwise) and incubated with gentle agitation at room temperature for 2-16 hours. The antibody was removed by washing the nitrocellulose sheet three times for 10 min in TTBS with shaking. Detection of bound antibody was achieved by incubating for 2-16 hours (gentle agitation) with goat anti-rabbit IgG coupled with horseradish peroxidase (Nordic Immunology) at a dilution of 1/5000 in TTBS plus 0.2%(w/v) skim milk powder. The nitrocellulose sheet was then washed four times (5 min intervals) with TTBS, followed by two 5 min washes in TBS (20mM Tris-HCl, 0.9%(w/v) NaCl). To detect the presence of the antigen-antibody complexes peroxidase substrate (9.9 mg 4-chloro-1-naphthol dissolved in 3.3 ml -20°C methanol added to 16.5 ml TBS containing 15 μ l hydrogen peroxide) was then added and allowed to incubate 10-15 min with shaking, as described by Hawkes *et al.* (1982).

2.14 M13 cloning and sequencing procedures

2.14.1 Preparation of M13 replicative form (RF) DNA

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

2.14.2 Cloning with M13mp18 and M13mp19

The M13 vectors, M13mp18 and M13mp19 (Messing and Vieira, 1982) were used for selective cloning of restriction enzyme generated DNA fragments. Stocks of M13 vectors cleaved with various enzyme combinations were stored at 4°C, after heat inactivation of enzymes. Plasmid DNA was cut with the appropriate enzyme combinations for subcloning into the M13 vectors. The ligation conditions used for blunt ends and cohesive ends were identical. The reaction mixtures consisted of the DNA to be cloned (100 ng) and the DNA vector (20 ng) in a final volume of 10 μ l of ligation buffer. Ligation with T4 DNA ligase was carried out overnight at 4°C.

2.14.3 Transfection of JM101

Strain JM101 was made competent for transformation as described in section 2.8. 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 μ l), 100 mM IPTG (40 μ l) and 2%(w/v) X-gal in N, N'-dimethyl formamide (40 μ l) 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.

2.14.4 Screening M13 vectors for inserts

White plaques were picked from X-gal, IPTG plates with sterile toothpicks and added to 1 ml 2x TY broth in microfuge tubes containing a 1:100 dilution of an overnight culture of JM101. These tubes were incubated for 5 hours at 37°C. The cells were pelleted by centrifugation (30 secs, Eppendorf) and 0.1 ml of supernatant was used to 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 method (section 2.9.1). After restriction enzyme digestion, DNA was examined on 1%(w/v) agarose gels.

2.14.5 Purification of single-stranded template DNA

M13 RF DNA containing appropriate inserts were reintroduced into JM101 and single white plaques from this transfection picked with sterile tooth picks to inoculate 2 ml 2 x TY broth containing 20 μ l of an overnight culture of JM101. After vigorous shaking at 37°C for 6 hours, the culture was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was transferred to clean tubes and recentrifuged for 5 min. A 1 ml aliquot of the supernatant from each tube was withdrawn and mixed in a fresh tube with 0.27 ml 20%(w/v) PEG, 2.5 M NaCl. These tubes were then incubated at room temperature for 15 min. The phage were pelleted by centrifugation for 5 min in an Eppendorf 5414 centrifuge and the supernatant discarded. Following another short spin (10 sec), the remainder of the PEG/NaCl supernatant was removed with a drawn out pasteur pipette. The pellets were 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 was added 6 μ l of 3 M sodium acetate, pH 5.0 and 400 μ l absolute ethanol. Single-stranded DNA was precipitated at -20°C overnight, followed by centrifugation for 15 min in an Eppendorf centrifuge. DNA pellets were washed once with 1 ml 70%(v/v) ethanol followed by centrifugation. After drying *in vacuo* the pellets were resuspended in 25 μ l TE buffer and stored at -20°C until required.

2.14.6 Dideoxy sequencing protocol

The method is based on that described by Sanger *et al.* (1977, 1980). Stock solutions of the four dNTPS and ddNTPs were 10mM 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 (0.1mM), ddCTP (0.1mM), ddGTP (0.3mM) and ddTTP (0.5mM). The deoxynucleotide mixes (A, C, G, T) were made for each of the four sequencing reactions, with [³²P]-dCTP, as follows:

Components	Mixes			
	A°	C°	G°	T°
0.5mM dATP	4 μ l	40 μ l	40 μ l	40 μ l
0.5mM dCTP	--	5 μ l	--	--
0.5mM dGTP	40 μ l	40 μ l	6 μ l	40 μ l
0.5mM dTTP	40 μ l	40 μ l	40 μ l	6 μ l
10xTE buffer	10 μ l	10 μ l	10 μ l	10 μ l

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 μ l	7 μ l	7 μ l	7 μ l
ddNTP	14 μ l	14 μ l	14 μ l	14 μ l

These were stored at -20°C until required for later use in sequencing reactions. The annealing of synthetic primer to template was achieved by incubating 6 μ l template, 1 μ l M13 primer, 1 μ l 10 x TM buffer (100mM Tris-HCl, pH 8.0, 50mM MgCl₂) and 2 μ l 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 μ l of annealed DNA. 5 μ Ci 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 μ l) was aliquoted into each of four tubes (one for each sequencing reaction) containing 2 μ l of annealed DNA. To the side of each tube was added 2 μ l Klenow fragment (0.125 units/ μ l TM buffer). These components were simultaneously brought together by a brief spin in an Eppendorf 5414 centrifuge and the reaction mixes incubated at 37°C for 13 min. Chase solution (2 μ l), consisting of 0.25 mM of each dNTP and 0.025 units

Klenow/ μ l, was added to the side of each tube and the chase reaction started by another brief spin. After 15 min at 37°C, 4 μ l formamide dye mix (95%(w/v) formamide, 0.1%(w/v) xylene cyanol, 0.1%(w/v) bromophenol blue, 10mM 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 μ l loaded onto 6% polyacrylamide denaturing gels (see below). For re-running, these samples were boiled for 60 secs prior to loading.

2.14.7 Double stranded sequencing

Plasmid DNA (2-4 μ g/ml) was diluted to a volume of 18 μ l with water. The DNA was denatured by the addition of 2 μ l of 2M NaOH and incubated for 5min. at RT. To this mix, 8 μ l of 5M ammonium acetate pH7.5 was added with 100 μ l 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 μ l of water. The DNA had 2 μ l of sequencing buffer (1X TM) and 1 μ l of primer added (0.5 pmol), and was heated to 65°C for 2min. and allowed to slowly cool to RT. The labelling and termination reactions were run exactly as described for M13 single stranded template DNA.

2.14.8 DNA sequencing gels

Polyacrylamide gels for DNA sequencing were prepared using glass plates 33x 39.4 cm and 33x 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 (89mM Tris base, 89mM boric acid, 2.5mM EDTA, pH 8.3), 420 μ l 25% ammonium persulfate and 11064 μ l TEMED (N,N,N',N'-tetramethyl-ethylene-diamine, Sigma). After thorough mixing this gel mix was poured into a clean gel sandwich and the comb inserted. Polymerization took place for 60 min at 37°C, with the gel in a horizontal position. The gel was mounted onto the sequencing apparatus and a waterjacket was attached to the outside plate of the gel. This consisted of a plastic bag wedged between two 0.3 cm thick spacers and a third plate. The plastic bag was filled with

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 using a constant voltage (700 V) for 15 min, which was increased to 1200 V (33 mA). After 4 hours the samples were reloaded into a second set of wells on the same gel. The gel was further electrophoresed, initially at 700 V, then 1200 V for 2.5 hour by which time the bromophenol blue dye front from the second loading, had reached the bottom of the gel. Plates were separated by heating one side of the gel with hot running water. Generally the polyacrylamide gel bound to the cold plate. Tissue paper was used along the borders of the gel to hold it to the plate during the fixation procedure which involved slowly washing the gel using 1.5 litres of 10%(v/v) acetic acid, 20%(v/v) ethanol in a 60 ml syringe. The gel was then dried at 100°C for 20 min. Plastic wrap was used to cover the gel before placing on film for autoradiography. Autoradiography was performed at room temperature, without the use of intensifying screens, for 16-24 hours.

2.14.9 Analysis of DNA sequences

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

2.15 Construction of protein fusions

The method described by Broome-Smith and Spratt (1986) was used to construct protein fusions of the DNase to β -lactmase. The vector pJBS633 used in this method was kindly supplied by Dr.J.Broome-Smith. Single stranded DNA for sequencing was packaged by the f1 phage variant IR1 (supplied by J.Broome-Smith) and isolated as described for M13 s/s templates. The oligo-deoxynucleotide (5'-CTCGTGCACCCAACTGA-3') to the mature β -lactamase protein was synthesized on an Applied Biosystems Model 381A DNA synthesizer.

Sequencing reactions with these fusion proteins were carried out as described for M13 dideoxy sequencing.

2.16 Animal experiments

2.16.1 Infant mouse cholera model

This method was first described by Ujiye *et al.* (1968) and was used to assess the virulence of *V.cholerae* strains. Infant mice were used at five to six days of age (weight, 2.4-2.7 g) and were removed from their parents about 6 hr before use, to permit the emptying of stomach contents. Mice (groups of 8) received 0.1 ml of bacterial suspension that was administered orally by means of a smooth-tipped hypodermic needle. After challenge, the mice were kept on tissue paper in plastic containers at 25°C. Forty-eight hours after challenge, the number of mice surviving within each group was noted.

Table 2.1: *Vibrio cholerae* strains

Strain	Biotype/Serotype	Genotype/Phenotype	Source
1621	El Tor Ogawa		J.E. Ogg
V62	El Tor Ogawa	<i>met, lys, rif</i>	J.E. Ogg
O17	El Tor Ogawa	Sm ^R	K. Bhaskaran
GN7007	EITor Ogawa	<i>spc201,met,trp::</i> Tn1	R.K. Holmes
MAK757	El Tor Ogawa	<i>leu, trp</i>	D. C. Deb
569B	Classical Inaba	Sm ^R , non-motile	K. Bhaskaran
CA401	Classical Inaba		C. Parker
154	Classical Inaba		D. C. Deb
RV69	Classical Ogawa	P ⁺ , <i>arg-1, his-1,</i> <i>ilv-1</i>	C.Parker
<i>V.cholerae</i> non-01			lab. stock
<i>V.costiculus</i>			lab. stock
<i>V. fluvialis</i>			lab. stock
<i>V.parahaemolyticus</i>			lab. stock
<i>V.mimicus</i>			lab. stock
<i>Aeromonas hydrophila</i> AE-18			lab. stock
<i>Aeromonas hydrophila</i> Th-4			lab. stock
<i>Aeromonas hydrophila</i> AH-1			lab. stock
<i>Aeromonas hydrophila</i> AH-2			lab. stock

Table 2.2: *Escherichia coli* strains

Strain	Genotype/Phenotype	Source/Reference
DH1	F ⁻ , <i>gyrA-96, recA-1, relA-1, endA-1, thi-1, hsdR-17, supE-44, lambda</i> ⁻	B. Bachmann
DS410	F ⁻ , <i>minA, minB, rpsL</i> ,	D. Sherratt
JM101	F'[<i>traD-36, proA,B, lacI^q, lacZ, ΔM15</i>], <i>supE, Δ[lac - proA,B], supE, thi -1</i>	A. Sivaprasad
C600	F ⁻ , <i>thr-1, leu-6, tonA-1, lacY-1, supE-44, thi-1</i>	J. Reeve
S17-1	RP4-2-Tc::Mu-Km::Tn7/ <i>pro, hsdR</i> U. Prierer	
SM10	RP4-2-Tc::Mu <i>thi, thr, leu, supE</i> U. Prierer	
MM294	<i>pro, endA-1, hsdR, supF</i> ,	B. Bachmann
WK6	Δ(<i>lac-proAB</i>), <i>galE, rpsL/F' lacI^q, ZΔM15, proA⁺B⁺</i>	W. Kramer
WK6mutS	Δ(<i>lac-proAB</i>), <i>galE, rpsL, mutS215::Tn10/F' lacI^q, ZΔM15, proA⁺B⁺</i>	R. Zell

Table 2.3: Plasmids and cloning vectors

Plasmid	Antibiotic marker	Reference
pBR322	Ap, Tc	Bolivar <i>et al.</i> , (1977)
pRK290	Tc	Ditta <i>et al.</i> , (1980)
pHC79Ap,	Tc	Hohn and Collins(1980)
pRU669	R _{ts} 1::Tn1725/Cm,Km	Ubben and Schmitt (1986)
pSUP205	Cm, Tc	Simon <i>et al.</i> , (1983)
pUC18	Ap	Vieira and Messing (1982)
pUC19	Ap	Vieira and Messing (1982)
M13mp18		Messing and Vieira (1982)
M13mp19		Messing and Vieira (1982)
pPH1Jl	Gm, Sp, Sm	Ruvkun <i>et al.</i> , (1982)
pJBS633	Km, Tc	Broome-Smith (1986)
pACYC184	Cm, Tc	Chang and Cohen (1978)
pBG50	Cm	Newland <i>et al.</i> , (1985)
pEG612	Ap	A. Brumby (pers. comm.)
pMa5-14	Ap	
pMc5-14	Cm	Stanssens <i>et al.</i> , (1986)

CHAPTER 3

Molecular Cloning and Characterization of the Deoxyribonuclease of *Vibrio cholerae*

3.1 Introduction

Vibrio cholerae produces a number of extracellular proteins. These include proteases, haemagglutinins, haemolysins, cholera toxin and deoxyribonucleases. Many of these extracellular proteins have been implicated as important virulence factors in *V.cholerae* (Schneider and Parker, 1978; Finkelstein and Hanne, 1982; Finkelstein, 1972; Manning, 1987). One of these extracellular proteins, the DNase, is of particular interest not only in the role it may play in the pathogenesis of the disease, but also in its involvement as a barrier to either transformation or transfection in *V.cholerae*, presumably due to degradation of the exogenously added DNA. Poor transformability associated with the production of an extracellular DNase has been described in *Serratia marcescens* (Timmis and Winkler, 1973; Reid *et al.*, 1982).

As the DNase belongs to this class of exported or extracellular proteins, it also provides a good working model for the analysis of proteins exported from Gram-negative bacteria. This chapter describes the cloning and molecular characterization of the gene encoding this enzyme, and the identification of its protein product.

3.2 Results

3.2.1 Cloning of the extracellular DNase of *Vibrio cholerae*

A cosmid gene bank derived from *V.cholerae* 017 was constructed using the vector pHC79 (Hohn and Collins,1980). Vector DNA was digested with *Bam*HI inactivating the Tc^R gene, and ligated to the genomic DNA partially digested with *Sau*3A. This was followed by *in vitro* packaging and transfection into the *E.coli* K-12 strain DH1, selecting for Ap^R colonies. 550 colonies (all Tc^S) were patched onto DNA agar containing Ap, and replicated onto nutrient agar containing Ap. After incubation overnight at 37°C, the DNA plates were then flooded with 1N HCl, and zones of hydrolysis due to DNase activity were able to be detected as regions of clearing in the precipitated DNA. One clone capable of DNA hydrolysis was detected amongst the 550 Ap^R,Tc^S clones (Figure 3.1). This colony was purified from the duplicate plate, and the plasmid in this strain was designated pPM1201. This plasmid contained a DNA insert of about 35 kb, and *E.coli* K-12 DH1 cells re-transformed with this plasmid retained DNase activity.

3.2.2 Subclones of pPM1201

Deletion derivatives of the plasmid pPM1201 were constructed by partial restriction endonuclease digestion followed by dilution and ligation. The restriction enzymes used were *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I (Figure 3.2). One deletion derivative pPM1202, still producing the DNase after digestion with *Eco*RI, contained the smallest fragment of *V.cholerae* DNA (Figure 3.3). Interestingly, the 1.55 kb *Eco*RI fragment in pPM1202 is not present in the original cosmid clone (see below). This is presumably due to an *Eco*RI site being introduced during the partial restriction endonuclease digestion and ligation procedure, or a deletion occurring within a larger *Eco*RI fragment; and the *Eco*RI site of this smaller DNA fragment ligating to the *Eco*RI site of the pHC79 vector. However, further deletion of the 1.55 kb cloned *V.cholerae* DNA gave rise to a 1.25 kb *Eco*RI/*Hind*III fragment present in the plasmid pPM1207 (see Figure 3.4).

Figure 3.1 Detection of the cloned DNase gene.

A nutrient agar plate containing Ap and 0.2% DNA (Sigma-Herring Sperm) was used to screen for DNase positive clones. Colonies represent *E.coli* DH1 with cosmid clones of *V.cholerae* 017 DNA. After overnight growth the DNA-agar plate was flooded with 1N HCl, precipitating the DNA. The colony showing a zone of clearing due to hydrolysis of the DNA, was identified as containing the DNase gene of *V.cholerae*. The arrow indicates the original cosmid clone DH1[pPM1201].

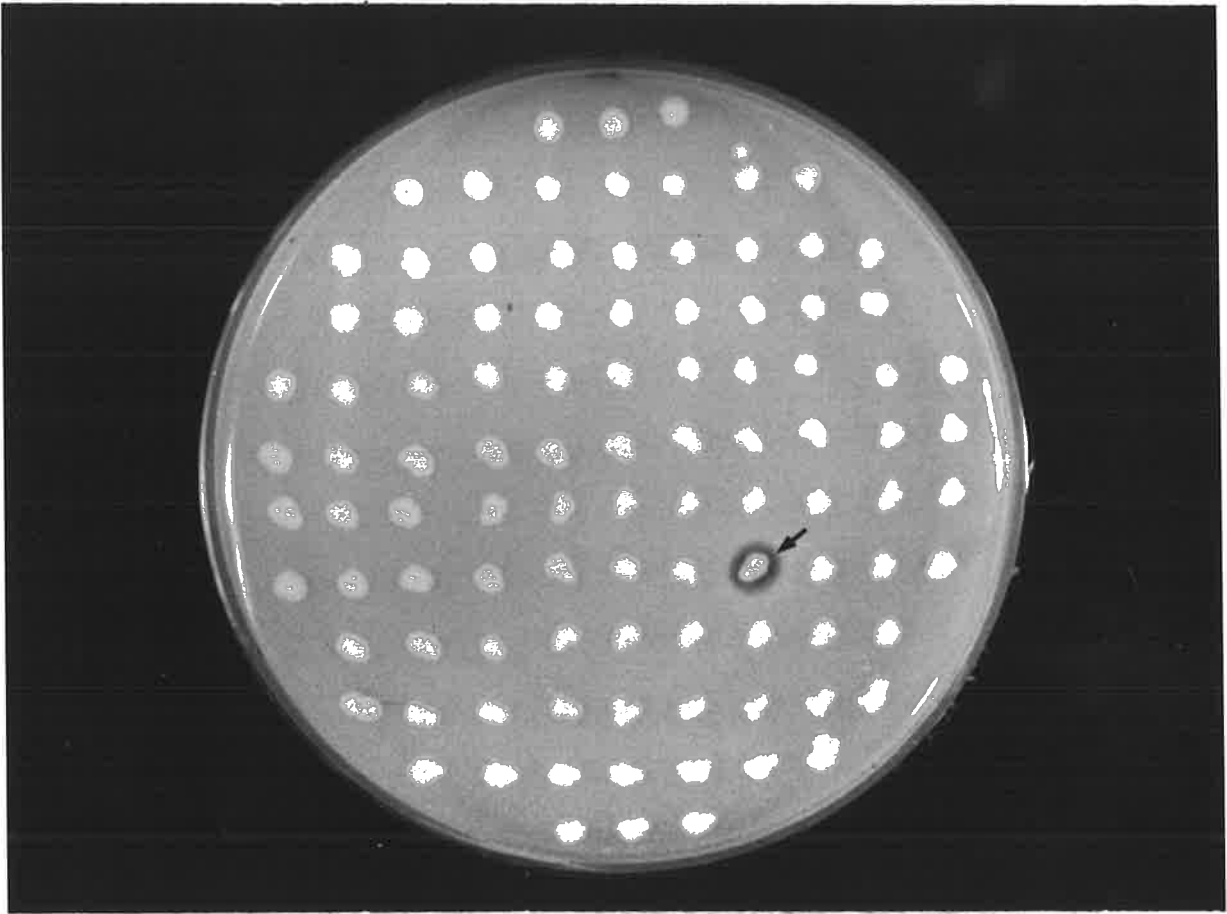
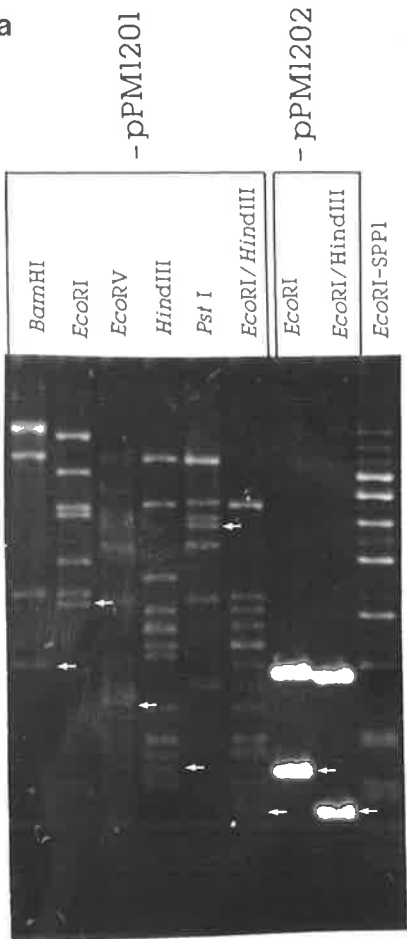


Figure 3.2 Southern hybridization analysis of the DNase positive plasmid pPM1201, and its deletion derivative pPM1202.

(a) DNA was digested with the restriction endonucleases indicated and electrophoresed on an agarose gel (0.8%). After transfer to a nitrocellulose filter, the block was probed with α -[³²P]-dCTP labelled *EcoRI/HindIII* fragment of pPM1202. The agarose gel shows that the 1.55 kb *EcoRI* DNA fragment of pPM1202 is not present in the cosmid clone pPM1201. The 1.25 kb *EcoRI/HindIII* DNA fragment is present in both the cosmid clone pPM1201 and pPM1202.

(b) The corresponding autoradiograph shows the *EcoRI* DNA fragment of pPM1202 to be present as a 3.55 kb *EcoRI* band in pPM1201, while the 1.25 kb *EcoRI/HindIII* fragment is present in both pPM1201 and pPM1202. The arrows indicate the restriction fragments on the cosmid clone which contain the *EcoRI/HindIII* DNA fragment encoding the DNase gene.

a



b

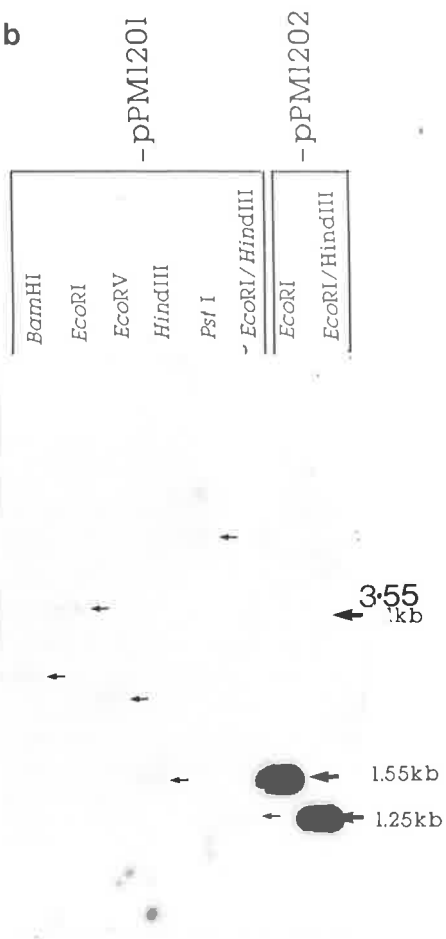
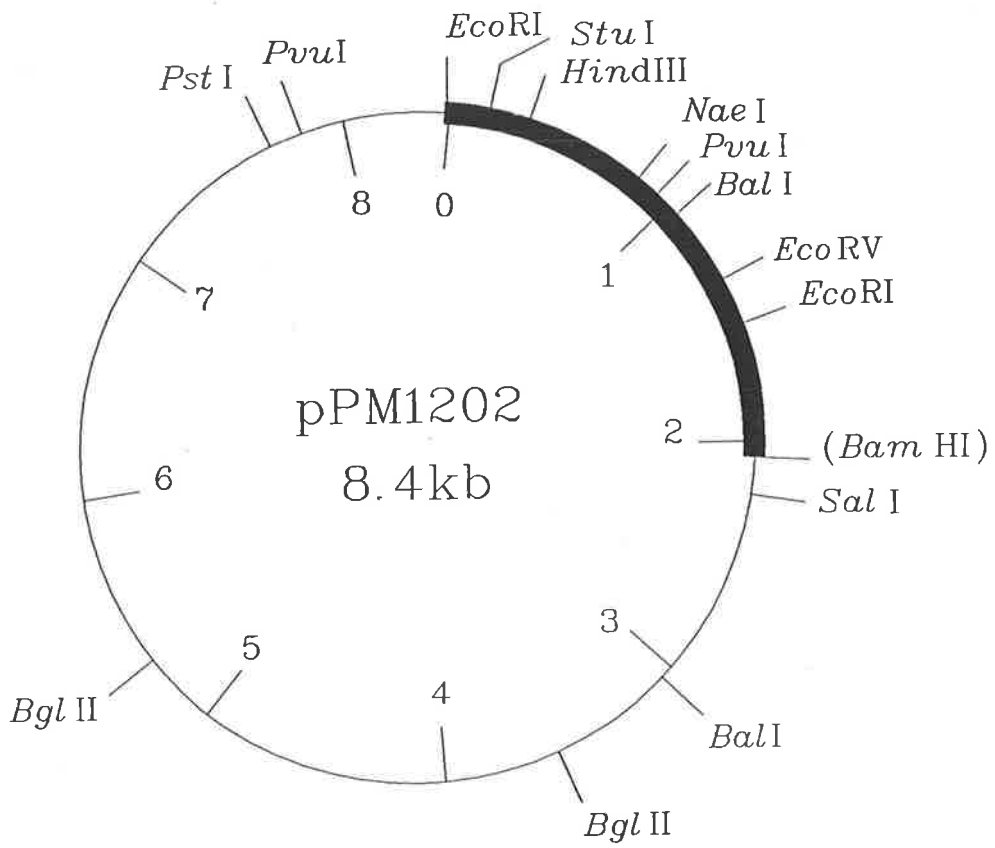


Figure 3.3 Restriction endonuclease cleavage map of pPM1202.

This plasmid originated from the partial digestion of the cosmid clone pPM1201 with *EcoRI* and subsequent re-ligation. The *EcoRI* sites were used to subclone this fragment which retained the DNase positive phenotype. The *Bam*HI site shown in brackets, was lost in the original cloning in which DNA partially digested with *Sau*3A was cloned into this site.



This plasmid retained the DNase positive phenotype. This 1.25 kb fragment is present in both the original cosmid clone and the 1.55 kb *EcoRI* cutdown (Figure 3.2).

There are no cleavage sites within the cloned *V.cholerae* DNA for the following enzymes: *AatII*, *AccI*, *BamHI*, *BclI*, *BglII*, *Clal*, *HpaI*, *KpnI*, *MluI*, *NdeI*, *NotI*, *NruI*, *PstI*, *SacI*, *Sall*, *Scal*, *SmaI*, *SstI*, *XbaI*, *XhoI* and *XmaI*.

3.2.3 Re-cloning of the DNase gene into other vectors

When the 1.55 kb *EcoRI* fragment of pPM1202 was re-cloned into the *EcoRI* site of the vector pACYC184, a DNase positive phenotype was obtained irrespective of its orientation, suggesting that the gene carried its own promoter. These plasmids were designated pPM1203 and pPM1204. The *EcoRI* fragment of pPM1202 was also cloned into the vector pUC18 in both orientations, to give the plasmids pPM1205 and pPM1254. Expression of the DNase was also independent of orientation in this vector, and did not require induction of the *lac* promoter with IPTG, thereby further establishing the lack a requirement for external promoters (Figure 3.4).

3.2.4 Transposon mutagenesis of pPM1202

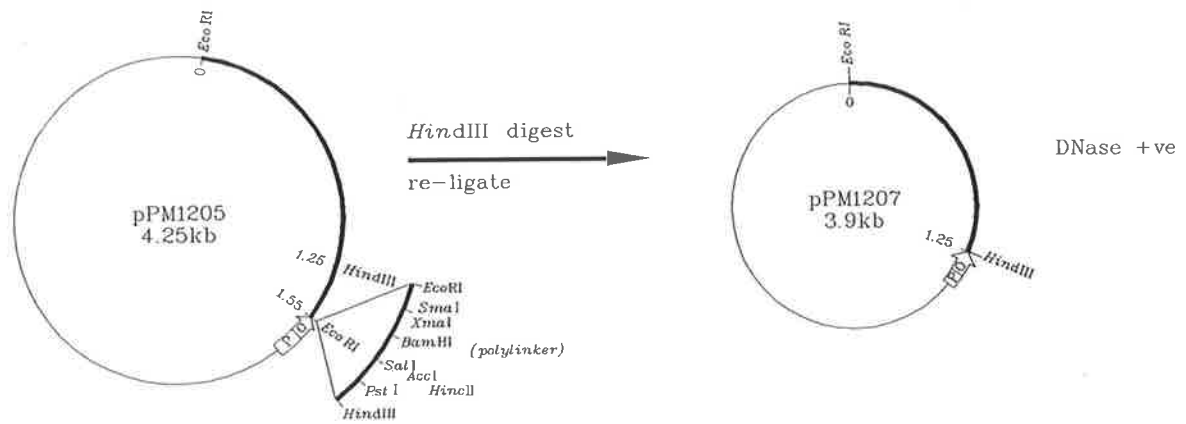
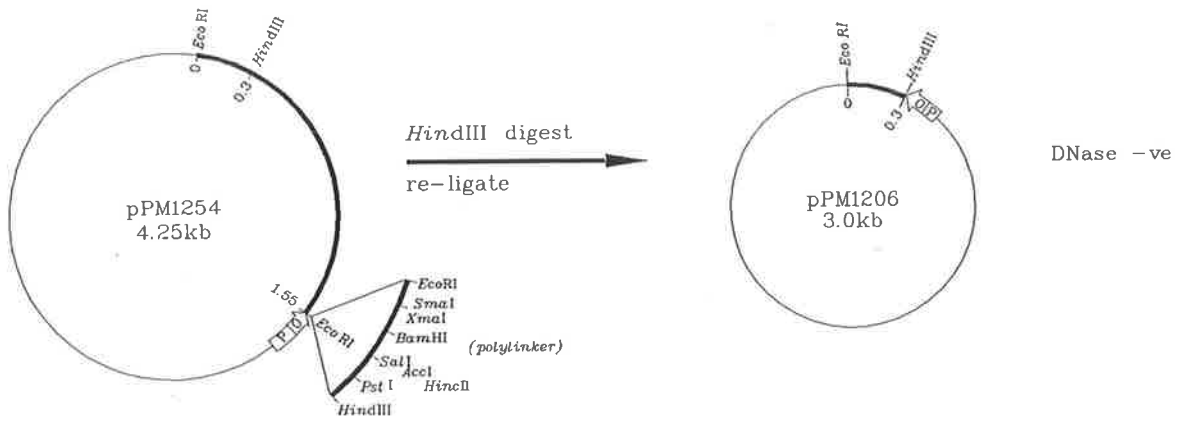
Plasmid pPM1202 was mutagenized with the transposon Tn1725 (Ubben and Schmitt, 1986). This transposon contains sites for the restriction endonuclease *EcoRI* 15 bp from each end, and thus allows a simple and accurate method of determining the point of insertion of the transposon in the DNA.

Tn1725 was transposed into pPM1202 from plasmid pRU669 ($R_{ts}1::Tn1725$) selecting for Cm^R derivatives of pPM1202. A total of 20 independent insertions were isolated and mapped by restriction analysis with both *EcoRI* and *HindIII*. These insertion derivatives showed either a positive or completely negative phenotype. None of the positive insertions found were located on the cloned fragment of DNA and therefore they were not further mapped. Small-scale DNA

Figure 3.4 Effect of gene orientation on DNase expression.

When the *EcoRI* fragment of pPM1202 was cloned in both orientations into pUC18 (plasmids pPM1205 and pPM1254), the cells expressed a DNase positive phenotype. . Expression of the DNase was also independent of induction of the external *lac* promoter contained in the vector.

Digestion of pPM1254 and pPM1205 with *HindIII* and re-ligation results in the deletion of a portion of the cloned DNA and the polylinker region of the vector. Cells harbouring pPM1207 with a 1.25 kb insert still express DNase activity.



preparations from these positive insertions were also particularly difficult to analyse because of the large amount of nuclease activity resulting in partial degradation of the isolated plasmid DNA. This was not seen with DNase-inactivating insertions. The insertions eliminating activity all mapped on the cloned DNA fragment within a region of 0.6 kb, giving a minimum estimate of 22 kDa for the DNase (Figure 3.5).

3.2.5 Linker insertion mutagenesis of the DNase gene

The plasmid pPM1207 was used to introduce various linker mutations into a number of restriction endonuclease sites present on the 1.25 kb insert DNA. *Pst*I linkers were introduced into the *Nae*I, *Ba*II and *Eco*RV sites individually. *Nae*I, *Ba*II and *Eco*RV are all restriction endonucleases that give rise to blunt-ended fragments. The *Pst*I linkers were ligated directly to these ends and the resulting plasmid derivatives were re-ligated. The *Pst*I linkers are 8 bp in length (dGCTGCAGC), and an insertion within the DNase gene would cause a frameshift mutation. Both *Nae*I and *Ba*II linker insertions resulted in a DNase negative phenotype, while the *Eco*RV linker insertion site retained DNase activity (Figure 3.5). The location and nature of the *Nae*I and *Ba*II insertions are consistent with the Tn1725 DNase negative mutations, while the *Eco*RV linker insertion positive phenotype defines the limit of the DNase gene to this restriction site. No further suitable restriction endonuclease sites were available at the *Hind*III end of the cloned DNA, with the maximum coding size of the DNase gene now being confined to a 1.07 kb DNA region, corresponding to a maximum protein size of 39 kDa.

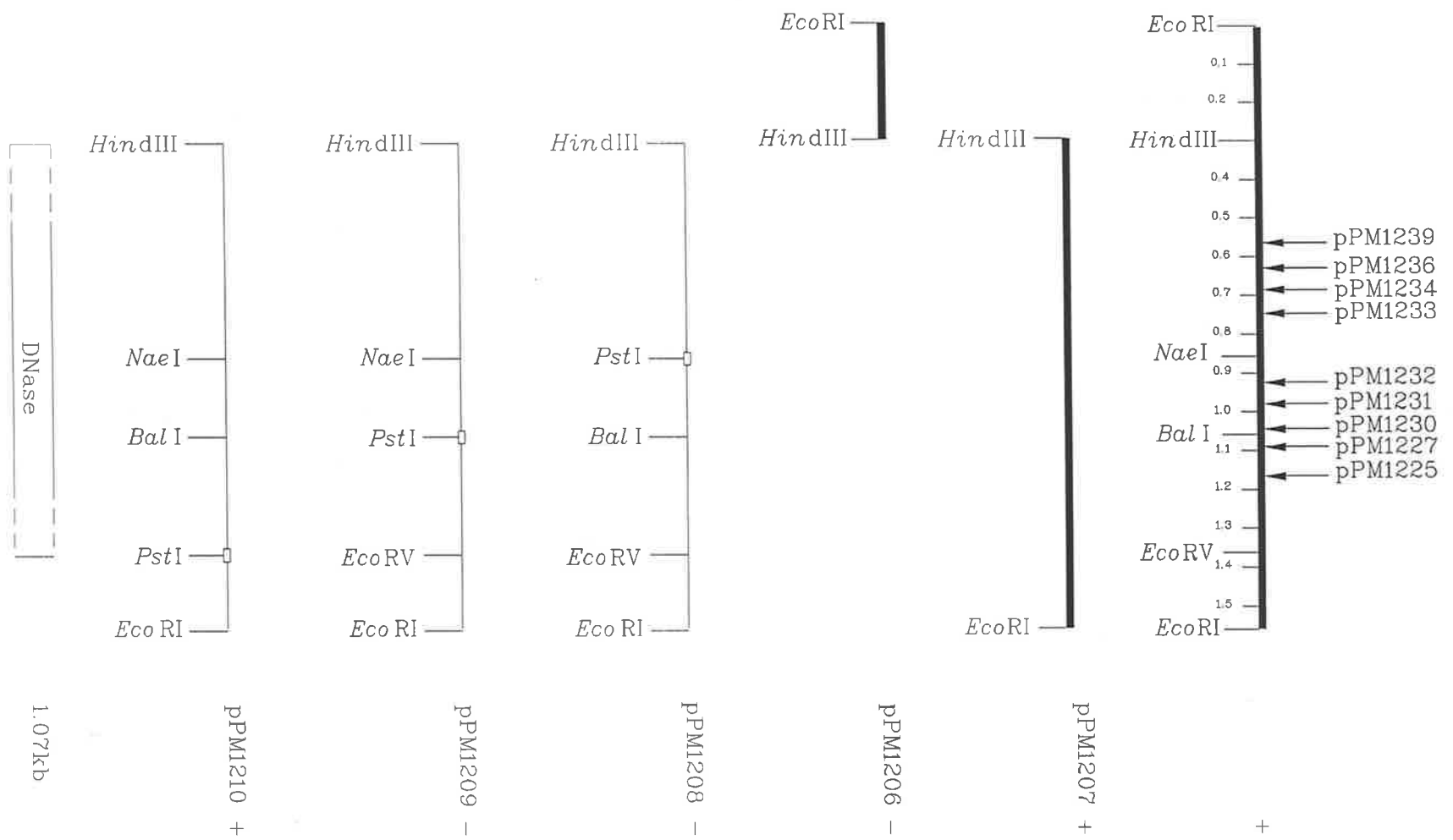
3.2.6 Analysis of plasmid encoded proteins in *E.coli* minicells

Several plasmids were introduced into the *E.coli* K-12 minicell-producing strain DS410 (Dougan and Sherratt, 1977). Minicells were purified from whole cells by centrifugation through sucrose gradients and subsequently the plasmid-encoded

Figure 3.5 Transposon and linker insertion mutagenesis of pPM1202 and pPM1207.

The top line indicates the sites of insertion of Tn1725 within each of the plasmids. These insertions were clustered within a 0.6 kb region result in a DNase negative phenotype. No transposon insertions that gave a positive phenotype mapped within the 1.55 kb *EcoRI* fragment.

Linker insertion mutagenesis of pPM1207 to give the plasmids pPM1208, pPM1209 and pPM1210, define the limit of the DNase at one end of the DNA fragment to the *EcoRV* site. The open boxes indicate the site of insertion of the *PstI* linkers. The DNase gene can be localized to a 1.07 kb region of DNA.



DNase activity

proteins were labelled with [³⁵S]-methionine (Kennedy *et al.*, 1977; Achtman *et al.*, 1979). These were visualised by autoradiography following electrophoresis in SDS through an 11-20% linear polyacrylamide gradient gel.

The plasmid pPM1202 was analysed in minicells, along with the Tn1725 DNase negative derivatives. Plasmid pPM1202 shows a protein band at about 24 kDa, which is very faint and probably reflects the fact that the DNase is poorly expressed. The corresponding band is not present in the pHc79 vector alone (Figure 3.6). The purpose of utilizing the transposon Tn1725 negative mutants was to detect the truncated protein products produced by the insertions in the structural gene. The size of these truncated protein products, would enable the direction of transcription and the start point of the gene to be determined. Unfortunately, no such products could be detected and this could be due either to the rapid degradation of these truncated forms by proteolytic enzymes, or merely not being visualized on the autoradiograph as the wild type gene itself appears to be expressed in very low amounts.

3.2.7 *In vitro* translation of pPM1207

The protein products of the plasmid pPM1207 and the cloning vector pUC18 were analyzed using a prokaryotic DNA-directed translation kit. This cell-free bacterial coupled transcription-translation system first described by DeVries and Zubay (1967) and later modified by Collins (1979), allows the expression *in vitro* of genes contained on bacterial plasmids, provided that the relevant control signals such as the Pribnow box for initiation of transcription, and Shine-Dalgarno sequence for translation are present. The system essentially utilizes an S-30 extract prepared from *E.coli* and the necessary nucleotides and amino acids for the transcription and translation of the plasmid encoded genes. The added radiolabelled [³⁵S]-methionine, allows the translated gene products to be visualized by autoradiography after polyacrylamide gel electrophoresis.

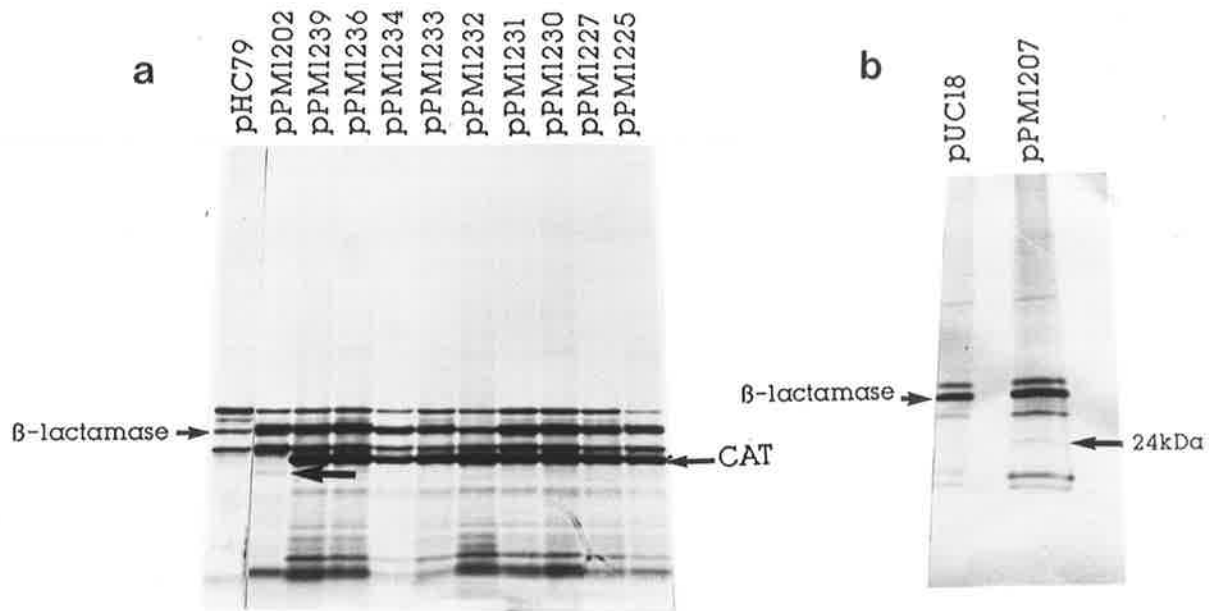
The plasmid pPM1207 shows a band at the 24 kDa size range although this is a minor band and presumably is due to the low expression of this gene, as is also seen in minicells (Figure 3.6). The track containing the pUC18 vector alone does

Figure 3.6 Expression of plasmid encoded proteins

(a) Plasmid encoded proteins in the *E.coli* K-12 minicell producing strain DS410. Minicells harbouring the various plasmids were purified on sucrose gradients, labelled with [³⁵S]-methionine and solubilized in sample buffer. The plasmid encoded proteins were visualized by autoradiography after electrophoresis on a 11-20% gradient polyacrylamide gel. The β -lactamase present in the vector in all the plasmids has a size of 30 kDa, while the chloramphenicol transacetylase (CAT) of the Tn1725 insertion plasmids has a size of 25 kDa. The 24 kDa protein is seen only in the track with pPM1202 which has a DNase positive phenotype.

(b) *In vitro* translation of plasmid encoded proteins.

Plasmid encoded proteins were labelled with [³⁵S]-methionine using a cell-free bacterial transcription-translation system. Samples were precipitated in 50% TCA and run in SDS on a 15% polyacrylamide gel. Bands were visualized by autoradiography. The 30 kDa protein represents the β -lactamase. The reaction containing pPM1207 shows a 24 kDa protein (the putative DNase) not present in the pUC18 vector.



not have the corresponding band, and is consistent with the data obtained from minicell analysis.

3.2.8 Functional identification of the DNase protein and its cellular location

Cytoplasm, periplasm, inner and outer membranes and extracellular fractions of *E.coli* K-12 containing the cloned *V.cholerae* gene were assayed for activity by spotting 50 μ l of each fraction onto DNA-containing (0.2%) agar plates. These plates were incubated overnight at 37°C and flooded with 1N HCl. Fractions containing the DNase resulted in a zone of clearing due to hydrolysis of the DNA in the agar (data not shown). Activity of the *V.cholerae* DNase was found only in the periplasmic fraction. Similar fractions were also assayed from *V.cholerae* 017 for DNase activity. In contrast to *E.coli* K-12, activity was detected only in the extracellular fraction, indicating that while the DNase is an extracellular protein in *V.cholerae*, it appears to be confined to the periplasmic space of *E.coli*.

To determine the actual size of the protein with the DNase activity, an assay for the DNase directly from a polyacrylamide gel was performed by using a modification of a method by Rosenthal and Lacks (1977). The periplasmic fractions were run on an SDS-polyacrylamide gel and the SDS washed from the gel by gentle agitation in 0.04M Tris-HCl pH7.6, 2mM MgCl₂ and 0.02% azide. The washing solution was changed at 30 min. intervals for 3hrs. The polyacrylamide gel was then placed onto a large DNA-agar plate and incubated overnight at 37°C. The polyacrylamide gel was taken from the DNA-agar plate which was subsequently flooded with 1N HCl. The distance of the bands of hydrolysis relative to the gel were measured and compared to a duplicate half of the same gel which had been fixed and stained in Coomassie blue.

E.coli strains harbouring plasmids with an active *V.cholerae* DNase showed a zone of hydrolysis corresponding to a protein size of 24 kDa. *E.coli* strains with DNase negative plasmids, or the plasmid-free parent strain DH1 showed no regions of hydrolysis on the DNA-agar. Interestingly, while the *E.coli* strain DH1 (*endA*) used here lacks a periplasmic DNase (Hanahan, 1983), the *E.coli* strain LE392 which does possess a periplasmic DNase (*endA*⁺), also showed a zone of

clearing at the 24 kDa region on the DNA-agar (Figure 3.7).

V.cholerae periplasmic fractions did not exhibit any zones of hydrolysis on the DNA-agar, while extracellular fractions only had a region of DNase activity at the 24 kDa position, identical to the size detected when it is confined to the periplasmic space of *E.coli* (Figure 3.7). However, with the addition of β -mercaptoethanol to the electrophoresis sample buffer all activity was lost. This implies that disulphide bridges are involved in the maintenance of a stable tertiary conformation of the protein.

The Coomassie blue stained portion of the polyacrylamide gel did not reveal any significant band(s) at the 24 kDa position of DNase positive clones, which was not also present in either DNase negative clones or plasmid-free *E.coli* DH1. The DNase protein therefore, either stains very poorly with Coomassie blue, or more likely, it is present in very small amounts due to a low level of expression of this gene.

3.2.9 Silver staining of cell fractions containing DNase activity

In an attempt to correlate the presence of a 24 kDa protein with the presence of DNase activity, both periplasmic fractions of the *E.coli* clones and extracellular fractions of *V.cholerae* were prepared and run on an SDS-polyacrylamide gel and stained with silver for greater sensitivity. The periplasmic fractions included *E.coli* DH1 containing the DNase positive plasmid pPM1207, the DNase negative plasmid pPM1208 and DH1 alone. The extracellular fractions of *V.cholerae* were precipitated with ammonium sulphate and resuspended in one hundredth the volume. Two ammonium sulphate fractions were used, 40% and 80%. The 40% fraction contained some DNase activity, but the majority of this activity was precipitated in the 80% fraction.

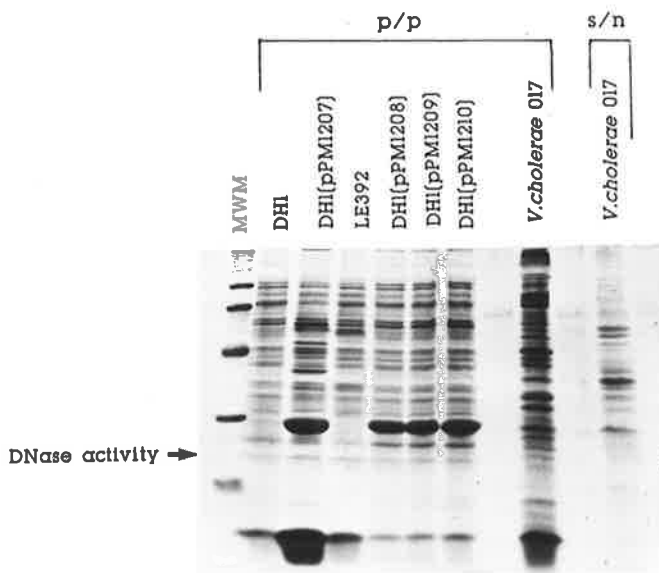
Silver staining of the periplasmic fractions of *E.coli* show a band at the 24 kDa position, which is present in the strain carrying pPM1207, as well as that carrying pPM1208. Plasmid free *E.coli* strain DH1 also has a protein band at this position, and so it is possible that this periplasmic protein co-migrates with the DNase protein, thereby masking it. The DNase must therefore be produced in small

Figure 3.7 Fractionation of cells to localize DNase activity.

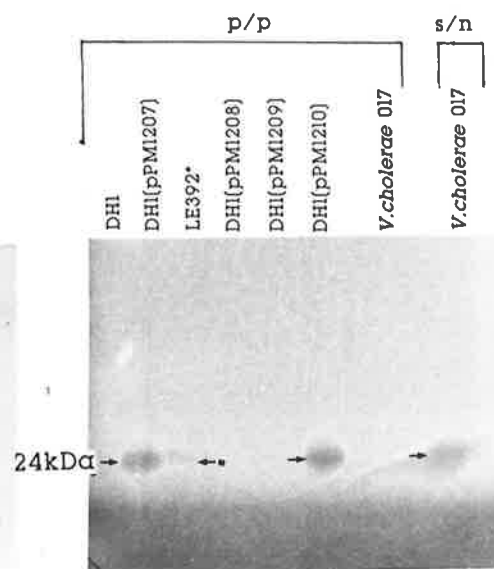
(a) Cell fractions of *E.coli* K-12 and *V.cholerae* 017 were electrophoresed in an SDS-polyacrylamide gel (15%), and stained with Coomassie blue G-250. The periplasmic fractions (p/p) represent the *E.coli* K-12 strain DH1 harbouring the indicated plasmids; the *E.coli* K-12 strain LE392 which has its own periplasmic DNase, and the *V.cholerae* El Tor strain 017. The extracellular fraction (s/n) was from the *V.cholerae* strain 017.

(b) A duplicate gel to that in panel A was incubated overnight on a DNA-agar plate and flooded with 1N HCl to detect DNase activity. The periplasmic fractions (p/p) of *E.coli* clones with an active DNase have a band of DNA hydrolysis at the 24 kDa region. A similar size band can be seen with the periplasmic DNase of *E.coli* LE392. The *V.cholerae* 017 strain also has a band of DNase activity of 24 kDa in the extracellular fraction (s/n).

a SDS-PAGE/COOMASSIE BLUE



b SDS-PAGE/DNA-AGAR OVERLAY



amounts as there does not appear to be a major protein band in this region specific for those strains where DNase activity is detected (Figure 3.8).

The *V.cholerae* extracellular fraction does show a protein in the 24 kDa region (Figure 3.8) corresponding to the region where DNase activity can be detected (Figure 3.7). Most of this protein is in the 80% ammonium sulphate fraction where the majority of the DNase activity is associated. This protein also appears to be a minor component of the extracellular fraction further suggesting that the DNase is not expressed at high levels in both *V.cholerae* and in *E.coli*, even when cloned into a multi-copy plasmid. The sensitivity of detection of the DNase, however, would appear to be greatly increased in the DNA-hydrolysis assay, as the DNase enzyme amplifies its presence by virtue of the rapid turnover of DNA molecules hydrolyzed per single DNase enzyme.

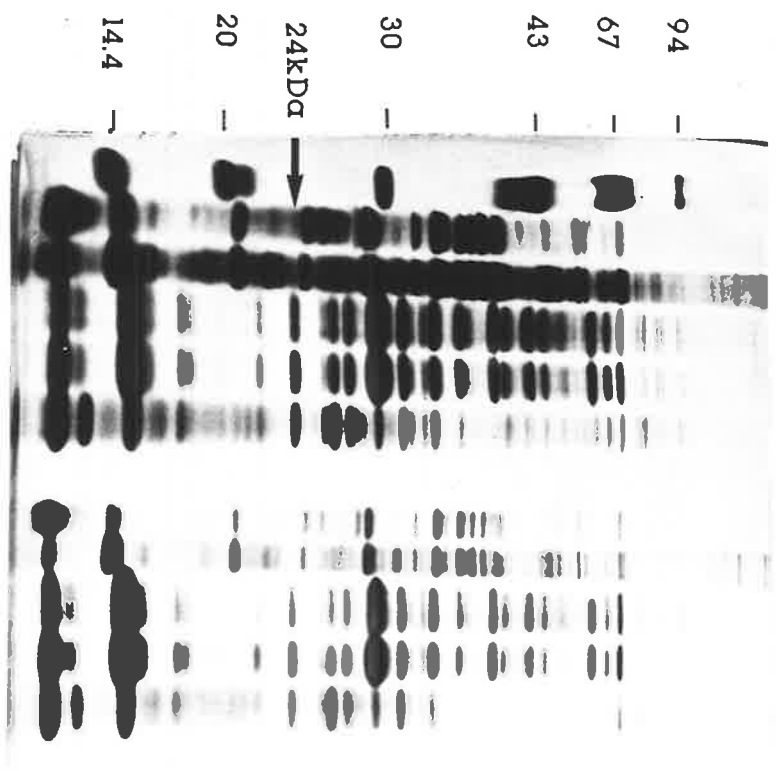
3.2.10 Expression of the DNase in periplasmic leaky mutants

A large zone of clearing around colonies is produced when *V.cholerae* is grown on DNA-agar plates because the DNase is secreted to the outside of the cell and can diffuse through the agar (Figure 3.9). However, *E.coli* clones containing the *V.cholerae* DNase show very small zones of hydrolysis, because the protein is confined to the periplasmic space and presumably, is only released upon cell lysis.

To facilitate the release of the DNase, the cloned gene was introduced into a periplasmic leaky mutant (*tolA,B*) of *E.coli* K-12 (Bernstein *et al.*, 1972; Anderson *et al.*, 1979). This mutant strain, E852, was isolated from the *endA* strain DH1 lacking the periplasmic DNase, so that expression of the cloned *V.cholerae* DNase would not be masked. The *tolA,B* mutant strain was selected by isolating *E.coli* that were resistant to colicin E1, essentially as described by Bernstein *et al.* (1972), and the cross-resistance to other group A colicins determined (Davies and Reeves, 1975). The mutant colonies were also tested for their sensitivity to deoxycholate. These checks were to distinguish between colicin-resistant mutants which had lost or altered their colicin receptor sites, and these specific colicin-tolerant (*tolA,B*) mutants which still possessed the adsorption sites. The

Figure 3.8 Silver stained polyacrylamide gel of cell fractions containing DNase activity.

Periplasmic fractions (p/p) of *E.coli* K-12 strain DH1 with the indicated plasmids; and 40% and 80% ammonium sulphate precipitated fractions of extracellular proteins (s/n) of *V.cholerae* 017 were run on a 15% SDS-polyacrylamide gel. The gel was silver stained for greater sensitivity. The left half of the gel was loaded with 100 μ l of each fraction, while the right half was loaded with 50 μ l. The arrow indicates a band of 24 kDa in the 80% ammonium sulphate extracellular fraction of *V.cholerae* 017 which correlates with the DNase activity detected in this fraction.



MWM

80%Amm.Sulph.

40%Amm.Sulph.

DH1(pPM1205)

DH1(pPM1207)

DH1

s/n

p/p

V.cholerae 017

E.coli DH1

80%Amm.Sulph.

40%Amm.Sulph.

DH1(pPM1205)

DH1(pPM1207)

DH1

s/n

p/p

V.cholerae 017

E.coli DH1

tolA,B mutants have been shown to be sensitive to the cationic detergent deoxycholate (Nagel De Zwaig and Luria, 1967; Davies and Reeves, 1975), as well as exhibiting a generalized loss of periplasmic proteins or "leaky" phenotype (Anderson *et al.*, 1979).

Figure 3.9 shows the increased zones of DNA hydrolysis observed, mimicking the excretion observed with *V.cholerae*. This further supports the periplasmic localization of the DNase in *E.coli* K-12 determined previously by assaying cell fractions.

3.2.11 Distribution of the DNase gene in El Tor and Classical strains of *V.cholerae*.

A number of *V.cholerae* El Tor and Classical strains were probed by Southern hybridization analysis with the plasmid pPM1207, in order to determine if the DNA coding for the DNase was present in all El Tor isolates, and if this were also true for Classical strains. All El Tor and Classical strains reveal a 3.55 kb *EcoRI* homologous DNA fragment as seen in the original cosmid clone pPM1201 and not the smaller 1.55 kb fragment detected in pPM1202 (Figure 3.10). This further supports the idea that the 1.55 kb *EcoRI* fragment generated during the cutdown of pPM1201 to give pPM1202 is not the true size of the fragment, but probably generated by a deletion internal to this fragment. However, while both El Tor and Classical strains have a 3.55 kb *EcoRI* fragment containing the DNase gene in common, the smaller 1.25 kb *EcoRI/HindIII* fragment is present only in the El Tor isolates from which this gene was originally cloned (Figure 3.10). The Classical isolates therefore appear to have lost the restriction endonuclease recognition site for *HindIII* but still retain the 3.55 kb DNA fragment when digested with *EcoRI* and *HindIII*. Since this 1.25 kb *EcoRI/HindIII* subclone was also present as a similar sized DNA fragment in the *V.cholerae* El Tor chromosome, most of the further work undertaken in the analysis of this gene involved the use of this cloned fragment.

Figure 3.9 DNA-agar plate of *V.cholerae*, and the *E.coli* DH1 strain and its *tolA,B* mutant harbouring pPM1207.

After overnight growth on DNA-agar the DNase activity was detected by precipitation of the unhydrolysed DNA with 1N HCl.

V.cholerae 017

V.cholerae 569B

DH1

DH1 (pPM1207)

E852

E852 (pPM1207)

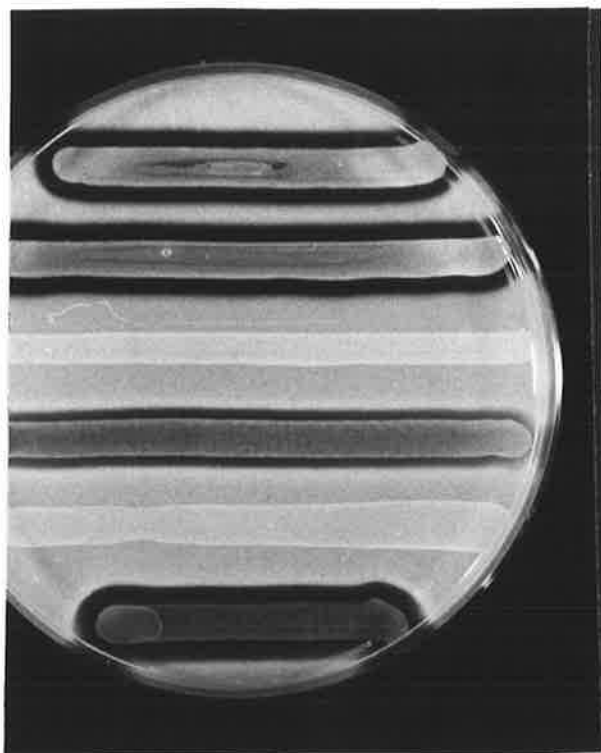
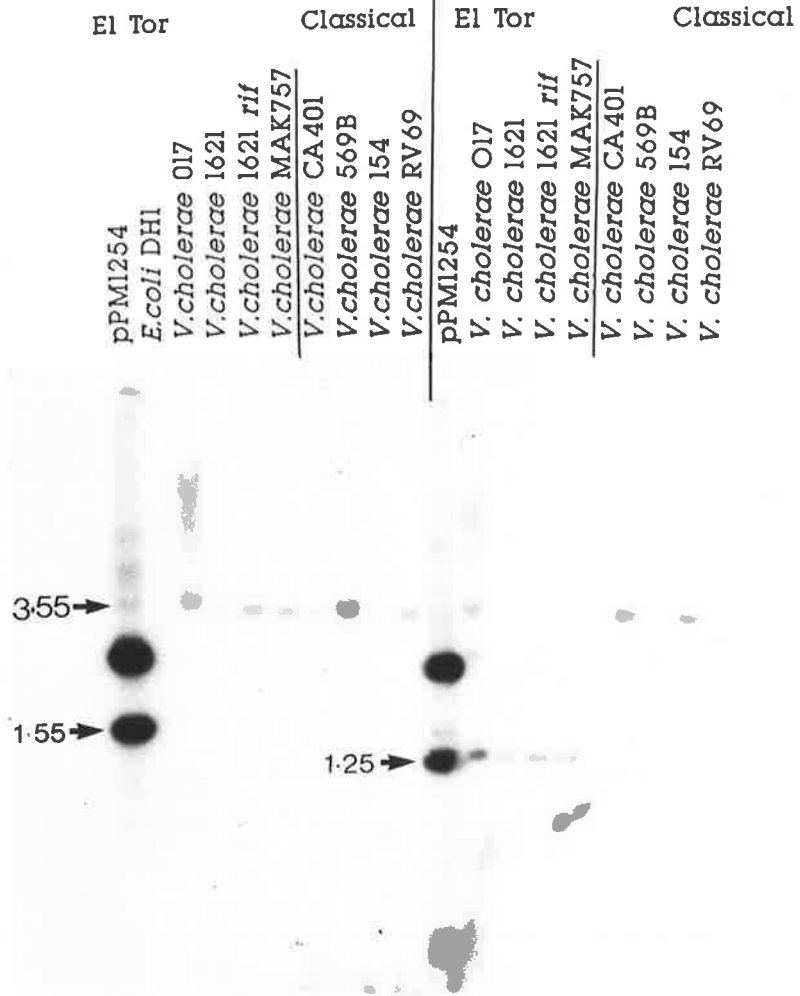


Figure 3.10 Southern hybridization analysis of whole genomic DNA of *V.cholerae* El Tor and Classical biotypes.

The DNA was digested with *EcoRI* or *EcoRI* and *HindIII*, transferred to nitrocellulose, and probed with pPM1254 nick-translated with α -[³²P]-dCTP. Both El Tor and Classical strains have a 3.55 kb *EcoRI* fragment, while the 1.25 kb *EcoRI/HindIII* fragment is present only in the El Tor isolates.

EcoRI

EcoRI + *HindIII*



3.2.12 Distribution of the DNase gene amongst the Vibrionaceae

The distribution of the *EcoRI/HindIII* DNA fragment encoding the DNase gene amongst bacteria belonging to the family Vibrionaceae was determined by Southern hybridization using DNA extracted from bacteria belonging to this group, and probing with pPM1207. DNA homologous to the DNase gene is not present in the *Aeromonas* strains tested, and is present in only one of the non-cholera Vibrios. *Vibrio parahaemolyticus* and *Vibrio fluvialis* do not contain any homologous DNA, but *Vibrio mimicus* does have a 3.55 kb homologous DNA fragment in its chromosome (Figure 3.11). This 3.55 kb fragment is the same as that seen in *V.cholerae* Classical strains, where the *HindIII* site does not appear to be present. The non-01 *V.cholerae* strain however, does have a 1.25 kb *EcoRI/HindIII* fragment similar to the El Tor isolates (Figure 3.11). Therefore, DNA homologous to the DNase gene appears to be restricted to the *V.cholerae* species, with the exception of the *V.mimicus*.

3.3 Conclusions

The gene coding for the extracellular DNase of *V.cholerae* 017 has been cloned via a cosmid gene bank, by screening clones capable of DNA hydrolysis on DNA agar plates. The cosmid vector containing this gene was designated pPM1201, with the deletion derivative pPM1207 containing the DNase gene on a 1.25kb *EcoRI/HindIII* DNA fragment.

Transposon mutagenesis and frameshifting linker insertion mutations have maximized the coding area of the DNase gene to a 1.07 kb segment of the cloned DNA. This coding capacity allows for a maximum size of 38 kDa, and a minimum of 22 kDa for the DNase, since transposon insertions covering 0.6 kb of this DNA all give rise to a DNase negative phenotype.

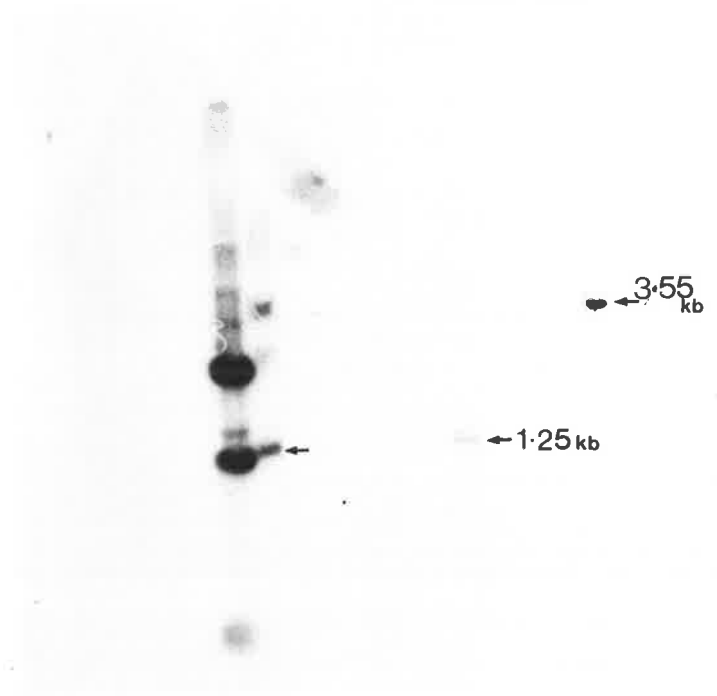
Both minicell analysis and *in vitro* protein translation, show a 24 kDa protein band present in clones expressing a DNase positive phenotype, while a band of this size comigrates with DNase activity as well. This fits well within the coding limits defined by mutations within this region appear to be well expressed when

Figure 3.11 Southern hybridization analysis of whole genomic DNA of other members of the Family Vibrionaceae.

The DNA was cleaved with *EcoRI* and *HindIII*, transferred to nitrocellulose, and probed with pPM1254 nick-translated with α -[³²P]-dCTP. The *V.cholerae* (non O1) strain has an homologous band present of 1.25 kb, while *V.mimicus* has an homologous band at 3.55 kb.

*Eco*RI and *Hind*III

- pPM1254
- V.cholerae* 017
- E.coli* DH1
- Aeromonas hydrophila* AE-18
- Aeromonas hydrophila* Th-4
- Aeromonas hydrophila* AH-1
- Aeromonas hydrophila* AH-2
- V.cholerae* non-01
- V.costicolus*
- V.parahaemolyticus*
- V.fluvialis*



compared to the larger amounts of β -lactamase or chloramphenicol transacetylase present in the plasmids or transposon Tn1725 respectively, also detected in these systems. However, the 24 kDa protein does not appear to be expressed well when compared to β -lactamase or chloramphenicol transacetylase present in the plasmids or transposon Tn1725, respectively, also detected in these systems. The DNase which is an extracellular protein in *V.cholerae*, remains localized in the periplasmic space when cloned in *E.coli* K-12. However, this is not surprising since a number of other extracellular proteins of *V.cholerae* such as the haemolysin (Manning *et al.*, 1984; Mercurio and Manning, 1985) and cholera toxin (Pearson and Mekalanos, 1982) are also periplasmic bound in *E.coli*. This is probably due to the inability of *E.coli* to recognize certain export signals on these proteins, or the lack of the necessary "export machinery" to secrete these proteins to the outside of the cell.

While the DNase remains in the periplasmic space of *E.coli*, it is exported to the outside of the cell in *V.cholerae*, with no DNase being detected in the periplasm. This has been determined by assaying for DNase activity in the cellular fractions of *V.cholerae*. The lack of any DNase being detected in the periplasm of *V.cholerae*, however, maybe due to the rapid export of such a periplasmic pool to the outside of the cell. Young and Broadbent (1982) observed an accumulation of both cholera toxin and a deoxyribonuclease in the periplasmic space of *V.cholerae* when cells are pretreated with the antibiotic lincomycin. The DNase detected in the extracellular fraction of *V.cholerae* has the same size (24 kDa) as that detected in the periplasm of *E.coli* when the cloned gene is introduced into these cells. The extracellular export of the DNase of *V.cholerae* can be mimicked in *E.coli*, if the DNase is introduced into *E.coli toIA,B* mutants (Bernstein *et al.*, 1972) which are leaky for periplasmic proteins (Anderson *et al.*, 1979).

Finally, the distribution of the DNase gene appears to be limited to the *V.cholerae* species amongst the Vibrionaceae, with the exception of the *V.mimicus*. Although El Tor and Classical biotypes of *V.cholerae* share a common 3.55 kb *EcoRI* fragment, the smaller 1.25 kb *EcoRI/HindIII* fragment encoding the DNase structural gene is present only in the El Tor biotype from which it was originally cloned. Strains of the Classical biotype and the non-cholera vibrio *V.mimicus* do not appear to have the *HindIII* restriction endonuclease recognition

site which generates the smaller 1.25 kb DNA fragment.

CHAPTER 4

Nucleotide sequence analysis and genetic organization of the DNase gene of *V.cholerae*

4.1 Introduction

The ever increasing knowledge of gene expression and regulation has resulted from the analysis of genes at their most basic level; the nucleotide sequence. Gene expression is controlled, in part, at the level of RNA transcription. RNA synthesis is regulated by a variety of specific signals encoded in the DNA, such as promoters which specify initial sites for interaction with the RNA polymerase (RNAP). Transcriptional regulation is achieved by modulating the efficiency with which the RNAP can recognize and interact with these sites. Moreover, a number of effector molecules are known which can positively or negatively influence the interaction between RNAP and DNA. These effector molecules recognize nucleotide sequence information in the DNA template which is adjacent to and/or overlaps with a site of RNAP action.

Translation, the second stage in gene expression, involves the interaction of ribosomes with the mRNA. A better understanding of the complex initiation process, has shed some light upon the translational regulation which can be exhibited at this level of gene expression. The observation of Shine and Dalgarno in 1974 that there was complementarity between the 3' end of 16S ribosomal RNA and the sequences 5' to the initiation codon AUG constituted an important breakthrough in the understanding of translational initiation (Shine and Dalgarno,

1974). The 16S sequence 5' ACCUCC 3' was postulated to interact with a variable mRNA domain just 5' to an initiation codon, with differing rates of translational initiation the result of both the degree of complementarity and the extent to which that mRNA domain is free of intramolecular constraints so that it might interact with 16S RNA.

The information on DNA sequences involved in the promotion and termination of RNA transcription as well as translation initiation, has led to the recognition of structural and functional similarities that occur among these sites. The most striking feature of a promoter region required for the transcription of mRNA, has been an area 10bp upstream of the mRNA startpoint known as the -10 region or Pribnow box (Pribnow, 1975a), containing a 7bp sequence, generally homologous to the sequence TATAATG (Pribnow, 1975b; Schaller *et al.*, 1975). Sequence similarity has also been noted among promoters in a region 35bp preceding the mRNA startpoint (called the -35 or recognition region) (Takanami *et al.*, 1976; Seeburg *et al.*, 1977). This TTGACA hexanucleotide has been implicated to function in the initial recognition of the promoter site by the RNA polymerase (Schaller *et al.*, 1975). The recognition of these and other important regions in the regulation and expression of genes has resulted directly from the analysis of nucleotide sequence data, which is now being steadily amassed due to recent advances in DNA sequencing technology.

This nucleotide sequence analysis can be extended to the class of exported proteins present in both prokaryotes and eukaryotes. These exported proteins usually have an N-terminal extension known as a signal peptide, which is cleaved when they subsequently traverse a membrane to another compartment of the cell (Blobel and Dobberstein, 1975a; Inouye *et al.*, 1977). The signal peptides themselves have several unique features (these will be discussed later in this chapter), and although at the DNA level the nucleotide sequence can be highly variable, the amino acids and their relative position are restricted. In this regard, an exported protein can usually be identified if the nucleotide sequence at the start of a gene encodes such a sequence (von Heijne, 1983).

The analysis of prokaryotic nucleotide sequences has until recently predominantly involved that of *E. coli* and its bacteriophages. In contrast, the nucleotide sequence of very few *V.cholerae* genes have been determined. The

recognition of the importance of a number of proteins of *V.cholerae* in the virulence of this organism has led to the cloning and sequencing of the genes encoding these virulence factors. These include the genes encoding cholera toxin (Lockman and Kaper, 1983; Mekalanos *et al.*, 1983), OmpV, an outer membrane protein of *V.cholerae* (Pohlner *et al.*, 1986a), the El Tor haemolysin (Alm *et al.*, 1988), the ToxR protein, responsible for the co-ordinate regulation of a number of virulence determinants (Miller *et al.*, 1987), and the genes for the biosynthesis of the O-Antigen of *V.cholerae* (P. Manning - personal communication).

Nucleotide sequence analysis has shown the cholera enterotoxin genes (*ctxAB*) to be highly conserved with respect to the heat-labile toxin genes (*eltAB*) of *E. coli* (Mekalanos *et al.*, 1983) which supports the homology found at the protein level and such data suggest that these functionally similar enterotoxins may have had a common ancestral origin. Upstream regions of *ctxAB* also have the repetitive 7bp sequence TTTTGAT, and these repeat sequences have been shown to be responsible for the regulation of the *ctxAB* operon via the transcriptional activator ToxR (Mekalanos *et al.*, 1983; Miller *et al.*, 1984). ToxR has also been shown to exhibit considerable amino acid sequence homology to a number of other bacterial transcriptional activators (Miller *et al.*, 1987).

In light of the data obtained from nucleotide sequence determination, a better understanding of how *V.cholerae* can regulate and express its gene products has been reached (Mekalanos *et al.*, 1983; Miller *et al.*, 1987; Taylor *et al.*, 1987a). Thus, the analysis of the nucleotide sequence of genes both individually and collectively can firstly, provide a great deal of information about the organism itself and secondly, contribute towards the elucidation of processes or virulence determinants common to a number of organisms. In some cases this can also be extended from prokaryotes to the higher order eukaryotes (e.g. the structure of the signal sequence).

In this chapter, the genetic organization of the gene encoding the extracellular DNase of *V.cholerae* is examined. The nucleotide sequence of the DNase gene was determined and primer extension analysis located the start point of the mRNA. The gene also appears to possess a signal sequence typical for an exported protein. A detailed analysis of the genetic organisation of this gene allows comparison with other exported proteins as well as other virulence factors.

It also allows the construction of specific and defined mutations within this gene, as well as enabling alterations to the control and level of expression of this gene.

4.2 Results

4.2.1 Generation of fragments for nucleotide sequencing

The gene encoding the extracellular DNase has been localized to a 1.07kb region on the plasmid pPM1207 (Chapter 3, Section 3.2.5). This plasmid, pPM1207, contains the 1.25kb *EcoRI/HindIII* DNA fragment which was used to generate a series of deletions from both the *EcoRI* and *HindIII* ends. These deletions were used as a series of overlapping fragments which covered the 1.25kb stretch of DNA from each end. In this manner, the gene could be sequenced in both directions; one strand being sequenced with the first set of deletions from the *EcoRI* site, and the other strand sequenced with the deletions from the *HindIII* site.

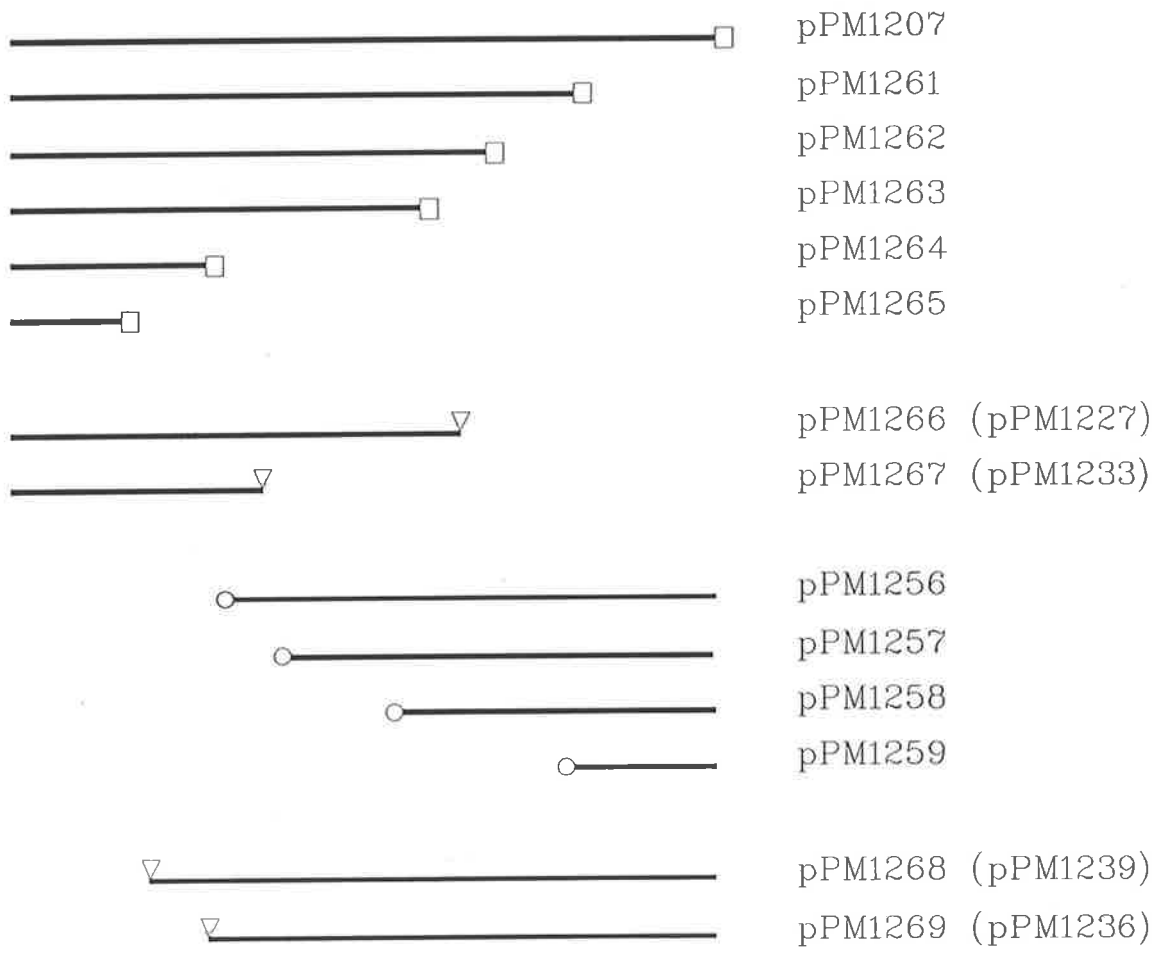
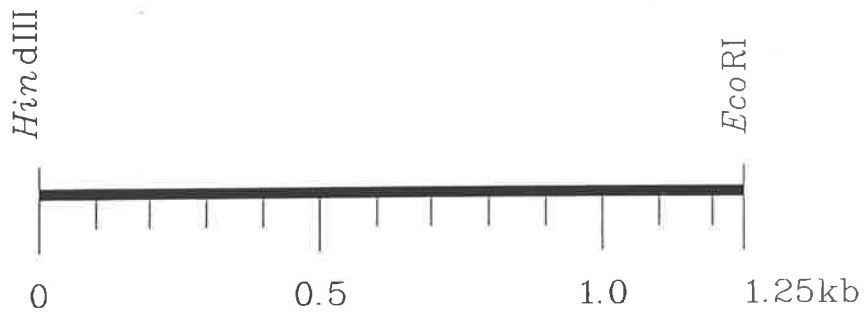
By varying incubation times, a series of deletions were generated from a given restriction endonuclease site on the plasmid pPM1207, using *Bal31* nuclease. The plasmid pPM1207 was first digested with *HindIII* and a time course of 1 to 15 minutes at 30°C in the presence of *Bal31* resulted in a series of deletions from this end. Before re-ligation, synthetic *HindIII* linkers were added, to enable the ends of the deletion to be defined precisely. The deletions from the *EcoRI* end of pPM1207 were obtained in a similar manner, with the *EcoRI* synthetic linkers being added before religation. The plasmids resulting from the deletion at the *HindIII* end of pPM1207 were designated pPM1256 through pPM1259, while those resulting from deletions at the *EcoRI* end were designated pPM1261 through pPM1265 (Figure 4.1).

The deletions obtained by *Bal31* nuclease generated a number of useful overlapping fragments. However, a number of gaps not covered by overlapping fragments were present in both directions. The insertion of Tn1725 into the cloned DNA (Chapter 3; Section 3.2.4; Figure 3.5), enabled the use of the *EcoRI* sites at either end of the transposon to obtain fragments commencing inside the gene. These fragments were removed as *EcoRI/HindIII* pieces and recloned into

Figure 4.1 Generation of overlapping fragments for nucleotide sequence determination.

Plasmid pPM1207 was digested with either *Hind*III or *Eco*RI, and deletions from either end were generated by varying the incubation time at 30°C in the presence of *Bal*31 nuclease. The extent of the deletions were defined by the addition of *Eco*RI or *Hind*III linkers to the ends before re-ligation. The boxes represent the *Eco*RI linkers, while the open circles represent the *Hind*III linkers.

To complete the series of overlapping fragments for sequencing, the regions not covered by the *Bal*31 nuclease deletions were supplemented by the use of fragments obtained from Tn1725 insertions in this region of DNA. The *Eco*RI sites present at either end of the transposon were used to obtain fragments commencing inside the gene. These fragments were removed as *Eco*RI or *Eco*RI/*Hind*III pieces and cloned into the vector pUC18. The open triangles represent the site of insertion of Tn1725 and the subsequent *Eco*RI site, while the plasmid number in brackets corresponds to the original plasmid from which these clones were derived (see Figure 3.5).



pUC18 to give the plasmids pPM1266 and pPM1267, while fragments needed in the other orientation were cloned as *EcoRI* pieces into pUC18 to give the plasmids pPM1268 and pPM1269 (Figure 4.1).

4.2.2 Nucleotide sequence determination

Sequencing reactions were carried out according to the dideoxy chain termination procedure of Sanger *et al.*, (1977). Randomly terminated chains were separated on ultra thin 6% polyacrylamide gels, in the presence of 8M Urea. Autoradiography was used to visualize DNA fragments.

The fragments generated for sequencing were cloned directionally into M13mp18 or M13mp19 (Yanisch-Perron *et al.*, 1985). The deletions from the *HindIII* end and the plasmids pPM1268 and pPM1269 from the Tn1725 insertion derivatives, were cloned into M13mp18. Deletions from the *EcoRI* end, and the Tn1725 insertion derivatives pPM1266 and pPM1267 were cloned into M13mp19 (Figure 4.2). The M13 universal sequencing primer (17-mer) was used for all sequencing reactions. In order to accurately sequence the cloned DNA region immediately adjacent to the polylinker site, the -40 M13 sequencing primer (17-mer) was also used (Figure 4.2).

The complete nucleotide sequence of 1,272 base pairs has been determined in both directions and shows a single open reading frame (ORF), within the limits of this DNA fragment (Figure 4.3). Features of this ORF will be discussed firstly with regard to the gene expression signals present on the DNA sequence, and secondly, the presence of a characteristic signal sequence at the start of this putative gene.

4.2.2.1 Nucleotide sequence of the DNase

The ORF begins 307bp after the *HindIII* site, and terminates 70bp before the *EcoRV* site. This corresponds to a protein of M_r26389. However, as might be predicted for an exported protein, there is a typical signal sequence of 20aa at the N-terminus. Cleavage of the signal sequence would give a mature protein of

Figure 4.2 Strategy employed for dideoxy sequencing of the 1,272 bp *EcoRI/HindIII* DNA fragment using M13.

Overlapping deletion fragments generated from the *EcoRI* end were cloned into M13mp19 and sequenced using the M13 universal sequencing primer (USP), while deletion fragments generated from the *HindIII* end were cloned into M13mp18. The 1.25 kb *EcoRI/HindIII* fragment from pPM1207 inserted into both M13mp19 and M13mp18, was also sequenced using the -40 sequencing primer to accurately determine the extreme ends of sequence at the junction of the polylinker cloning site.

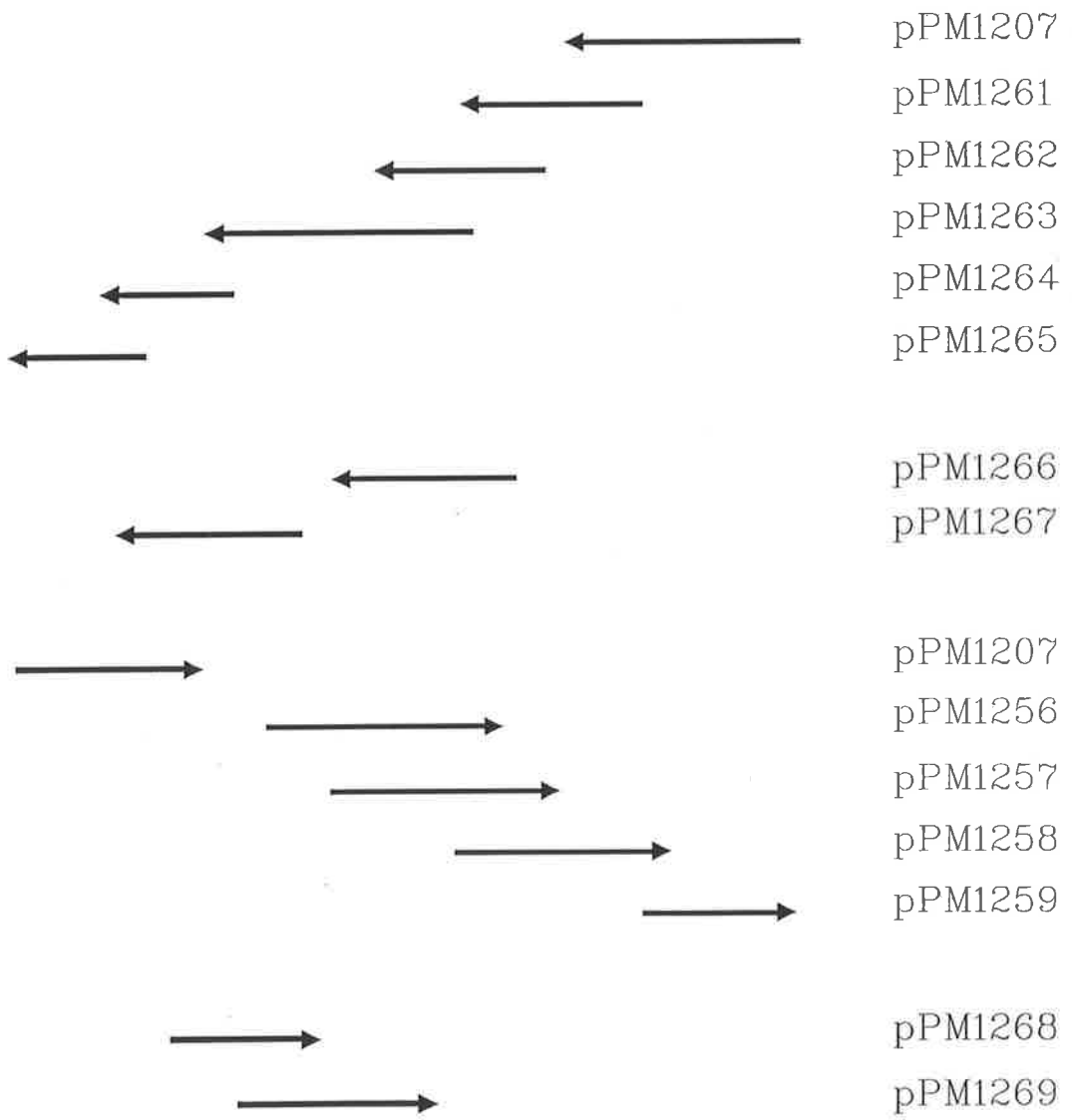
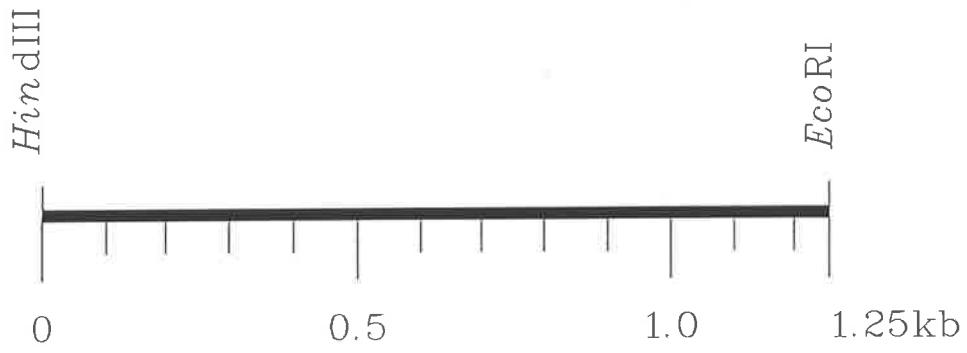


Figure 4.3 Nucleotide sequence of the 1,272 bp *Hind*III/*Eco*RI fragment of cloned *V.cholerae* DNA.

The *E.coli* K-12 consensus promoter sequences for -43 (A's), -35 (TTGACA) and -10 (TATAAT) regions are shown for comparison with the putative DNase promoter. The transcriptional start points are shown by two upward arrows. The transcriptional terminator (nt 1002 to 1022; see also Figure 4.4) is also indicated.

The termination codon is indicated by asterisks. The nt are numbered on the right and the aa within the ORF are numbered below the sequence starting from the initiation codon (Met). The likely cleavage site of the signal sequence (Ala-Ala) is indicated by the larger upward arrow.

M₂₄₁₆₃ which is in excellent agreement with the size of the protein found by both minicell analysis and *in vitro* protein translation data, as well as the size of the protein detected via a functional DNase assay (Chapter 3). The characteristics of this signal sequence will be discussed in more detail in Section 4.2.4, while the remainder of this section will deal with the expression signals present, adjacent to the ORF.

A potential promoter can be detected at the *Hind*III proximal end of the ORF by virtue of the homology detected between this region and *E.coli* promoter consensus sequences (Rosenberg and Court, 1979; Hawley and McClure, 1983), (Figure 4.3). While the homology to these consensus sequences and their relative spacing is suggestive of a promoter structure, the degree of homology and spacing cannot predict the "strength" or efficiency of promoters (Deuschle *et al.*, 1986). In fact, comparison of a synthetic "consensus promoter" to other known promoters has put this consensus promoter in the class of inefficient or poorly expressed promoters (Deuschle *et al.*, 1986). Promoter function therefore also appears to be influenced by structures and information outside the classical promoter region (between +1 and -36, +1 being the first nucleotide transcribed). Promoters having the most efficient transcriptional initiation signals, namely those of the coliphages T5 and T7 also show homology around position -43 and between +1 and +20 (Gentz and Bujard, 1985; Kammerer *et al.*, 1986).

Based on the analysis of conserved sequences of efficient promoters (Gentz and Bujard, 1985), a promoter sequence can be re-defined as extending from position +20 to -50 (Kammerer *et al.*, 1986). This region has been subdivided into a "core" consisting of the -10 and -35 region, an upstream region centred around position -43 consisting of a block of A's, and a downstream region consisting of the conserved pentamer TTTGA and a stretch of purines between +9 and +18. In principle therefore, promoters with similar "core" regions may not necessarily have identical signal strengths, since they may differ at these additional sites.

The importance of the predictive value of these "classical" RNAP recognition sites, however, should not be underestimated. The extensive studies of structural homologies and consensus sequences (Rosenberg and Court, 1979; Hawley and McClure, 1983) do support the importance to promoter function of the conserved sequence elements. In addition, over 100 promoter mutations have been

documented with nearly all of the altered base pairs in the mutants conforming to the general rule that down-mutations decrease homology and up-mutations increase homology to the consensus sequence of this core region (Hawley and McClure, 1983)

The -10 region of the ORF shows perfect homology to the consensus sequence proposed for *E. coli* promoter sequences (Figure 4.3). Within the large group of studied promoters, variation occurs from this hexamer sequence, TATAAT, with a strong bias being displayed for four of these nucleotides. This hexamer sequence can therefore be re-written as TAtAaT, with the capital letters representing bases which are conserved in over 60% of the promoter regions studied (Siebenlist *et al.*, 1980; Hawley and McClure, 1983). The importance of these bases in promoter function has been well documented with base specific mutations in these regions resulting in marked decreases in promoter activity (Rosenberg and Court, 1979; Hawley and McClure, 1983). The last base of this hexamer, the T, is the strongest conserved element and is almost invariant (Hawley and McClure, 1983). This is thought to be one of the regions where the critical contacts between RNAP and the promoter occur, and an induced local melting or strand separation of the promoter allows further interaction of the RNA-polymerase with the DNA (Dubendorff *et al.*, 1987).

The -35 region has been implicated in the initial recognition of the promoter site by the RNA polymerase, prior to formation of the tightly bound initiation complex (Schaller *et al.*, 1975). By following the same pattern of conservation of nucleotides in various promoters (Hawley and McClure, 1983), the -35 recognition site can be written as TTGACa, with the capital letters representing nucleotides which are over 60% conserved. The -35 region preceding the ORF in this case is less conserved than its -10 counterpart (Figure 4.3). The conserved hexamer TTGACa is represented by the sequence ICAACG, where 3 of the 6 bases are identical.

Base changes that increase homology to this -35 consensus sequence result in promoter-up mutations (Hawley and McClure, 1983), and it would be expected that similar changes in the -35 region of the ORF would result in a more efficient promoter. The base changes resulting from promoter-down mutations which occur in the -35 region, however, are more difficult to interpret. Most of these

mutations occur in promoters that are positively regulated and thus the mutation may be affecting the positive regulation system rather than the direct interaction of RNAP.

Although the nucleotide sequences upstream of the -35 region in most promoters are generally A/T rich (Rosenberg and Court, 1979), conserved sequence homologies are not observed. Instead, the prominent feature of this upstream region (USR), is the predominance of A's centred around position -43 (Gentz and Bujard, 1985). The USR of the ORF is rich in A's and this extends from the -40 region to just past the -50 region. The downstream region (DSR) of the ORF, (i.e. the transcribed sequence) does not have the conserved pentamer TTTGA or the stretch of purines between the +9 and +18 residues.

The information derived from the nucleotide sequence, taken collectively, is highly suggestive of a functional promoter preceding the ORF. Utilizing the above-mentioned parameters (and sequence homology), the prediction of a promoter region can be very accurate (Siebenlist *et al.*, 1980; Rosenberg and Court, 1979; Hawley and McClure, 1983; Gentz and Bujard, 1985). However, it should be stressed that although such a promoter region can be recognized, nucleotide sequence and structural data are not sufficient to predict the "strengths" of these promoters.

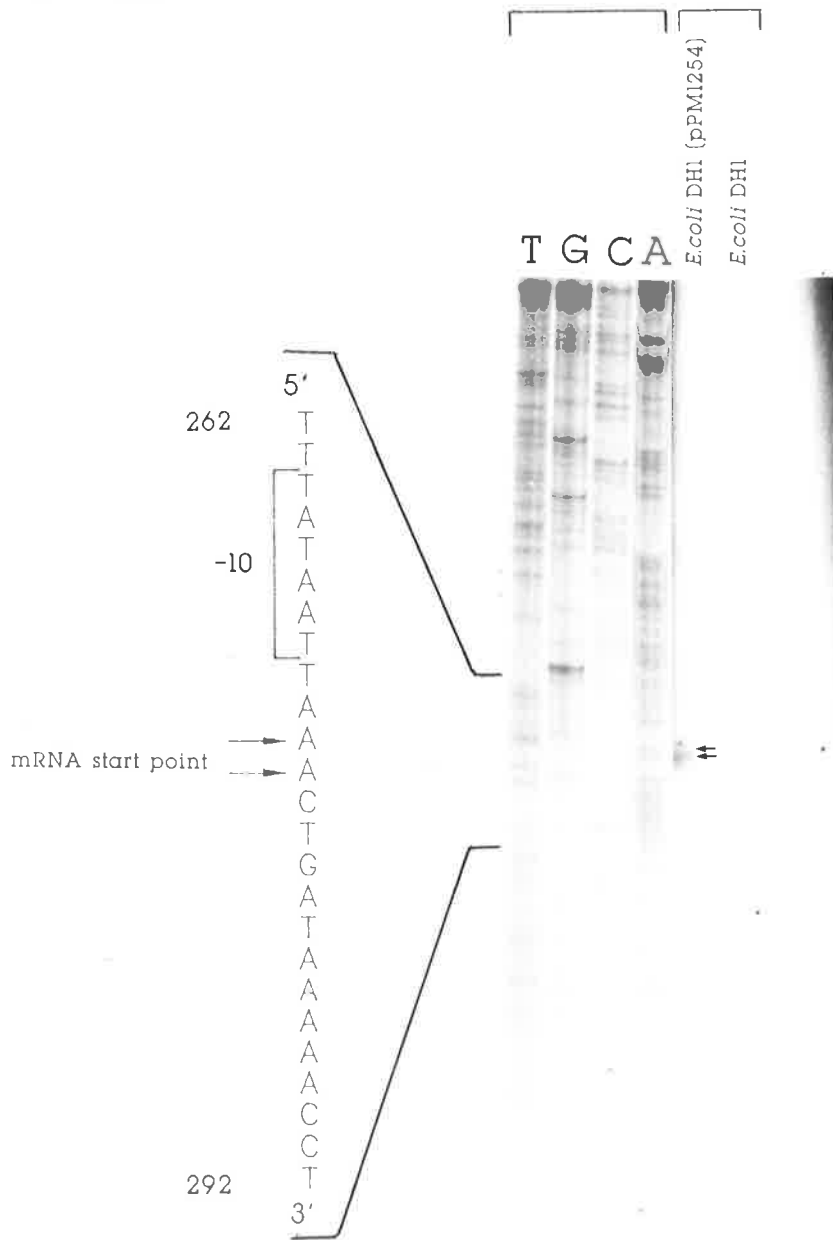
4.2.2.2 Primer extension

To identify the site of transcriptional initiation, and to confirm the predicted promoter of the putative DNase gene, primer analysis was performed. Cellular RNA from the *E.coli* strain harbouring pPM1207 to enrich for DNase transcripts was used as a source of mRNA. A 24nt synthetic primer (3'TCAGTACGGTTTTTACTTCGCCAC 5') complementary to nt 383 through 406 of the sequenced 1.25kb DNA fragment (Figure 4.3) was 5' [³²P]-labelled, annealed to the clone pPM1207, and a sequencing ladder was prepared by the double-stranded DNA dideoxy method. The primer was annealed to the RNA and extended by AMV reverse transcriptase. The extension products were displayed on a sequencing gel (Figure 4.4). The minor products could be due to minor transcriptional initiation sites, but are more likely due to the premature stops of the

Figure 4.4 Primer extension mapping of the DNase promoter

The autoradiograph of a 6% polyacrylamide gel used to analyze a DNA primer extended by reverse transcriptase is shown. Equivalent amounts of [³²P]-end-labelled DNA primer were used for both the primer extension and the sequencing ladder. The sequencing ladder was generated by double-stranded dideoxy sequencing of pPM1254 with Klenow. For the primer extension reaction, 20μg of total RNA isolated from *E.coli* DH1 carrying pPM1254 was used, with the control reaction utilizing total RNA from *E.coli* DH1 alone. The numbers correspond to the nucleotide position and the arrows indicate the position of the start sites of the mRNA (see Figure 4.3).

24hours exposure 96 hours exposure



reverse transcriptase along the RNA before reaching the 5' end of the full size transcript. The major start sites are at position A₂₇₅ and A₂₇₆. These start sites are consistent with the location of the predicted promoter (Figure 4.3). The presence of two start sites can be accounted for by the slippage or flexibility often seen with RNA polymerase when an initiation site is rich in A's (Rosenberg and Court, 1979).

4.2.2.3 Terminators

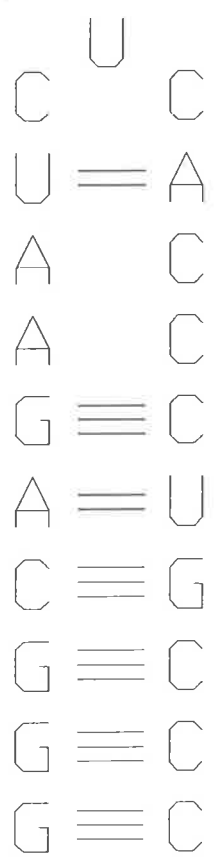
Termination of transcription occurs at the ends of genes and operons and is important in preventing transcription from one region of the chromosome from interfering with that of another. Transcription termination also occurs within genes and operons and the associated sites, terminators, have three common features; an inverted repeat sequence, U residues are found in the terminus of the RNA transcript, and G/C rich sequences are found preceding the stop site. These terminators offer a wide range in overall efficiency (i.e. strength) and dependence on the protein termination factor, Rho.

An inverted repeat sequence can be found in all termination sites (Rosenberg and Court, 1979) with the potential ability to form stable base-paired stem and loop type structures. The sequence immediately preceding the ORF, displays such a region of hyphenated dyad symmetry (inverted repeat sequence), and the formation of the potential stem and loop structure has a free energy of -14.4kcal/mol (Tinocco *et al.*, 1973), thereby making such a structure energetically favourable (Figure 4.5). This region is rich in G/C sequences, and although the G/C base pairs are important in stabilizing the intramolecular stem and loop structure, they may also aid in termination by impeding RNA polymerase movement on the template (Gilbert, 1976). Most of the terminated RNA transcripts end in a run of 4 to 8 U residues, 20 (± 4) nucleotides beyond the centre of the dyad symmetry (Rosenberg and Court, 1979). These U's are thought to aid in the release of the transcript. The proposed transcriptional terminator following the ORF (Figure 4.5) has a run of 4 consecutive U's with the transcript ending 20 nucleotides from the centre of the inverted repeat sequence.

Rho-dependent terminators generally do not encompass all of these elements in their termination sites, and the RNAP does not terminate unless Rho is present,

Figure 4.5 A possible transcriptional terminator corresponding to nt 998 to 1032 of the sequence in Figure 4.3.

This structure has a free energy of -14.4kcal/Mol (Tinoco *et al.*,1973), and represents a possible Rho- independent terminator. The termination codon of the gene is indicated by asterisks.



5' UGA U

CUUGUAUUUU 3'

although a substantial pause in the movement of the RNAP is observed in the absence of Rho (Rosenberg *et al.*, 1978). On the other hand, RNAP usually stops completely at Rho-independent termination sites and releases the transcript without the requirement for added Rho protein (Rosenberg and Court, 1979). The potential terminator site described in Figure 4.5, thus, appears to be typical of a Rho-independent transcriptional terminator.

4.2.2.4 Translation Initiation

The ribosome binding site and translation of this protein should also be considered. A ribosome binding site consists essentially of an initiation codon, a Shine-Dalgarno sequence (complementary to the 3' end of 16S RNA), and appropriate spacing between the two. The sequence TCTACGT just prior to the initiation codon of the ORF, shows poor homology to the conserved sequence (AGGAGGT), which may explain the low levels of gene product expressed (Chapter 3).

Mutations in the Shine Dalgarno region have been shown to drastically affect the level of initiation of translation, and closer homology to this consensus sequence (Shine and Dalgarno, 1974) usually corresponds to ribosome binding sites that are "stronger" (Gold *et al.*, 1981; Kozak, 1983). The spacing of the Shine-Dalgarno sequence is 7 nucleotides prior to the initiation codon of the ORF, and this correlates well with the average spacing of 7 nucleotides usually seen (spacings less than 5 and longer than 9 nucleotides are rare). Reducing this spacing results in mutations that affect translation (Gold *et al.*, 1981; Kozak, 1983). These structural and sequence similarities seen in ribosome binding sites, however, follow the same rule as promoter regions where other factors are also involved, as the initiation codon and polypurine tract (AGGAGG) are not the only elements involved in the translation initiation process.

4.2.3 Signal sequence of the DNase

The existence of precursors for secretory proteins was first demonstrated by Milstein *et al.*, (1972). They showed that immunoglobulin light chains were produced from precursors of slightly larger molecular weights. The signal hypothesis which offered a precise description for the translation steps of secretory proteins was proposed by Blobel and Dobberstein (1975a,b).

In prokaryotes, the existence of the signal peptide was first shown for the major outer membrane lipoprotein (Halegoua *et al.*, 1976; Inouye *et al.*, 1977). Since then the existence of signal peptides has been shown for many other prokaryotic secreted proteins. A comparison of the general structure of different signal peptides demonstrates that, independent of their origin, they share a number of common features, and these can be related to their possible functional roles in protein secretion (Inouye and Halegoua, 1980; von Heijne, 1983).

The rules which govern the structure of a signal peptide extend to five common features. These features will be listed, and the signal peptide of the DNase can then be discussed in terms of these elements.

1. One to three positively charged amino acids are in the first five residues in the amino-terminal region of the signal peptide (amino-terminal basic region).
2. A hydrophobic sequence of 9-20 amino acids follow the amino-terminal basic region (hydrophobic domain).
3. One to two proline and/or glycine residues are usually present within the hydrophobic domain.
4. The presence of a serine and/or threonine residue(s) within the hydrophobic domain, divides it into two regions of different degrees of hydrophobicity.
5. An alanine or glycine residue (serine to a lesser extent) is present at the carboxy terminus of the signal peptide (cleavage site).

In every known prokaryotic signal peptide, there is, without exception, one to five positively charged amino acid residues (lysine and/or arginine and occasionally histidine) located at the amino-terminal position (von Heijne, 1983; Watson, 1984). The role of these positive charges is thought to be for the initial interaction between the signal peptide and the inner surface of the negatively charged cytoplasmic membrane. Figure 4.6 shows the proposed signal sequence and cleavage site of the DNase, and compares it with other known signal sequences of extracellular proteins and the outer membrane protein *ompV*, of *V. cholerae*. The DNase (ORF) has a positively charged arginine residue at the fifth position, while the other *V. cholerae* exported proteins have one or two positively charged lysine residues within the first five amino acids. The hydrophobic domain requires at least 9 hydrophobic residues, i.e. sufficient to span a membrane. The *V. cholerae* signal peptides in Figure 4.6 all fit this rule, with at least 15 consecutive hydrophobic amino acids.

Glycine and proline are known to be involved in the β -turn structure of proteins and are often referred to as helix breaking residues. A helix breaking residue frequently occurs 4 to 8 residues before the cleavage site (von Heijne, 1983). The DNase and OmpV have a proline and glycine residue, respectively, within this hydrophobic core. The proline is 6 residues before the cleavage site, while the glycine in OmpV is at the fifth residue from the cleavage site. Both cholera toxin subunits A and B, and the haemolysin of *V. cholerae* do not have a proline or glycine residue present in this region. The exact role of these amino acids in the hydrophobic domain is not clear, although mutations that alter or delete the glycine at position 7 of prolipoprotein show a reduction in the efficiency of signal peptide cleavage by the signal peptidase (Inouye *et al.*, 1984). The "helix-bending" property of these residues may serve to bring the cleavage recognition site into juxtaposition with the signal peptidase for efficient cleavage of the signal peptide.

The fourth conserved feature of signal peptides is the presence of a serine or threonine residue that divides the hydrophobic domain into an extremely hydrophobic centre, and a less hydrophobic portion towards the cleavage site. This feature is common to all the *V. cholerae* signal peptides in Figure 4.6, but the structural function of these residues is not well understood, although, it has been

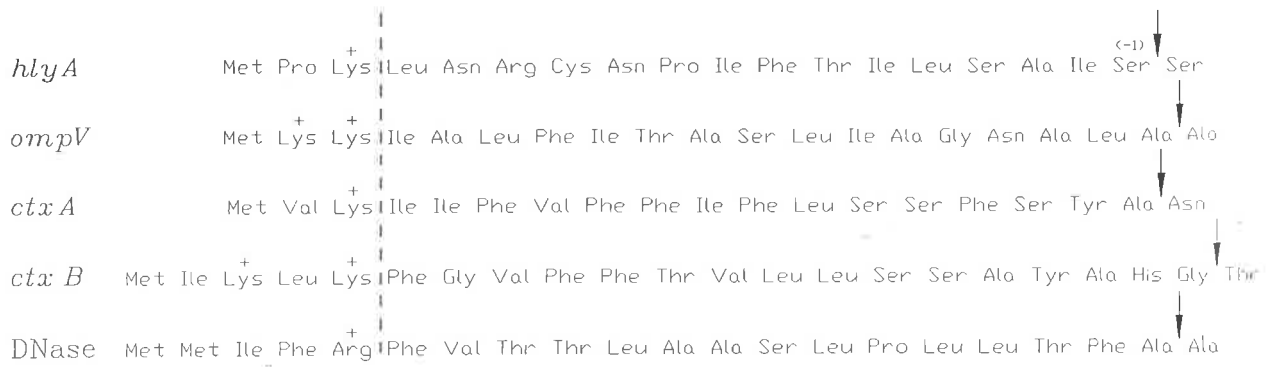
Figure 4.6 Signal sequences of some exported proteins of *V.cholerae*

The signal sequences of some of the exported proteins of *V.cholerae* have been determined. All have at least one positively charged residue at the N-terminal end, a hydrophobic core, and a cleavage site at either an ala, ser, or gly residue, consistent with the rules of von Heijne, (1983). The cleavage site of OmpV (Pohlner *et al.*,1986) and cholera toxin A and B (Mekalanos *et al.*, 1983) have all been determined by N-terminal analysis of the mature protein, while the DNase and HlyA (Alm *et al.*,1988) show the proposed cleavage sites.

N-TERMINAL
POSITIVE CHARGED
RESIDUE

HYDROPHOBIC CORE

CLEAVAGE
SITE



suggested that they are involved in the formation of a specific conformation necessary for the translocation event (Wickner, 1983). The final aspect of a signal peptide to be considered is the striking prevalence of amino acids with small, uncharged side chains at the cleavage site. This position (-1) is filled by alanine in most cases, with glycine being found to a lesser extent, and finally serine in a small proportion of cases (Watson, 1984). The DNase, OmpV and cholera toxin subunit A all have an alanine cleavage site, while cholera toxin subunit B has a glycine residue at position -1, and the haemolysin a serine (Figure 4.5). This rather strict requirement at the cleavage site may reflect signal peptidase substrate specificity.

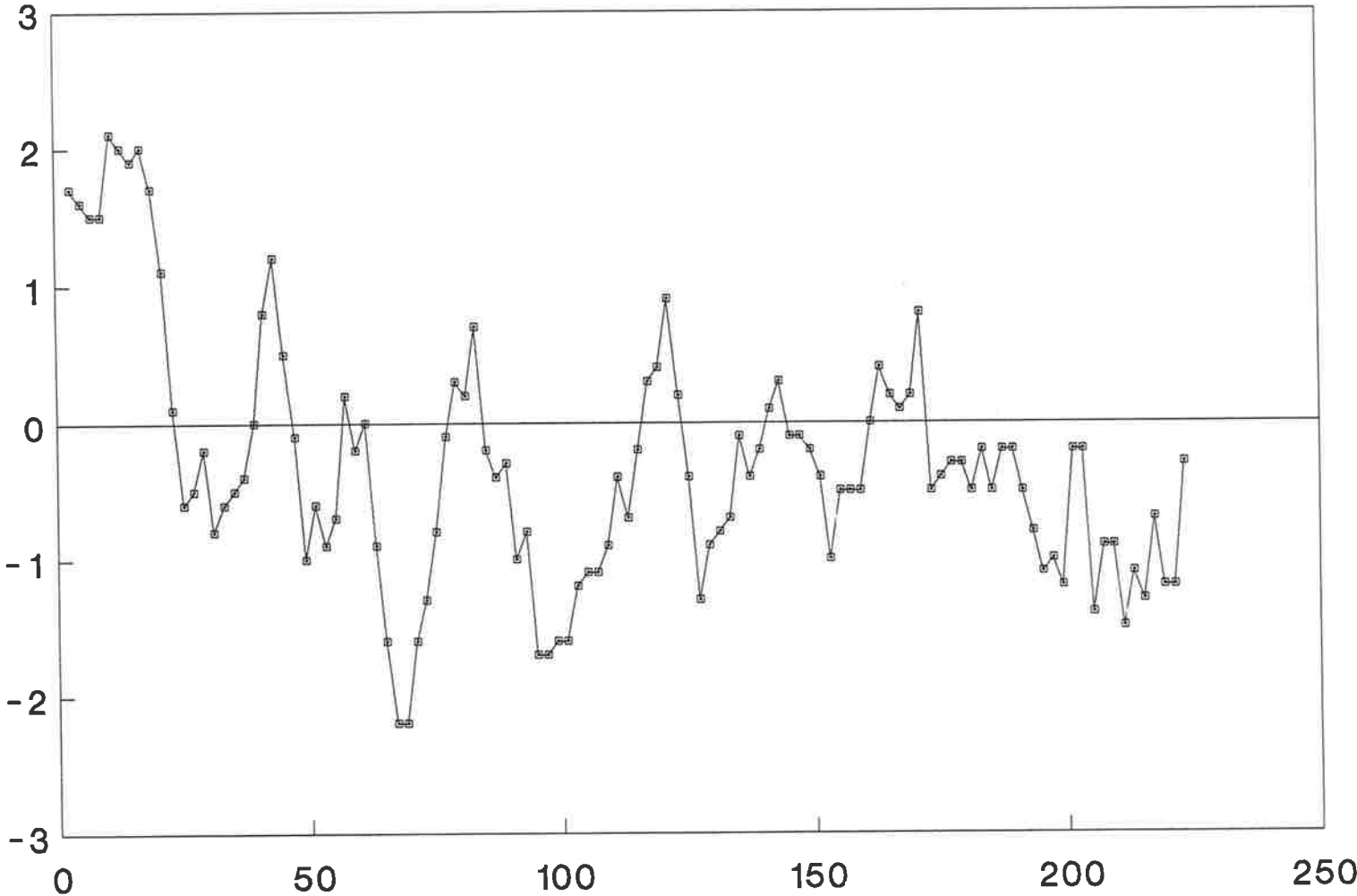
While the proposed signal sequence of the DNase, and the other known signal peptides of *V. cholerae* (Figure 4.6) conform to the rules set for prokaryotic signal peptides (von Heijne, 1983), the exact cleavage site of the DNase signal peptide has yet to be confirmed experimentally and can only be predicted. The other 4 signal peptides and their cleavage sites illustrated in Figure 4.6 have been determined by sequencing of the amino-terminal portion of the mature protein. The low amounts of DNase protein detected in either *V. cholerae*, or the recombinant plasmid pPM1207 (Chapter 3, Section 3.2.9) has made the purification of significant amounts of this protein, necessary for N-terminal amino acid analysis, difficult.

An hydropathy plot of the DNase protein, according to Kyte and Doolittle (1982), (Figure 4.7), suggests that the protein is very hydrophilic, as expected for a water-soluble protein. This is in agreement with the location of the DNase protein in *V. cholerae* where it is extracellular, and its periplasmic location in *E.coli*. There is, however, a region of marked hydrophobicity at the amino-terminal portion of the proposed pro-DNase protein. This region corresponds to the hydrophobic domain of the predicted signal peptide of the DNase protein, while the end of this region at about aa 20, correlates to the predicted cleavage site and N-terminus of the mature protein (Figure 4.7). A predicted secondary structure according to Chou and Fasman (1974a, 1974b, 1978) is shown in Figure 4.8. In particular, the arrangement of the Cys residues is interesting. Cys₉₄ and Cys₁₀₄ as well as Cys₂₀₇ and Cys₂₂₈ are closely linked to turn regions and in close proximity to each other, and it seems possible that they may be crosslinked. The other four residues are

Figure 4.7 Hydropathic nature of the pro-DNase

The predicted amino acid sequence of the precursor form of the DNase was analyzed according to Kyte and Doolittle (1982) using a window of nine amino acids. The protein is markedly hydrophilic as would be expected for a water-soluble protein. The region of hydrophobicity at the N-terminus of this protein is contained within the proposed signal sequence.





Hydrophobic

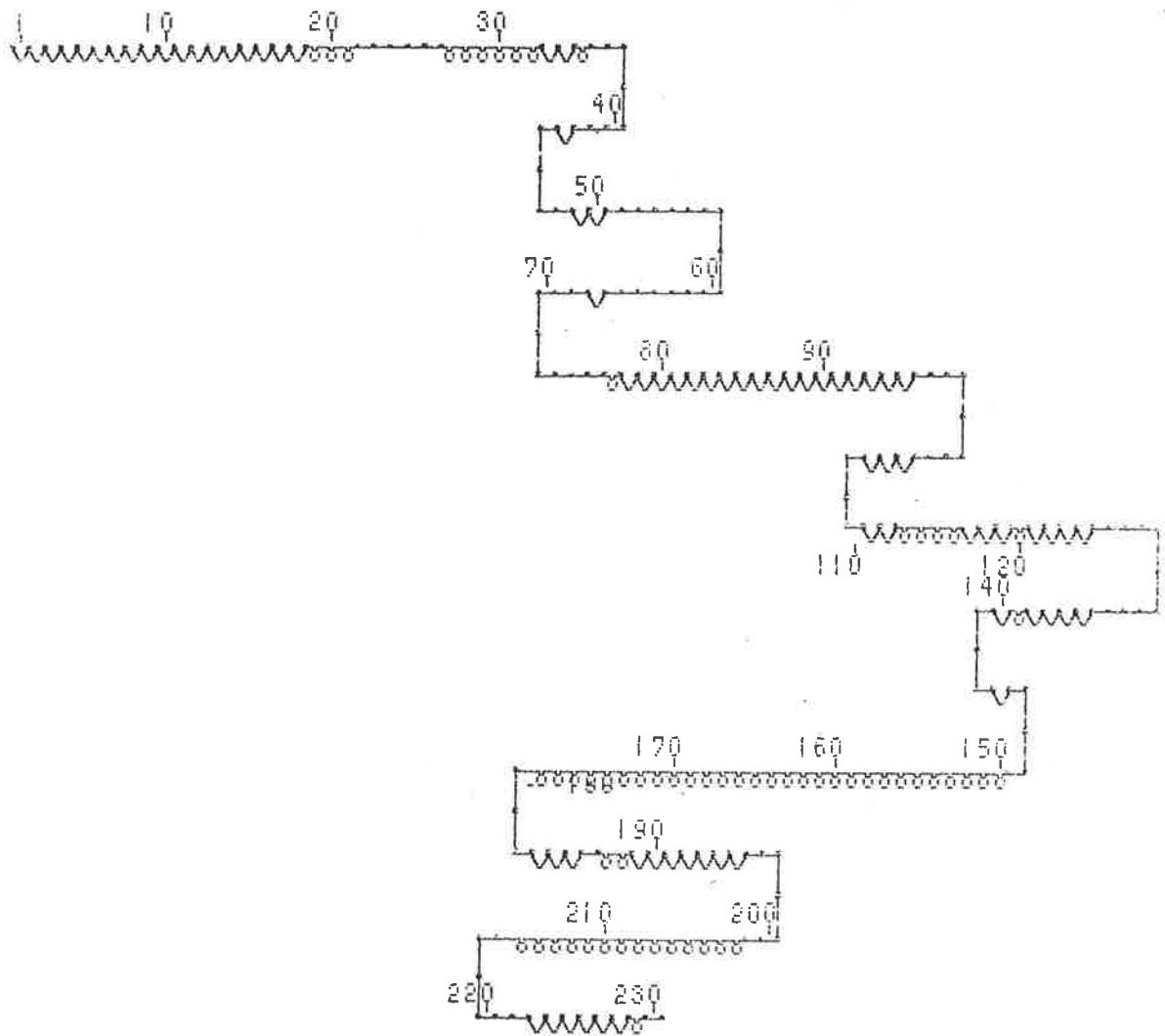


Hydrophilic

Figure 4.8 Predicted secondary structure of the DNase protein according to Chou and Fasman (1978)

The DNase does not display any helix-turn-helix domains which are common to DNA-binding proteins (McKay and Steitz, 1981; Ohlendorf *et al.*, 1983)

α helix	
β sheet	
β turn	
random coil	



also closely linked to turn regions, but while Cys₄₅, Cys₄₇ and Cys₆₃ are in close proximity, Cys₁₅₀ is located a good distance from these three cysteine residues in this proposed secondary structure. Possibly they are involved in the formation of the tertiary structure of this protein, as the reduction of disulphide bonds with β -mercaptoethanol results in the loss of activity of the DNase (Section 3.2.8).

4.2.4 Recombinant system for the overexpression of the DNase

With the aims of obtaining high levels of expression, a system was constructed in which the gene encoding the DNase of *V.cholerae* was placed under the control of the strong IPTG-inducible *tac* promoter (Amann *et al.*, 1983; DeBoer *et al.*, 1983). Nucleotide sequence analysis (Section 4.2.2) shows the DNase to have a very poor potential Shine-Dalgarno site, and it was thought this may have been the reason for the low level of protein expressed by the DNase gene. For this reason, a vector was used which contained both the *tac* promoter and a consensus Shine-Dalgarno sequence. This expression vector, pEG612, was constructed and obtained from Anthony Brumby (Department of Biochemistry, Adelaide University). The expression vector pEG612 utilizes the *NdeI* cloning site, to clone the N-terminal portion of a gene at its startpoint of translation (AUG), into this site, creating a gene fusion with the strong promoter and ribosome binding site of the vector. Gene fusions constructed using this vector have resulted in the high level expression of phage encoded proteins (A. Brumby - personal communication).

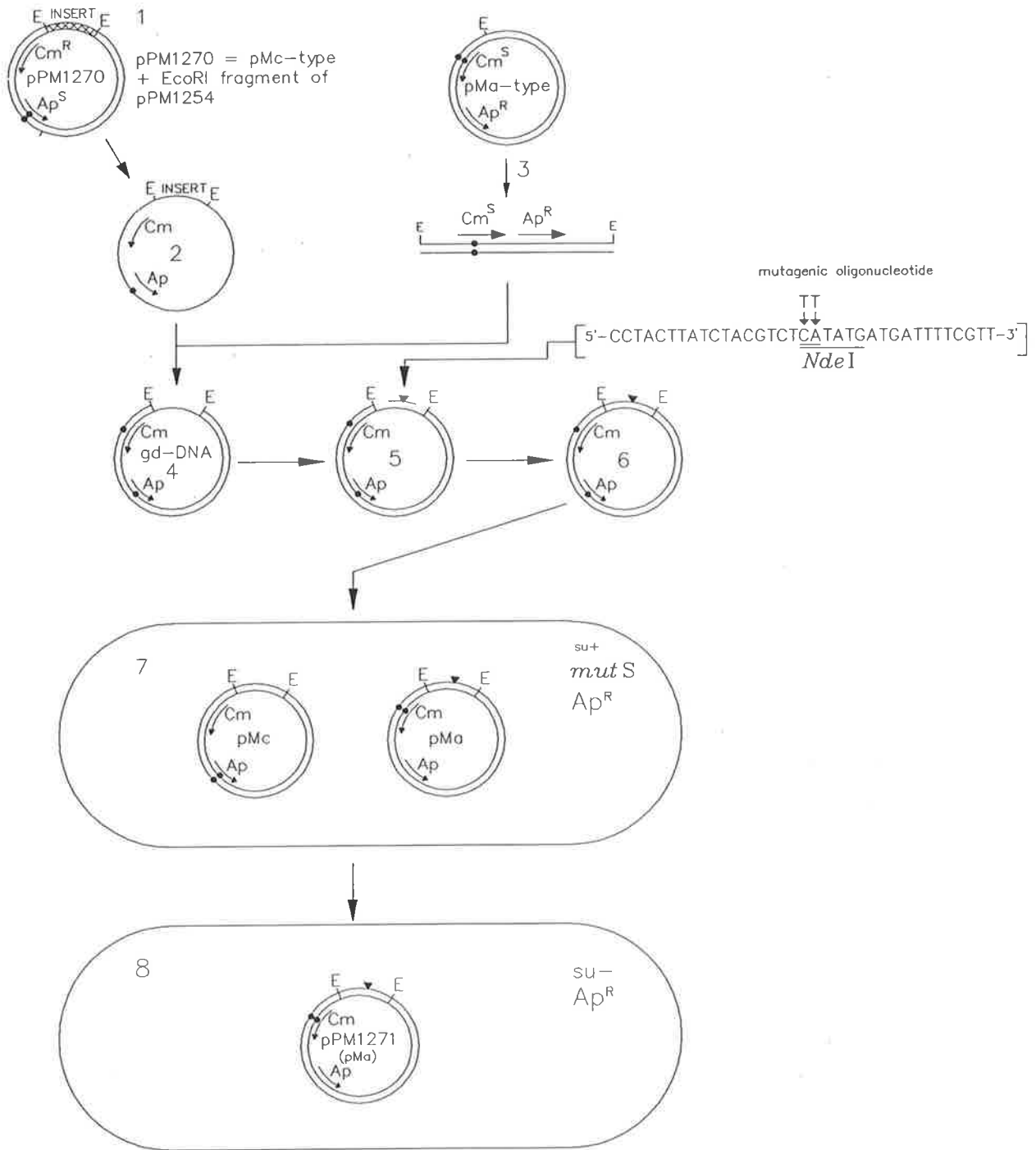
No restriction endonuclease cleavage sites are present near the translation initiation site of the DNase ORF, and this necessitated the construction of an *NdeI* site by site-directed oligodeoxynucleotide mutagenesis. The oligodeoxynucleotide 5'CTACTTATCTACGTCTCATATGATGATTTTTTCG 3' (33-mer) was used to mutagenize nt 289 through 321 to introduce an *NdeI* site (Figure 4.9). Plasmids with the introduced *NdeI* site could be identified by digesting with *NdeI* as there are no other sites for this enzyme in the cloned DNA. The resulting plasmid pPM1271, was then used as a source of the DNase gene to clone into the expression vector pEG612. The *NdeI*/*EcoRV* fragment of pPM1271 was cloned

Figure 4.9 Site-directed oligonucleotide mutagenesis to introduce an *Nde*I site at the start of the DNase

Schematic representation of a single mutagenesis round. The amber mutations in the Ap and Cm selectable markers are shown by closed circles. The mutation itself is shown by the replacement of two T's with a CA in the oligonucleotide to create an *Nde*I site, and is indicated by the arrowhead.

The individual steps of the process are as follows:

1. Cloning of the *Eco*RI DNA fragment of pPM1254 into pMc5-14. This vector carries an amber mutation in the Ap^R gene and specifies Cm^R.
2. Preparation of s/s DNA of this recombinant from pseudoviral particles.
3. Preparation of a restriction fragment from the complementary pMa-type phasmid. pMa-type vectors contain the wild type Ap^R gene with an amber mutation in the Cm^R gene.
4. Construction of gapped-duplex DNA (gdDNA) by *in vitro* DNA/DNA hybridization. In the gdDNA the target sequences are exposed as s/s DNA.
5. Annealing of the synthetic oligonucleotide to the gdDNA.
6. Filling in the remaining gaps and sealing of the nicks by a simultaneous *in vitro* DNA polymerase/DNA ligase reaction.
7. Transformation of a *mutS* host, i.e. a strain deficient in mismatch repair and su⁺, selecting for Cm^R. This results in production of mixed phasmid progeny.
8. Elimination of progeny deriving from the template strand (pMc-type) by retransformation into a suppressor free (su⁻) host. Selection for Ap^R results in enrichment of the progeny derived from the gapped strand, i.e. the strand from which the mutagenic oligonucleotide has been incorporated. (For further details of this procedure; see Materials and Methods).

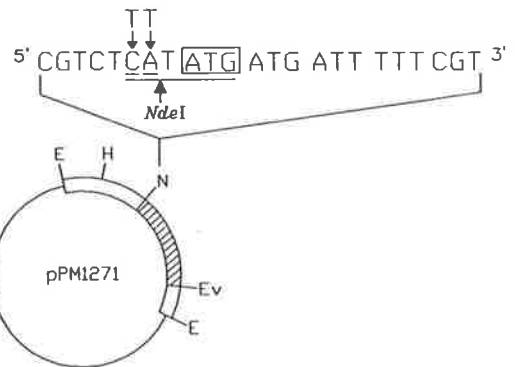
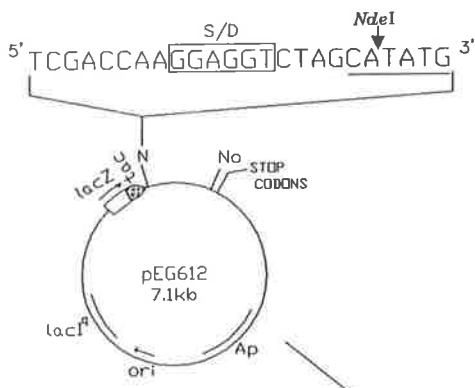


into pEG612 to give the recombinant plasmid pPM1272 (Figure 4.10).

Induction of the DNase on pPM1272 was achieved by growing cells harbouring this plasmid until early exponential phase (OD_{650} of 0.2) at 30°C. The temperature was then shifted to 42°C with the addition of 100mM IPTG, and grown for a further 3 hours. The effect of raising the temperature was to increase copy number of the plasmid as the vector pEG612 also exhibited thermo-inducible runaway replication. The combined effect of a strong transcriptional and translational signal, with the increased gene dosage provided by the runaway replication of the plasmid, was expected to give a significant increase in the level of protein detected. However, analysis of fractions of cells harbouring the plasmid pPM1272, showed no significant increase in amounts of protein. The plasmid pPM1272 was shown to be under the control of the *tac* promoter, as cells without the added inducer IPTG, did not express the DNase, while the addition of IPTG to the plasmid pPM1272 gave a DNase positive phenotype. Cloning of the *NdeI/EcoRV* fragment into the *NdeI* and *HincII* sites of pUC19 where there are no transcriptional or translational signals present upstream, resulted in no expression of the gene. Therefore, while both the transcriptional and translational signals supplied by the expression vector pEG612 are recognized, when the DNase gene minus its own signals is fused to them, the protein product is not overexpressed as expected. The reason for this is not known, but it may be that the mRNA transcript resulting from this gene fusion is unstable. If the DNase mRNA is unstable a possible method of overcoming this would be to construct a protein fusion, by fusing the DNase downstream of a protein which itself is highly expressed. Such a system has proved successful in overexpressing a number of other proteins from a wide variety of bacteria, utilizing the C-terminus of the 26 kDa glutathione S-transferase of the parasitic helminth *Schistosoma japonicum*, as a fusion point (Smith and Johnson, 1988). This, or any alternate strategies have however, at present not been employed.

Figure 4.10 Construction of a gene fusion of the DNase with pEG612.

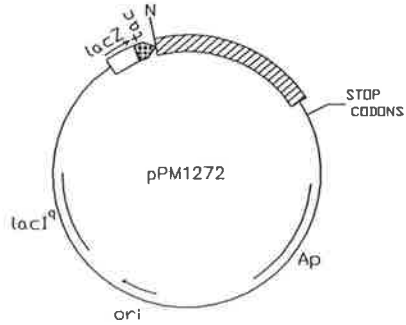
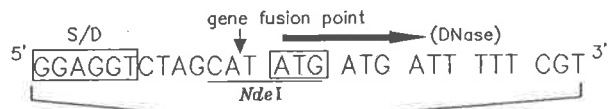
The introduced *Nde*I site at the translational start site of the DNase in pPM1271, was used to fuse the structural gene to the strong transcriptional and translational signals of pEG612. The sequence above pPM1271 shows the change of the dinucleotide TT to CA to create the *Nde*I used to clone into pEG612. The sequence above pPM1272 shows the fusion point of the DNase to pEG612.



digest with NotI
endfill
digest with *NdeI*

digest with *NdeI* and *EcoRV*

ligate



4.3 Conclusion

Determination of the nucleotide sequence of the 1.25kb *V.cholerae* DNA fragment present in pPM1207 has identified a 690 bp ORF which could code for a 26 kDa protein. This ORF has a potential signal sequence, and cleavage of this signal peptide would give a mature protein size of 24 kDa, which is in agreement with the expected size of the DNase. The *EcoRV* site of pPM1207 lies outside this region as expected since a mutation at this site has been shown to retain DNase activity (Chapter 3; Section 3.2.5). As predicted the *BalI* and *NaeI* sites lie within the ORF since insertion of the 8bp *PstI* linkers at both the sites resulted in a DNase negative phenotype (Chapter 3; Section 3.2.5).

The nucleotide sequence upstream of the ORF reveals a potential promoter structure with perfect homology at the -10 position but less conserved at the -35 RNA polymerase recognition region. The promoter has been shown to be functional by fusion of an inactive Ap^R gene downstream of this region, and this will be discussed in Chapter 6. Downstream of this promoter, and 7bp prior to the initiation codon of the ORF is a potential Shine-Dalgarno sequence, but this has low complementarity to the consensus sequence. The lack of a strong consensus S-D sequence could account for the low level of expression observed with this gene. The positions of the promoter and Shine-Dalgarno regions are supported by primer extension analysis, which puts the start of the message at nucleotide position A_{275} and A_{276} (Figure 4.3). The two start sites for the mRNA transcript are not surprising, as this region has three consecutive A's, and RNA polymerase is known to have some flexibility at regions rich in A's (Rosenberg and Court, 1979).

The DNase gene appears to have a good signal sequence which can be expected for an exported protein. This signal sequence was identified by use of the rules proposed by Inouye *et al.*, (1977), Inouye and Halegoua, (1980), and the refined rules of von Heijne, (1983). This signal sequence also appears to be functional, as the DNase is exported from the cytoplasm of *E.coli* into the periplasmic space (although in *V.cholerae* the DNase is exported still further into the extracellular medium). An hydropathy plot of the protein (Kyte and Doolittle, 1982) shows it to be markedly hydrophilic, indicative of a water-soluble protein,

with the only region of hydrophobicity occurring at the N-terminus and probably representing part of the proposed signal peptide. The secondary structure predicted from this protein (Chou and Fasman, 1974a, 1974b, 1978) however, does not exhibit the helix-turn-helix domains usually seen with DNA-binding proteins. This could reflect the fact that the DNase does not interact with the DNA in a specific way as seen with transcriptional activator and repressor proteins (McKay and Steitz, 1981; Ohlendorf *et al.*, 1983). Computer homology searches of both the nucleotide and amino acid sequence of the DNase using the GenBank and EMBL Gene/Protein Sequence Database, has failed to find any other genes with significant homology.

Utilizing information gained from nucleotide sequence analysis, an attempt was made to overproduce the DNase protein. Normal expression of the cloned DNase is very low, and overexpression of this protein could enable its purification, which might allow the N-terminal sequence analysis of the mature protein to be determined to define the signal peptidase cleavage site within the DNase; the production of antisera specific for this protein; and the examination of its enzymatic and physical properties. The expression vector used, consisted of a strong inducible promoter and strong ribosome binding site, which was fused to the translation initiation site of the DNase. Although the vector expression signals were utilized by the DNase gene, an overproduction of the protein was not seen. The reason for this is not known, but may be related to the stability of the mRNA. If this is the case, future work involving the overexpression of this gene may require alternate strategies.

CHAPTER 5

Construction and characterization of defined mutations in the *Vibrio cholerae* chromosome.

5.1 Introduction

Pathogenic bacteria in both man and animals exhibit a number of virulence properties that facilitate the disease causing process. These virulence determinants include the ability to avoid, resist or inactivate host defenses (see Mims, 1982), cell surface structures that are responsible for adherence (Gaastra and DeGraaf, 1982) and probably the best studied and earliest recognized bacterial pathogenesis factor, the production of potent exotoxins (Holmgren and Lonroth, 1980).

With the advent of recombinant DNA technology, rapid advances have been made in understanding the molecular organization of bacterial pathogens. This has been especially true in the analysis of enterotoxin structure and function (So *et al.*, 1976, 1978; Moseley and Falkow, 1980; Pearson and Mekalanos, 1982). The cloning of a bacterial virulence factor, however, does not in itself determine how the factor contributes to the infection of the host.

The analysis of microbial pathogenicity, therefore can efficiently proceed by constructing specific mutants in the organism of interest, and evaluating these mutants for virulence in either an animal host or in a model infection system. Although such a concept is not at all original, it means that the true power of the recent advances in DNA technology lies as much in the construction and analysis of well-defined mutants as in the isolation and sequencing of genes. This can be

best illustrated with the isolation of the first non-toxinogenic *V.cholerae* strain, Texas Star-SR, by Honda and Finkelstein (1979). This strain was isolated with a mutation in one of the cholera toxin structural genes. However, because the strain was subjected to heavy mutagenesis with the chemical mutagen NTG, other phenotypic alterations unrelated to toxin production were present in this mutant, which have included other virulence determinants since the resultant strain was a poor colonizer. Additionally, as the nature of the mutation in the toxin structural gene was unknown, reversion to a toxinogenic strain was also a possibility. The subsequent cloning of the genes encoding cholera toxin (Pearson and Mekalanos, 1982; Gennaro *et al.*, 1982), allowed the introduction of defined mutations in the cholera toxin genes, and the incorporation of these specific deletions into the chromosome of an otherwise wild-type *V.cholerae* strain (Mekalanos *et al.*, 1983; Kaper *et al.*, 1984b), enabling such strains to be evaluated as possible vaccine candidates.

The increased application of genetic analysis to the study of bacterial pathogens has resulted in the discovery of new factors, as has been described for *toxR* and *toxS*, which are responsible for the coordinate regulation of many virulence determinants in *V.cholerae* (Miller and Mekalanos, 1984; Peterson *et al.*, 1988).

This approach is not limited to the study of the effects on virulence. For example, defined mutations introduced into an organism can be used to study a number of other cellular processes. This chapter describes the construction of a DNase negative *V.cholerae* strain in order to evaluate the role it may play in the pathogenesis of the disease, but also looks at a possible secondary role the DNase may play in the overall physiology of the organism.

Extracellular nucleases have been shown to act as a barrier to the uptake of exogenous DNA molecules, in both transformation and transfection in *S.marcescens* (Timmis and Winkler, 1973; Reid *et al.*, 1982). The construction of mutants deleted specifically for the DNase of *V.cholerae* has allowed the role the DNase may play in this process also to be assessed. These defined mutations have also led to the subsequent recognition of two distinct DNases being elaborated by *V.cholerae*.

5.2 Results

5.2.1 Construction of DNase negative *Vibrio cholerae* strains

5.2.1.1 Chemical mutagenesis with EMS

Attempts to isolate a DNase negative *V.cholerae* strain were originally carried out using the chemical mutagen ethyl methanesulfonate (EMS). EMS is an alkylating agent which is quite specific in its action, producing primarily GC-AT transition mutations. This mutagen was chosen over NTG, because although it has a similar mutational effect, it is less likely to yield multiple mutations. Mutants defective for DNase production were screened by looking for colonies without a zone of clearing on DNA-agar plates after the addition of 1N HCl. Approximately 10,000 colonies were screened, but although a high proportion of these cells were auxotrophs, no DNase negative *V.cholerae* mutants were isolated.

5.2.1.2 Transposon mutagenesis of *Vibrio cholerae* with Tn5

Since the isolation of a *V.cholerae* DNase negative mutant by chemical mutagenesis proved unsuccessful, transposon insertion mutagenesis of the *V.cholerae* chromosome was attempted. Advantages of using transposable elements to isolate such mutants are several-fold. Insertion mutations usually result in totally eliminating the expression of the gene; their phenotype is rarely leaky. They are easy to map as the insertion of a drug-resistance transposon enables a mutation to be located either by changes in the restriction pattern of a DNA fragment or by genetically mapping the site of drug resistance. Finally, they are easy to isolate and are almost always the result of only a single insertional event, so having secondary mutations in the same strains is not a problem. For these reasons, mutagenesis of the *V.cholerae* chromosome was carried out using the transposon Tn5. Insertions of Tn5 onto the chromosome were isolated by selecting for Km^R after introduction of the replication-defective (F'_{ts}/ac::Tn5) plasmid that harboured the transposon. Each Km^R colony that grew was the

result of a transposon-induced mutation, so in theory, fewer colonies would need to be screened by this method in order to obtain the lesion in the desired DNase gene, as opposed to the chemical mutagenesis method. Over 8,000 colonies were screened in a manner similar to that described for EMS mutagenesis. Whilst mutants for other extracellular proteins such as the haemolysin were obtained, no DNase negative *V.cholerae* mutants were isolated.

5.2.1.3 Transposon mutagenesis of *V.cholerae* with *TnphoA*

A modification of the transposon insertion mutagenesis method was attempted, using the transposon *TnphoA* (Manoil and Beckwith, 1985). This transposon *TnphoA* encodes for Km^R , and utilizes the alkaline phosphatase gene (which is deleted for its signal sequence) to select for exported proteins. In-frame insertions of *TnphoA* downstream of an exported protein results in hybrid proteins composed of alkaline phosphatase fused to amino-terminal sequences of these proteins. Such a hybrid gives alkaline phosphatase activity if the protein fused to alkaline phosphatase contributes sequences that promote export and thus compensate for the missing alkaline phosphatase signal peptide. The frequency of an in-frame *TnphoA* insertion into an exported protein is about 1% (Manoil and Beckwith, 1985), so that if 100 *TnphoA* insertions with alkaline phosphatase activity were screened, this would represent 10,000 colonies screened by the Tn5 insertion mutagenesis method if isolating a lesion in an exported gene, as this former method highly enriches for this class of mutant. In fact, 250 colonies were isolated which had Km^R and alkaline phosphatase activity. A number of mutations in extracellular proteins were isolated, including an extracellular protease(s), but no DNase negative phenotypes were among these.

The failure to obtain a DNase negative *V.cholerae* by any of these methods suggests that more than one gene may have been involved in DNase production, and the isolation of a DNase negative mutant was not simply a matter of screening for an insertion in a single gene.

5.2.2 Site-specific recombination of a DNase negative mutant into the *Vibrio cholerae* chromosome.

5.2.2.1 Insertion of a Km^R cartridge into the DNase gene.

To ascertain if a mutation in a single gene was sufficient to isolate a DNase negative mutant of *V.cholerae*, a mutation in the cloned DNase gene was constructed *in vitro*, and recombined back into the *V.cholerae* chromosome.

The system chosen was that utilized by Mekalanos *et al.*, (1983), and Kaper *et al.*, (1984), to introduce cholera toxin deletions back into *V.cholerae* by *in vivo* recombination. This system made use of the broad host range cloning vehicle, pRK290, developed by Ditta and co-workers (1980). This plasmid is a deletion derivative of the larger plasmid RK2, it is no longer capable of self-conjugal transfer, but still retains a Tc^R phenotype. It has unique cloning sites for the restriction endonucleases *EcoRI* and *BglII*.

To clone into the vector pRK290, a mutation within the DNase gene was constructed in the plasmid pPM1254. This mutation consisted of the insertion of *PstI* linkers into the *BaII* site within the gene. The plasmid pPM1209, Chapter 3; Figure 3.5, which contained *PstI* linkers at this site was not suitable, as the gene was contained on an *EcoRI/HindIII* fragment and could not be cloned directly into pRK290.

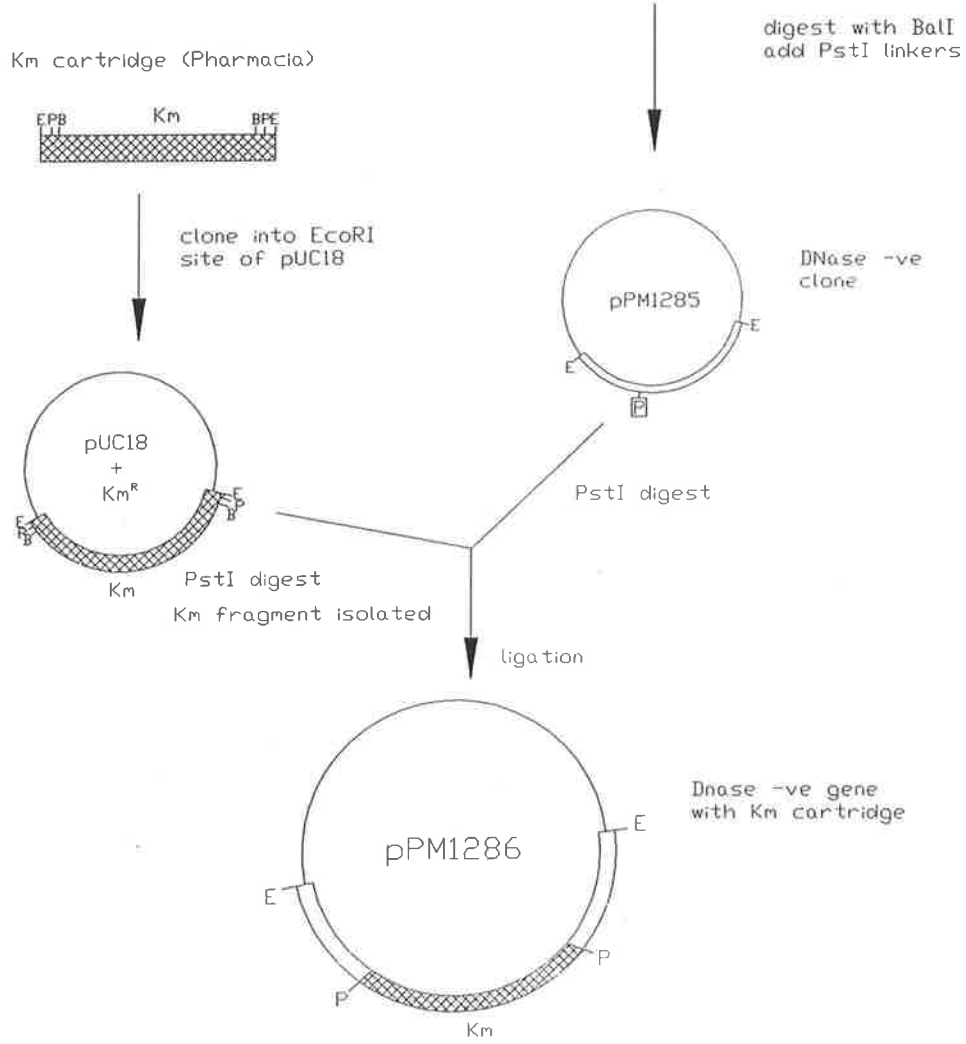
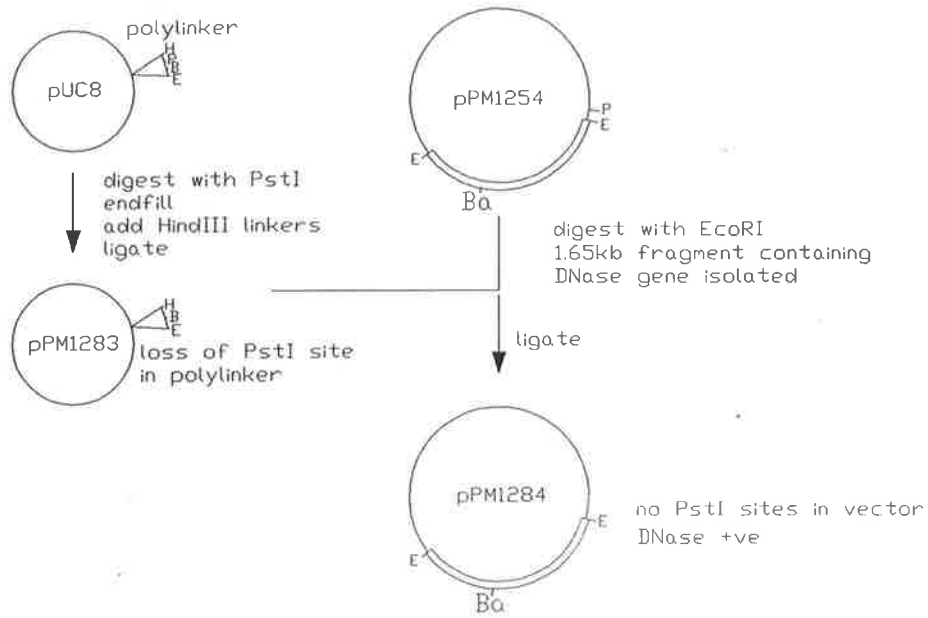
While the *PstI* linkers introduced a mutation into the DNase, the introduced *PstI* site was used to insert an antibiotic resistance between the gene, to select for the recombination of this mutant into the *V.cholerae* chromosome by the acquisition of an antibiotic resistance, rather than screening for a DNase negative phenotype. The antibiotic resistance gene inserted was a Km^R of 1500bp. This cartridge was first cloned into the *EcoRI* site of pUC18, and the $\times PstI$ sites also present on the ends of this cartridge were used to transfer the Km^R gene into the *PstI* site of pPM1285 to give the plasmid pPM1286 (Figure 5.1).

Figure 5.1 Construction of the DNase negative, Km^R plasmid pPM1286.

Plasmid pUC8 was digested with *Pst*I, and *Hind*III linkers added to eliminate the *Pst*I site in the polylinker region, to give plasmid pPM1283. The *Eco*RI fragment of the DNase was cloned into this newly constructed vector to give pPM1284. The absence of a *Pst*I site in pPM1284 allowed the introduction of *Pst*I linkers into the *Bal*I site of the structural gene of the DNase. The open box indicates the added *Pst*I linker. The resulting DNase negative plasmid pPM1285, was then inserted with the Km^R gene into the created *Pst*I site to give pPM1286. The Km^R cartridge was obtained as a 1500bp *Eco*RI fragment, and subsequent cloning into pUC18 allowed it to be isolated as a similar sized *Pst*I DNA fragment due to the presence of internal *Pst*I sites within the gene cartridge.

The restriction endonuclease sites are as follows:

Ba:*Bal*I; **B:***Bam*HI; **E:***Eco*RI; **H:***Hind*III; **P:***Pst*I



5.2.2.2 Subcloning into plasmid pRK290

As plasmid DNA cannot be transformed into *V.cholerae* and pRK290 cannot self-transfer, a helper plasmid is required to mobilize pRK290 into *V.cholerae*. However, helper plasmids usually carry a number of antibiotic resistances themselves, and so to simplify the transfer system, a mobilizable derivative of pRK290 was constructed. This was done by cloning the mobilization (*mob*) region of the plasmid RP-4 (Simon *et al.*, 1983) into the *Bgl*II site of pRK290. The *mob* region is present as a *Bam*HI fragment in plasmid pSUP205 (Simon *et al.*, 1983), and cloning of this fragment into the *Bgl*II site of pRK290 results in a hybrid *Xho*II site being created, with the loss of both *Bam*HI and *Bgl*II recognition sites (Figure 5.2). This plasmid, pPM1287, can be efficiently mobilized from the *E.coli* K-12 strain S17-1, which contains the transfer genes of RP-4 integrated into the chromosome (Simon *et al.*, 1983).

The *Eco*RI fragment containing the inactivated DNase gene (Section 5.2.2.1) was cloned into pPM1287, to give pPM1288. This resulted in a Km^R, Tc^R, DNase negative plasmid, which could be efficiently mobilized from *E.coli* S17-1 into any other strain (Figure 5.2).

5.2.2.3 Mobilization of pPM1288 from *E.coli* into *V.cholerae*

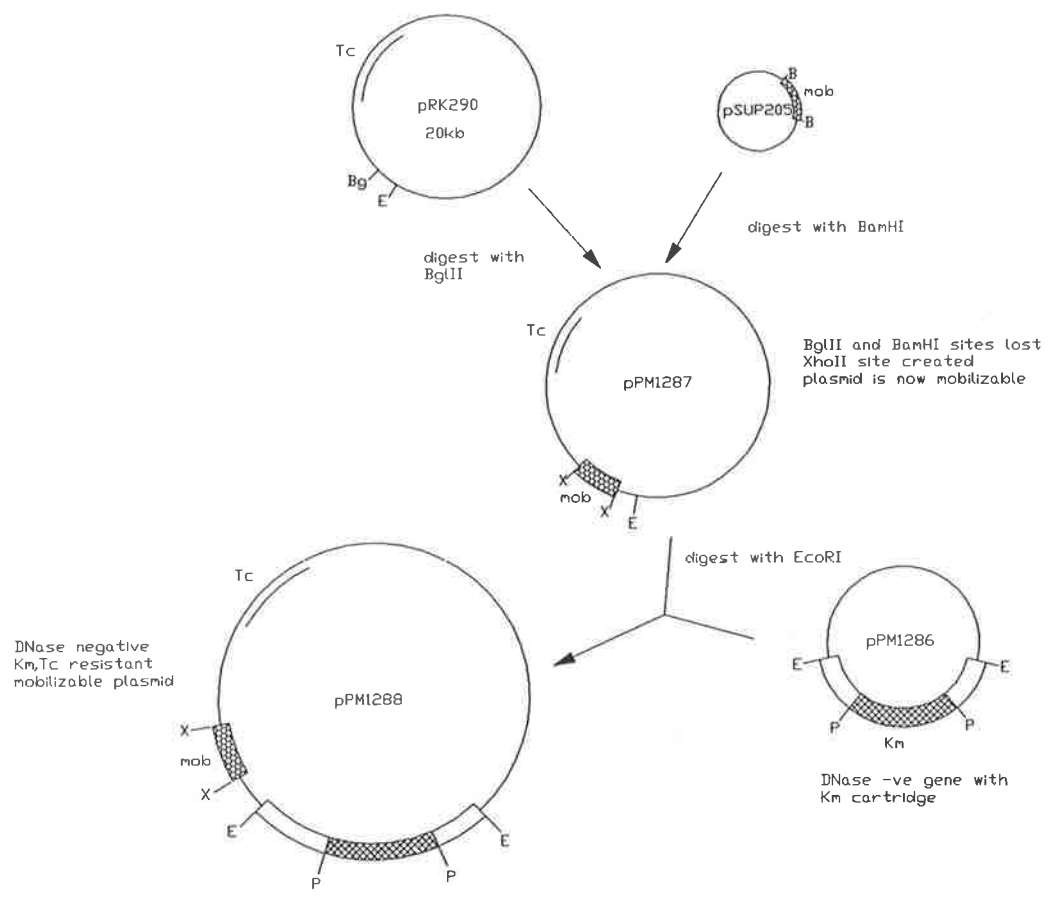
Plasmid pPM1288 was transferred to *V.cholerae* strain V62 (El Tor, Ogawa) by selection for *rif*, Km^R, and Tc^R conjugants. V62 is a spontaneous *rif* mutant of the *V.cholerae* strain 1621. Plasmid pPM1288 was also transferred to the *V.cholerae* strain GN7007 (Green *et al.*, 1983) which is Ap^R by virtue of its Tn1 insertion between *met* and *trp*. This Ap resistance was used as another means to select exconjugants which had received the plasmid pPM1288, by plating for Ap^R, Km^R and Tc^R colonies. *V.cholerae* strains which had obtained plasmid pPM1288 were purified for single colonies, and tested for sensitivity to *V.cholerae* specific bacteriophage and agglutination with *V.cholerae* specific antiserum.

Figure 5.2 Construction of the DNase negative, Km^R, mobilizable plasmid pPM1288

The mobilizable derivative of pRK290 (pPM1287) was constructed by cloning the *mob* region of RP4 from pSUP205 into the *Bgl*III site of the vector. The *mob* region isolated from pSUP205 as a 1.9kb *Bam*HI fragment and cloned directly into the *Bgl*III site of pRK290 with the resultant loss of both restriction endonuclease recognition sites, and the creation of an *Xho*II site to give pPM1287. The DNase negative (Km^R) gene was subcloned from pPM1286 into the mobilizable pRK290 plasmid (pPM1287) to give the plasmid pPM1288. The plasmid pPM1288 can be efficiently mobilized into *V.cholerae*, from the *E.coli* strain S17-1 containing the RP4 transfer genes.

The restriction endonuclease sites are as follows:

B:*Bam*HI; **Bg:***Bgl*III; **E:***Eco*RI; **X:***Xho*II



5.2.2.4 Construction of a *V.cholerae* strain with a DNase negative gene.

The Gm^R plasmid pPH1JI (Ruvkun and Ausubel, 1981) was transferred to strain V62 [pPM1288] and GN7007 [pPM1288] by conjugation. This plasmid belongs to the incompatibility group IncP, which is the same as that of the plasmid RK2. Since pPM1288 is a derivative of RK2 (pRK290 is a deletion of RK2), this plasmid still retains its IncP incompatibility, and cannot co-exist in the same cell with pPH1JI. By selecting for Gm^R, pPM1288 is not maintained in either V62 or GN7007, and *V.cholerae* cells retaining Km^R must have undergone recombination with the DNase negative, Km^R construct (Figure 5.3). One hundred transconjugants were selected which were either *rif*,Gm^R,Km^R (V62) or Ap^R,Gm^R,Km^R (GN7007). All of the colonies were Tc^S, and since Km^R could not be due to pPM1288 which had been lost, a recombination event via the homologous DNA provided by the DNase gene has must have inserted the Km^R into the chromosome of V62 and GN7007 (Figure 5.3). These strains were designated V553 and V554, respectively.

Southern hybridization analysis was performed on several *V.cholerae* isolates retaining Km^R but not Tc^R. This was in order to see if the *in vitro* constructed DNase mutation had inserted into the *V.cholerae* genome, as expected. Whole genomic DNA was isolated from both the *rif*,Gm^R,Tc^S and Ap^R,Gm^R,Tc^S colonies and probed with the plasmid pPM1207. Since the 1500bp Km^R cartridge inserted into the DNase gene has no *EcoRI* sites within it, the *EcoRI* fragment in *V.cholerae* with the recombined gene would be 1500bp larger. Likewise, a *PstI* digest would yield two fragments not present in the wild type gene, as a *PstI* linker was introduced internal to the DNase gene into which the Km^R cartridge was cloned. The altered restriction pattern of the *V.cholerae* Km^R, Tc^S mutants, confirms that the altered DNase gene has in fact replaced the wild-type gene on the *V.cholerae* chromosome (Figure 5.4).

However, when these *V.cholerae* mutants are plated on DNA-agar plates they have a DNase positive phenotype (see Figure 5.10). This is despite a mutation being present in the DNase gene, and can only be explained if there is a second DNase present. The presence of another DNase would also account for the failure to obtain a DNase negative *V.cholerae* mutant by chemical or transposon

Figure 5.3 Introduction of the defined DNase negative, Km^R mutation into the chromosome of *V.cholerae* 1621, *rif* (V62).

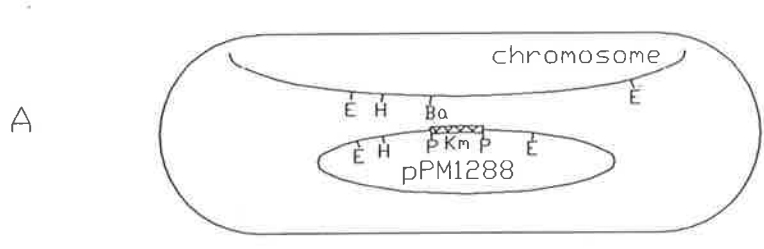
(A) Plasmid pPM1288 (Tc^R,Km^R) was mobilized from *E.coli* strain S17-1 into *V.cholerae* strain, V62. Resulting transconjugants were *rif*,Km^R,Tc^R.

(B) Plasmid pPH1JI (Gm^R), belonging to the IncP group, was transferred by conjugation into *V.cholerae* V62 [pPM1288]. *rif*, Gm^R,Km^R colonies were selected and screened for Tc sensitivity.

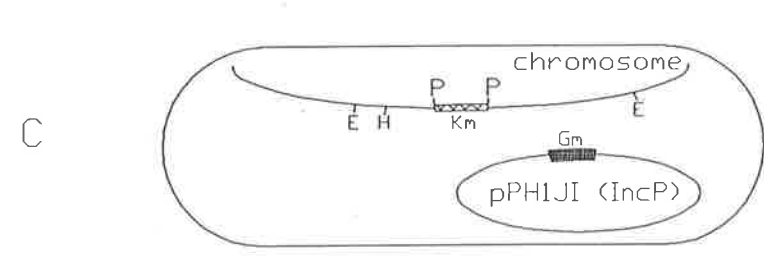
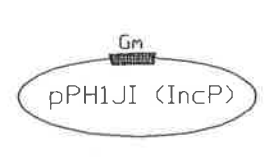
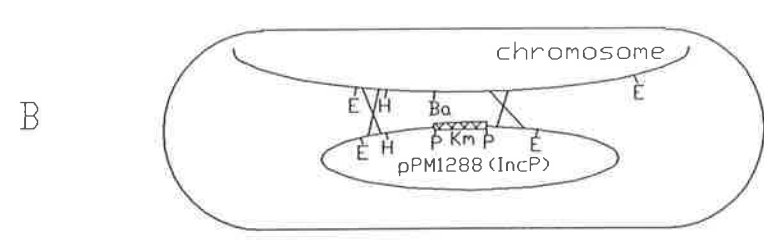
(C) *V.cholerae* cells in which the DNase negative mutation had recombined into the chromosome were isolated. This strain was designated V553.

The restriction endonuclease sites are as follows:

Ba:*Ball*; **E:***EcoRI*; **H:***HindIII*; **P:***PstI*



V62



V553

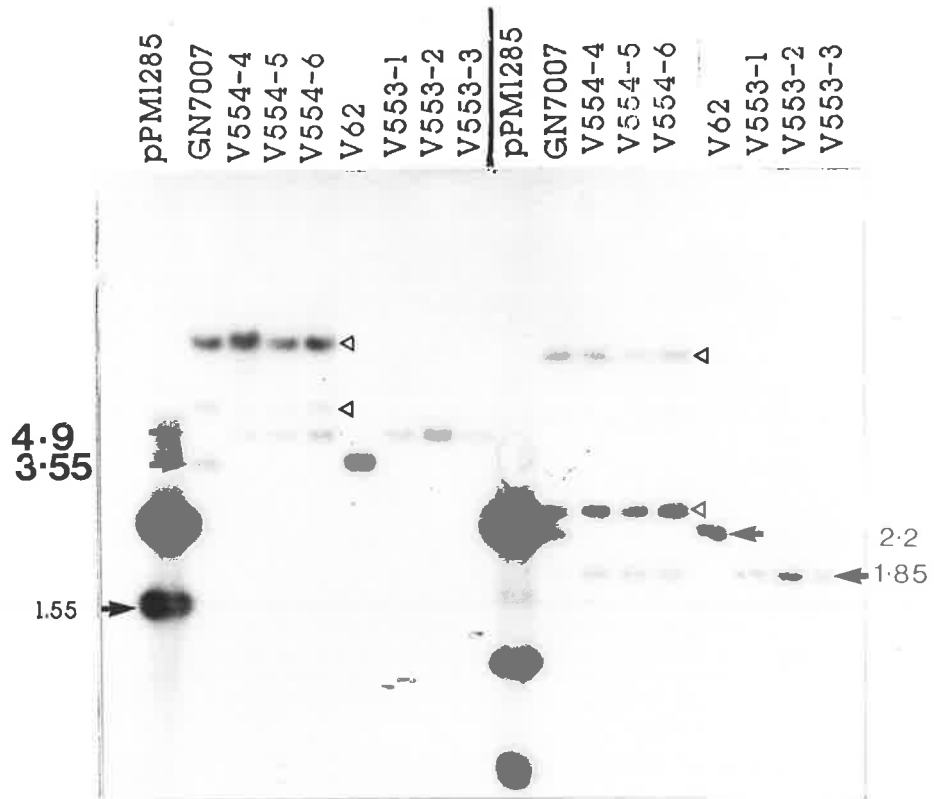
Figure 5.4 Southern hybridization analysis of recombinants of *V.cholerae* strains V62 (*rif* Km^RTc^SGm^R) and GN7007 (Ap^RKm^RTc^SGm^R).

Whole genomic DNA was digested with *Eco*RI or *Eco*RI and *Pst*I, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α -[³²P]-dCTP nick translated pPM1207, washed and subjected to autoradiography.

The arrows at 3.55kb indicate the expected size of the DNA fragments in the wild type strain, and the increase in size (4.9kb) with recombinants when digested with *Eco*RI. A decrease in size is seen when digested with *Eco*RI and *Pst*I from 2.9kb in the wild type strains to 1.85kb in the recombinants. Recombinants of V62 are labelled V553-1 to 3, while recombinants GN7007 are labelled V554-4 to 6. The extra bands (indicated with the open arrow heads) appearing in GN7007 and its recombinants V554-4 to 6, are due to homology of the Ap gene of the inserted Tn1 with the Ap gene present on the plasmid pPM1207.

EcoRI

EcoRI + *PstI*



mutagenesis, as a mutation in this gene would be masked by the other DNase. The presence of more than one nuclease has been seen in *Streptococcus* species (Wannamaker, 1958), *Streptococcus* species (Davis *et al.*, 1977) and *Serratia marcescens* (Eaves and Jeffries, 1963).

5.2.3 Site-specific recombination of a second DNase negative mutant into the *Vibrio cholerae* chromosome.

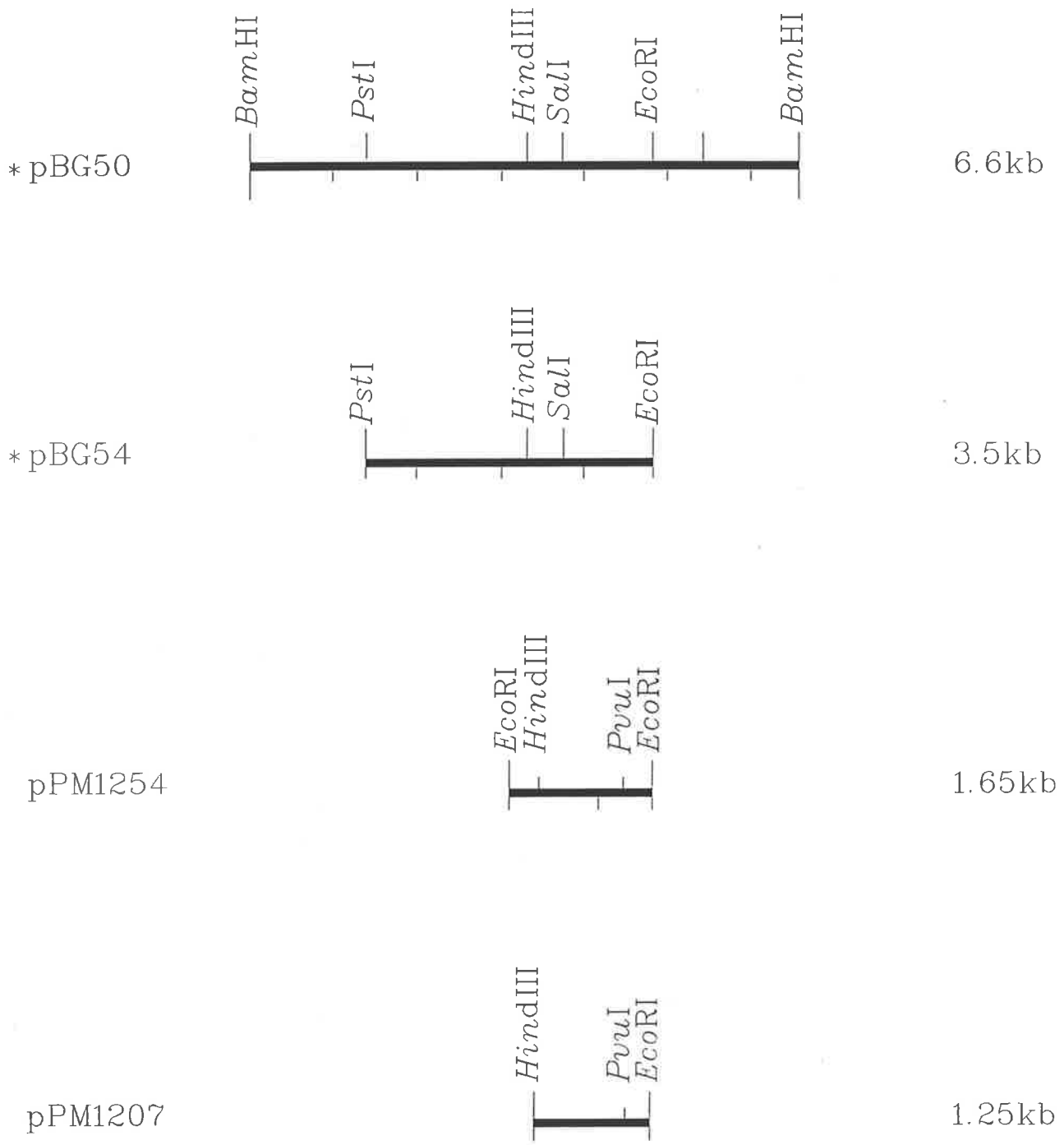
The gene encoding a DNase was also cloned by Newland and co-workers (1985) from a *V.cholerae* El Tor strain. This DNase (DNase-2), however, appears to be distinct from the DNase (DNase-1) described in Chapter 3. The restriction map of this gene, is different to that of the DNase-1 described here (Figure 5.5). These authors also describe a 100kDa protein present in minicells, compared with the 24kDa protein of DNase-1. Therefore, it appeared that there were in fact two different DNases, and a clone of this second DNase (DNase-2) was obtained by the kind permission of Professor R.K. Holmes.

The clone of DNase-2 was present as a 6.6kb *Bam*HI fragment in the vector pACYC184, and designated pBG50. However, when the activity of DNase-2 on plasmid pBG50 was compared with that of pPM1203 (the 1.55kb *Eco*RI fragment of DNase-1 cloned into pACYC184; Section 3.2.3), zones of clearing could be detected around colonies after overnight incubation, with pPM1203 (DNase-1), but no sign of DNase activity was detected with colonies containing pBG50 (DNase-2) until after at least 48 hours. Since both these clones were in identical plasmid vectors, and in the same genetic background (*E.coli* DH1), it appears that the DNase encoded on pBG50 is not as active in this type of qualitative assay as that of pPM1203, or that because of its larger size it does not leak out of DH1 as readily as DNase-1. Furthermore, this could explain why the second DNase was not isolated during the cosmid cloning of the first DNase, as colonies containing cosmid clones were incubated overnight only, before screening for DNase producing cells.

Figure 5.5 Comparison of the two cloned extracellular DNases of *Vibrio cholerae*.

The DNase cloned here in this study is present on a 1.25kb *EcoRI/HindIII* fragment on the plasmid pPM1207. The plasmid pBG54 represents the smallest fragment encoding the DNase cloned by Newland *et al.*, (1985). Alignment of the common *EcoRI* site, shows there is no restriction site homology between the two DNases.

*from: Newland *et al.*, (1985)



* From: Newland *et al.*, (1985)

5.2.3.2 Subcloning into plasmid pRK290

Another mobilizable derivative of pRK290 was constructed by cloning the *mob* region of pSUP205 (Simon *et al.*, 1985) into its *EcoRI* site. The *Bam*HI fragment, harbouring DNase-2 was then cloned into the *Bgl*II site of pPM1289 losing both the *Bam*HI and *Bgl*II sites and creating an *Xho*II site (Figure 5.6).

5.2.3.3 Insertion of an Ap^R resistance cartridge into DNase-2.

To determine if DNase-2 was responsible for the DNase activity still seen in *V.cholerae* mutants in the DNase-1 gene, a mutation was constructed by introducing the Ap^R cartridge into the structural gene of DNase-2.

The Ap^R gene cartridge was constructed by digesting pBR322 with *Ha*ell. The Ap^R gene (β -lactamase) was present on a 1.7kb fragment. This fragment was isolated and *Hind*III linkers were added. This 1.7kb fragment was then digested with *Hind*III, as there was a *Hind*III site about 250bp internal from one of the ends, resulting in a 1.4kb *Hind*III fragment containing the Ap^R gene. This Ap^R gene cartridge was then cloned into the *Hind*III site of DNase-2, to give the plasmid pPM1291 (Figure 5.7). Such a construct inactivated the second DNase.

5.2.3.4 Mobilization of pPM1291 from *E.coli* into *V.cholerae*.

Plasmid pPM1291 was transferred to *V.cholerae* strain V62 by selection for *rif*, Km^R and Tc^R exconjugants. Plasmid pPM1291 was also transferred to V553 (*rif*, Km^R; i.e. V62 with a mutation in the DNase-1 gene). The *V.cholerae* strain GN7007 was not used in this instance, because its Ap^R due to a *Tn1* insertion, could not be used to select for exconjugants, as the plasmid pPM1291 also carried Ap^R. *V.cholerae* colonies which contained plasmid pPM1291 were purified and tested for sensitivity to *V.cholerae* specific bacteriophages and agglutination by *V.cholerae* specific antiserum.

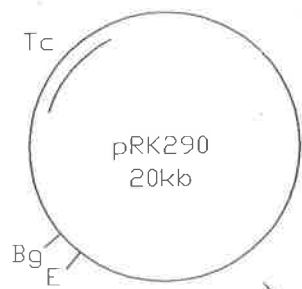
Figure 5.6 Subcloning the second DNase (DNase-2) into the mobilizable derivative of pRK290 (pPM1289).

Plasmid pRK290 was made mobilizable by cloning the *mob* region of RP4, present in pSUP205 as a *Bam*HI fragment, into the *Eco*RI site of pRK290 to give the plasmid pPM1289. Plasmids which had obtained this *mob* region, were selected by their ability to be mobilized from *E.coli* strain S17-1 (containing the complementing RP4 transfer genes).

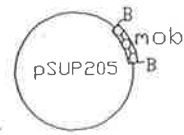
The *Bam*HI fragment from pBG50 encoding DNase-2 was cloned into the *Bg*III site of pPM1289, with the resultant loss of both *Bam*HI and *Bg*III sites, to give the plasmid pPM1290.

The restriction endonuclease sites are as follows:

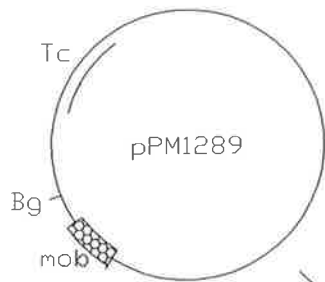
B:*Bam*HI; **Bg:***Bg*III; **H:***Hind*III; **P:***Pst*I; **X:***Xho*I



digest with EcoRI
endfill

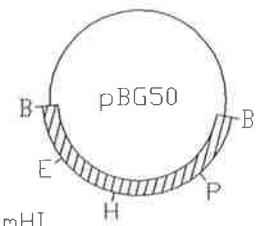


digest with BamHI
endfill

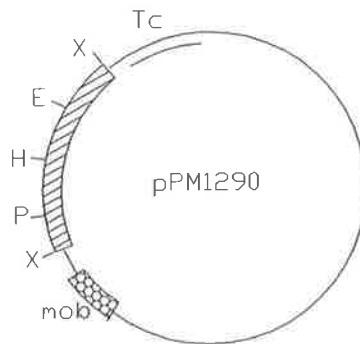


loss of EcoRI sites

digest with BglII



digest with BamHI



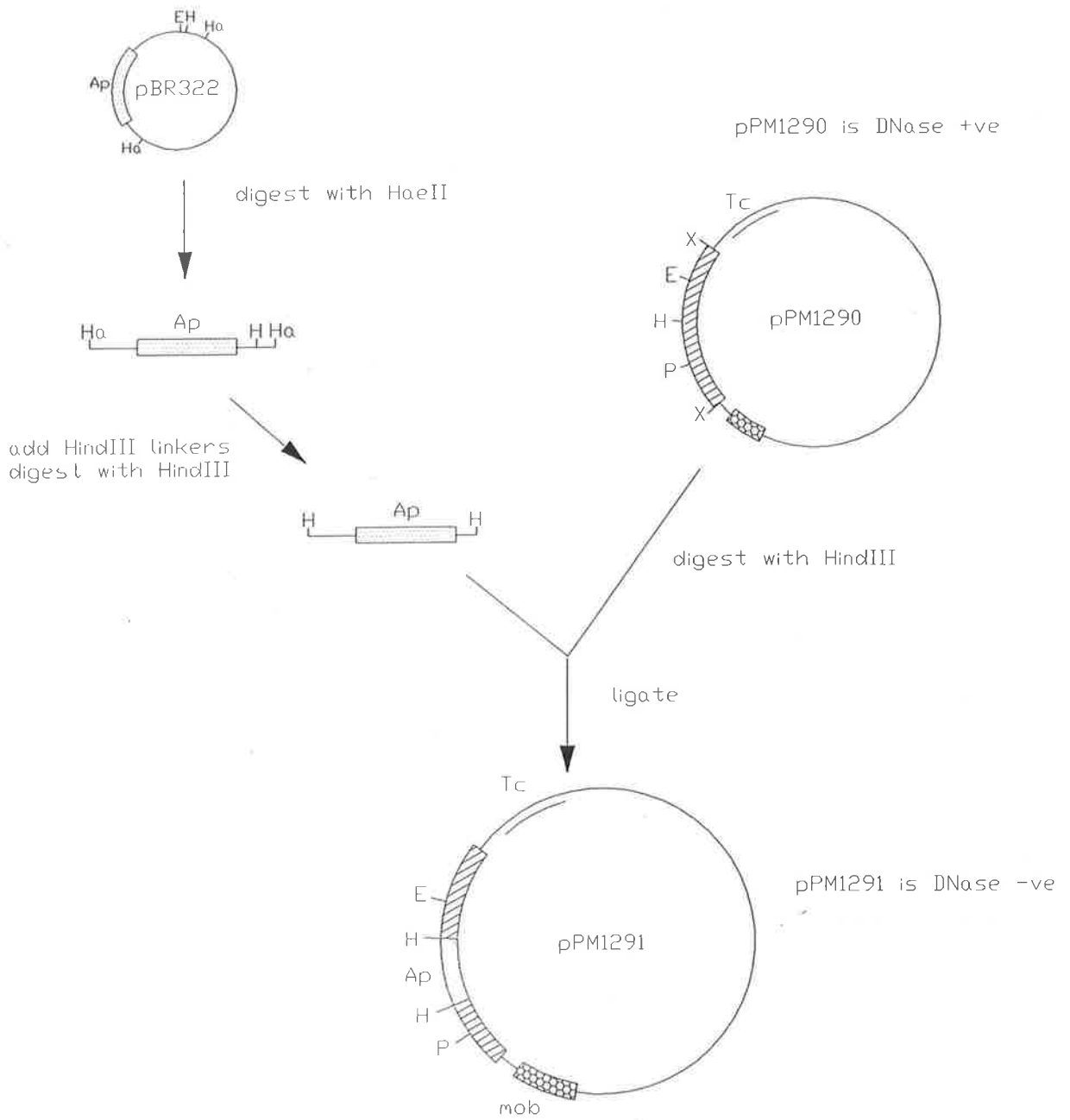
loss of BglII and BamHI
site
DNase +ve

Figure 5.7 Construction of an antibiotic resistance insertion mutation in DNase-2.

The Ap^R gene was isolated from plasmid pBR322 on an *Hae*II fragment. The addition of *Hind*III linkers, enabled this fragment to be cloned into the *Hind*III site of the DNase gene thereby inactivating it. The resultant plasmid was pPM1291.

The restriction endonuclease sites are as follows:

B:*Bam*HI; **E:***Eco*RI; **H:***Hind*III; **P:***Pst*I; **Ha:***Ha*II; **X:***Xho*II



5.2.3.5 Construction of a *V.cholerae* strain with two DNase negative genes.

V.cholerae strains V553 and V62 with the DNase-2 mutation were selected for in the same manner as described in Section 5.2.2.4 (Figure 5.8). Two different types of mutants were constructed. The first had a mutation in the DNase-2 only, recombined into the chromosome (V557), and selected by plating for *rif*,*Ap*^R,*Tc*^S colonies. The second contained the mutation in the DNase-2 gene, as well as a mutation in DNase-1 and were selected by plating for *rif*,*Km*^R,*Ap*^R,*Tc*^S colonies, (strain V555).

These strains were analyzed by Southern hybridization analysis to see that the DNase-2 had undergone recombination and replaced the wild-type gene in the *V.cholerae* chromosome. Whole genomic DNA was isolated from both the *rif*,*Ap*^R,*Tc*^S colonies, and the *rif*,*Ap*^R,*Km*^R,*Tc*^S colonies and probed with pBG50. Strains with the DNase-2 mutation, would give a 1.4kb larger *Bam*HI fragment than the wild-type gene, as a result of insertion of the 1.4kb *Hind*III fragment encoding *Ap*^R. Likewise, an *Eco*RI/*Pst*I digest would result in a number of fragments different from the wild-type gene, because the introduced *Ap*^R cartridge has an extra *Pst*I site. Figure 5.9 shows the altered pattern of *V.cholerae* *Ap*^R,*Tc*^S (V553) and *Km*^R,*Ap*^R,*Tc*^S (V554) mutants, which confirms that the altered DNase-2 gene has replaced its wild-type gene on the chromosome.

When the *V.cholerae* mutant (*Ap*^R,*Tc*^S) with a mutation in DNase-2 only, is plated onto a DNA-agar plate, this strain still has a DNase positive phenotype, presumably due to DNase-1. The *V.cholerae* strain (*Km*^R,*Ap*^R,*Tc*^S) with mutations in both of the DNases however, has a completely DNase negative phenotype (Figure 5.10). It therefore appears that a mutation in only one of these DNases can still produce a DNase positive phenotype in *V.cholerae*.

Figure 5.8 Introduction of a defined mutation of the DNase-2 (Ap^R) into the *V.cholerae* strain V553 harbouring a defined mutation in the DNase-1 (Km^R).

(A) Plasmid pPM1291 was mobilized from *E.coli* strain S17-1 [pPM1291] into the *V.cholerae* strain V553. Resulting transconjugants were *rif*, Km^R, Ap^R, Tc^R.

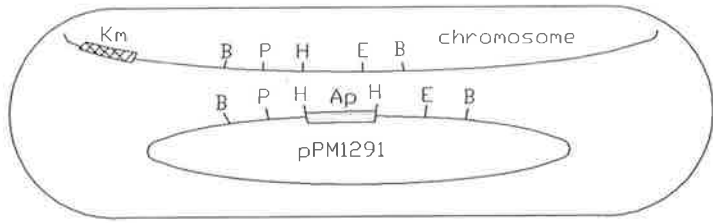
(B) Plasmid pPH1JI (Gm^R) belonging to the IncP group, was transferred by conjugation into *V.cholerae* V553 [pPM1291]. Gm^R, *rif*, Km^R, Ap^R colonies were selected and screened for Tc^S.

(C) *V.cholerae* in which the second DNase negative mutation had recombined into the chromosome were isolated. This strain was designated V555.

The restriction endonuclease sites are as follows:

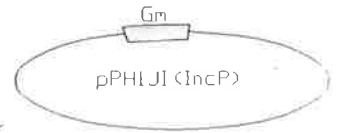
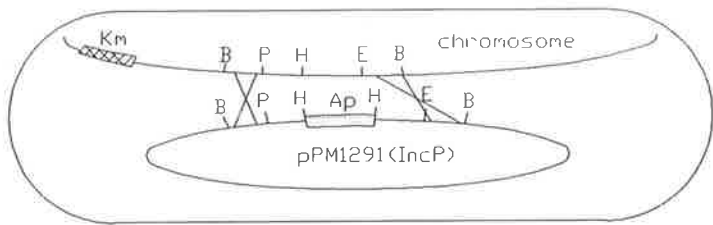
B:*Bam*HI; **E:***Eco*RI; **H:***Hind*III; **P:***Pst*I.

A

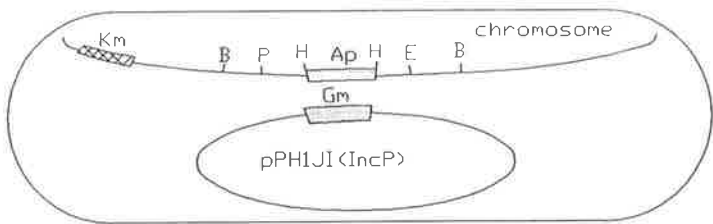


V553

B



C



V555

Figure 5.9 Southern hybridization analysis of *V.cholerae* recombinants V557 and V555.

Whole genomic DNA was digested as indicated, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α -[³²P]-dCTP nicked translated pBG50.

The arrow at 6.6kb indicates the expected size of the DNA fragment in V62, while the arrow at 8.0kb shows the resultant increase in size with recombinants when digested with *Bam*HI. The *Eco*RI, and *Eco*RI and *Pst*I digests also show changes in DNA fragment sizes with the recombinants when compared with the wild type strain. V557-1 and 2 are recombinants with a single mutation in DNase-2, while V555-3 and 4 represent *V.cholerae* strains with mutations in both DNase-1 and DNase-2.

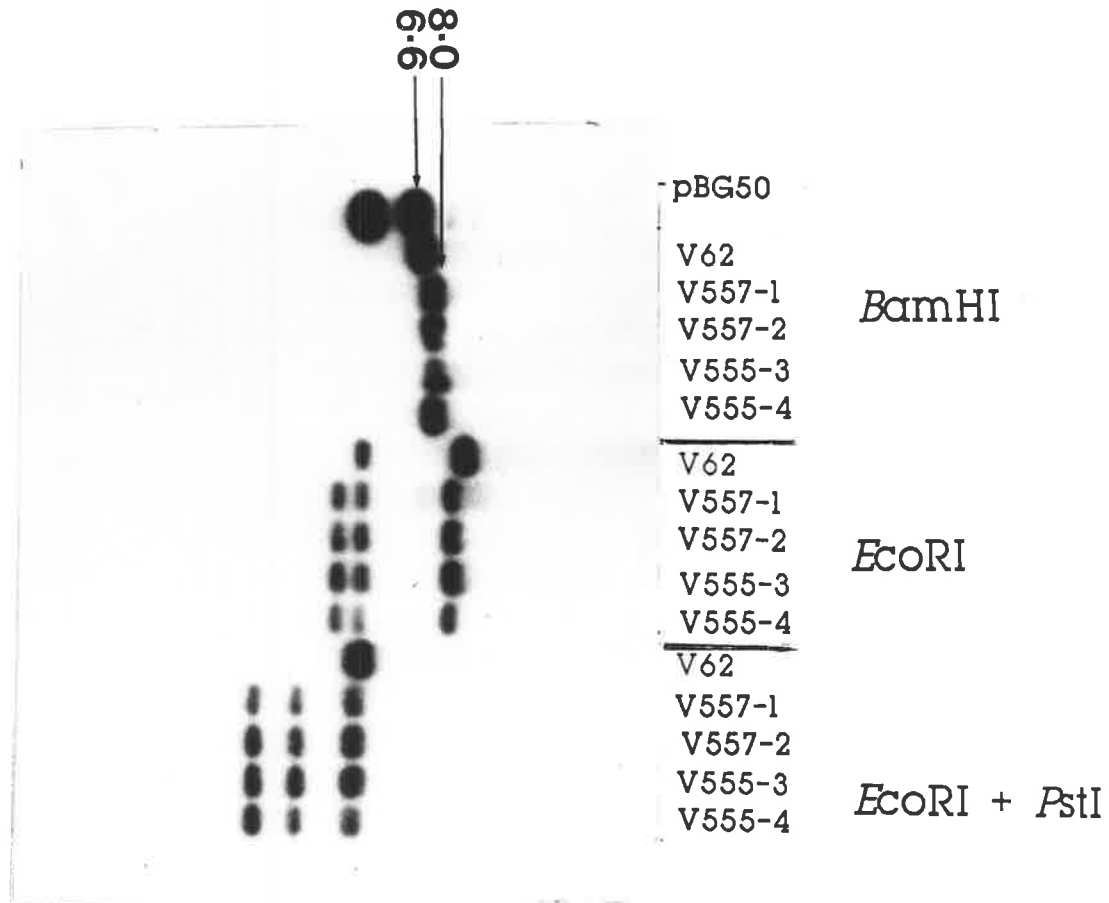
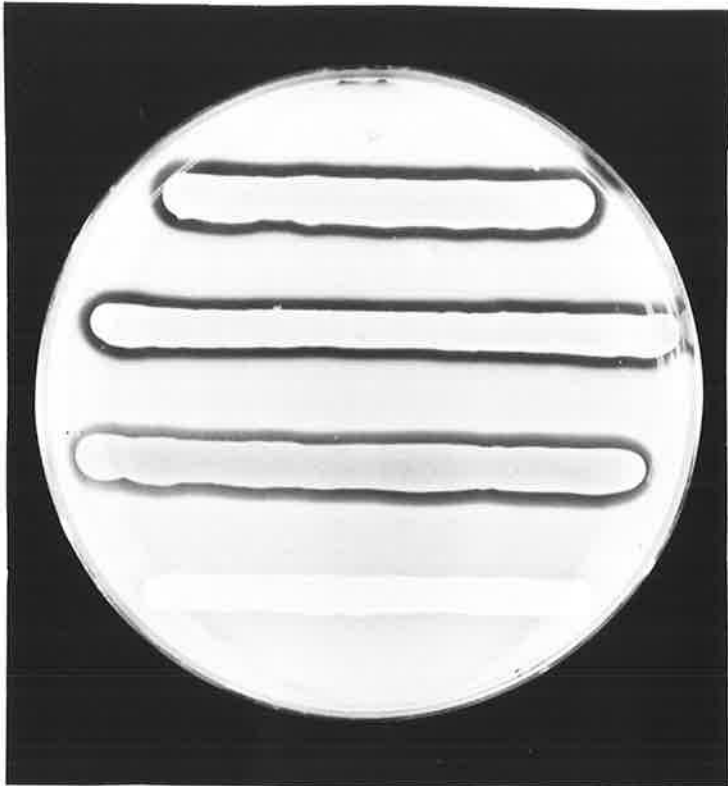


Figure 5.10 DNA-agar plate of V62 and its mutant derivatives.

V.cholerae strains were grown overnight on DNA-agar and the plate flooded with 1N HCl. DNase activity was seen as zones of clearing. V62 represents the wild type *V.cholerae*, V553 is the mutation in DNase-1 (Km^R), V557 the mutation in DNase-2 (Ap^R), while V555 has a mutation in both DNases (Km^R, Ap^R). A totally DNase negative phenotype is only seen when both DNases are inactivated.



V62

V553

V557

V752

5.2.4 Distribution of the second DNase in El Tor and Classical strains of *V.cholerae*

To determine if this second DNase was present in both El Tor and Classical strains, a number of strains were probed with pBG50 by Southern hybridization analysis. As seen with the DNase-1 (Chapter 3; Section 3.2.11), DNA homologous to this second DNase is present in both El Tor and Classical strains (Figure 5.11). However, as was noted with DNase-1, there was a restriction site difference in these fragments between the biotypes. The *Bam*HI fragment (6.6kb) is larger (13.5kb) in the Classical strains when compared to the El Tor strains. The 3.5kb *Eco*RI/*Pst*I fragment, which is the maximum determined size of DNase-2 (Newland *et al.*, 1985) on the other hand, is identical in all strains (Figure 5.11). It therefore appears that DNA homologous to this second DNase is found in both biotypes of *V.cholerae* O1.

5.2.5 Distribution of the DNase-2 amongst the Vibrionaceae.

Whole genomic DNA from bacteria belonging to the family Vibrionaceae, was probed with pBG50 by Southern hybridization, to determine if DNA homologous to this *Bam*HI fragment was present in these other species. The plasmid pPM1254 was also included to see if the two DNases shared any homology (Figure 5.12). No homology exists between the two DNases, but the probe detects a larger *Bam*HI fragment in the *V.cholerae* non-O1 strain and also in *V.mimicus*. This distribution of homologous DNA within the Vibrionaceae is identical to that of the DNase-1 (Chapter 3; Section 3.2.12). However, some homology could be detected in the *V.parahaemolyticus* and *V.fluvialis* species. Presumably this homologous DNA also represents a DNase.

Figure 5.11 Southern hybridization analysis of selected *V.cholerae* Classical and El Tor strains.

Whole genomic DNA was digested as indicated, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α -[³²P]-dCTP nick translated pBG50.

The arrows indicate the fragment containing the DNase-2 cloned from the *V.cholerae* El Tor strain 26-3. The *EcoRI/PstI* DNA fragment is identical in both El Tor and Classical strains, while the larger *BamHI* fragment varies between biotype strains.

*Bam*HI

*Eco*RI + *Pst*I

El Tor

Classical

El Tor

Classical

pBG50

E.coli DH1

V.cholerae 017

V.cholerae 1621

V.cholerae 1621 *rif* (V62)

V.cholerae MAK757

V.cholerae CA401

V.cholerae 569B

V.cholerae 154

V.cholerae RV69

pBG50

V.cholerae 017

V.cholerae 1621

V.cholerae 1621 *rif* (V62)

V.cholerae MAK757

V.cholerae CA401

V.cholerae 569B

V.cholerae 154

V.cholerae RV69

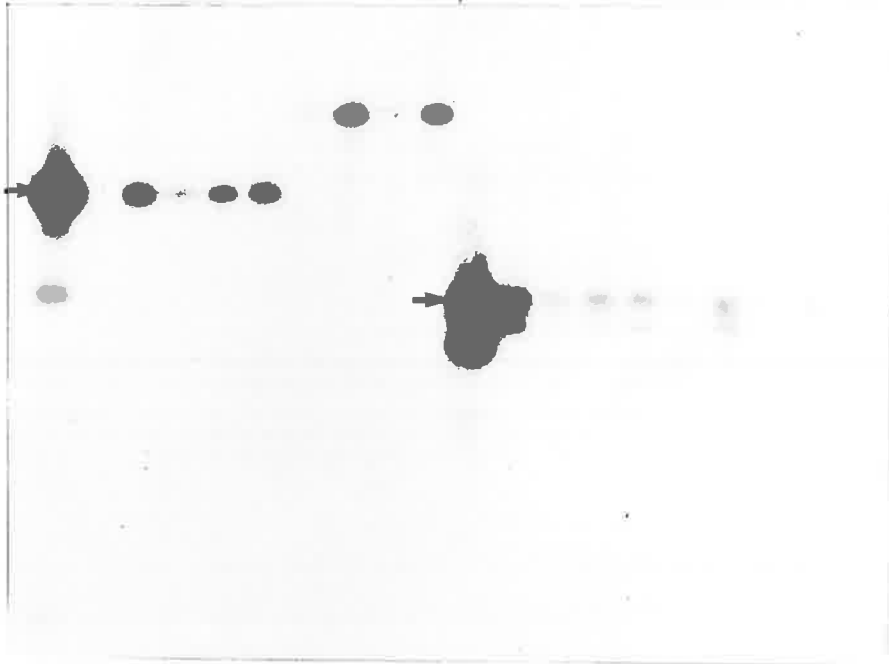
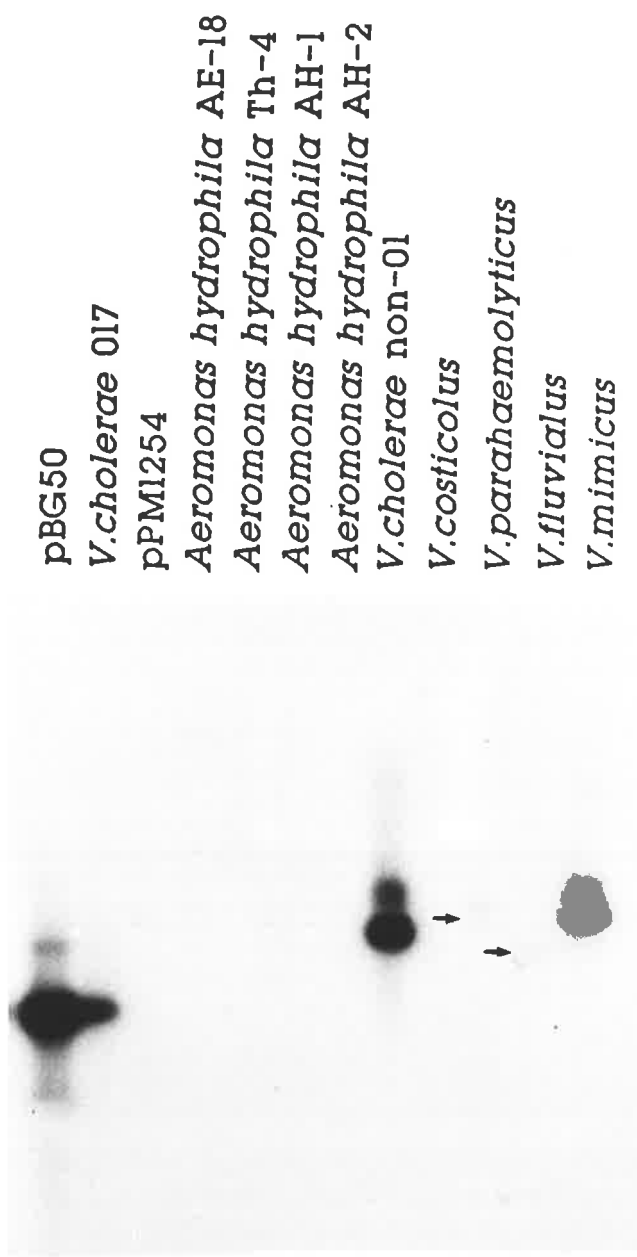


Figure 5.12 Southern hybridization analysis of bacteria belonging to the family Vibrionaceae.

Whole genomic DNA was digested with *Bam*HI, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α -[³²P]-dCTP nick translated pBG50.

Homology can be seen in *V.mimicus* and a *V.cholerae* non-01 strain. The arrows indicate bands of low homology that are detected in *V.paraahaemolyticus* and *V.fluvialis*. No homology is seen between pPM1254 (containing DNase-1) and pBG50 (DNase-2).

BamHI



pBG50

V.cholerae 017

pPM1254

Aeromonas hydrophila AE-18

Aeromonas hydrophila Th-4

Aeromonas hydrophila AH-1

Aeromonas hydrophila AH-2

V.cholerae non-01

V.costicolus

V.parahaemolyticus

V.fluvialis

V.mimicus

5.2.6.1 Construction of an antibiotic resistance free DNase negative *V.cholerae* strain.

5.2.6.2 Recombination of an antibiotic resistance free mutation into the DNase-1 structural gene of V555.

In order to fully evaluate the effect of the DNase negative mutations in *V.cholerae* on transformation by exogenous DNA, the antibiotic resistance cartridges had to be eliminated from the chromosome. A *V.cholerae* strain with a number of stable antibiotic resistance markers is of limited use when trying to select for transformants with plasmids carrying those same markers.

Plasmid pPM1285, which carries the *Pst*I linker insertional inactivation in the DNase-1 gene (see Figure 5.1), was used to clone the *Eco*RI fragment into pPM1287 (pRK290 with the *mob* region of RP4) to give pPM1292. This plasmid was mobilized into the DNase negative strain V555 as described previously (Section 5.2.2.3). Loss of this plasmid was achieved by the introduction of the plasmid pPH1JI (Gm^R) belonging to the same incompatibility group (IncP) as pPM1292, and selecting for Gm^R colonies. *V.cholerae* strains harbouring this constructed DNase-1 negative mutation were selected for as *rif*, Ap^R (Km^S , Tc^S) colonies (Figure 5.13). However, unlike the previous methods where recombination could be selected for directly by the acquisition of an antibiotic resistance, these colonies had to be screened for loss of an antibiotic resistance, namely Km^R . Of the 2,000 colonies screened, only 6 had lost Km^R . These 6 *rif*, Km^S , Ap^R , Tc^S colonies were purified and tested for sensitivity to *V.cholerae* specific bacteriophages and agglutination by *V.cholerae* specific antiserum. All were identical and one example of this strain was designated V556.

5.2.6.3 Recombination of an antibiotic resistance free mutation into the DNase-2.

The remaining antibiotic resistance on the chromosome was due to the Ap^R gene inserted within the DNase-2 gene. To eliminate this marker, another mutation was

Figure 5.13 Introduction of an antibiotic resistance free mutation in DNase-1, into the DNase negative *V.cholerae* strain V555 (Km^R,Ap^R).

(A) To replace the Km^R cartridge present in the structural gene of DNase-1, an antibiotic resistance free mutation of the first DNase was cloned into pPM1287 to give the plasmid pPM1292. This mutation of DNase-1 present on pPM1285 was constructed previously as shown in Figure 5.1. The open box indicates the added *Pst*I linkers to the DNase structural gene.

(B) Plasmid pPM1292 was mobilized from *E.coli* strain S17-1 into *V.cholerae* strain V555.

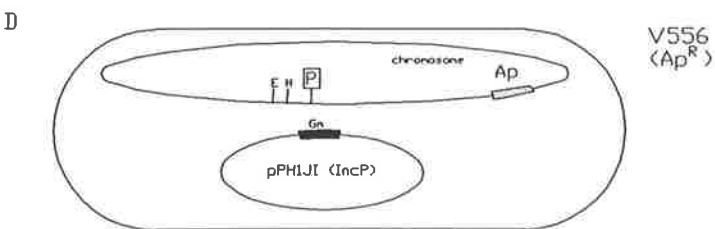
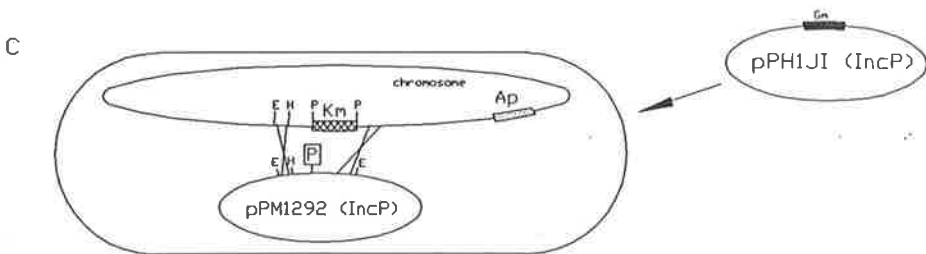
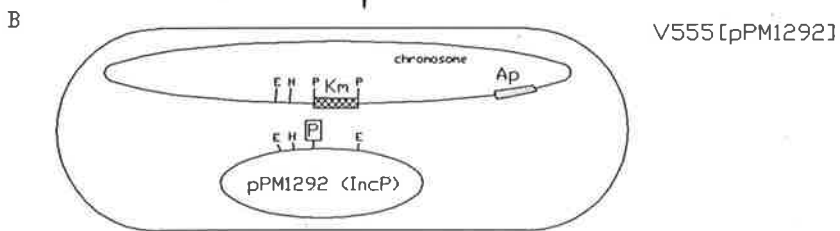
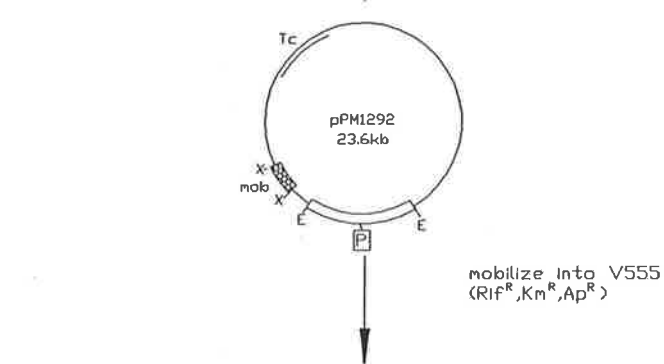
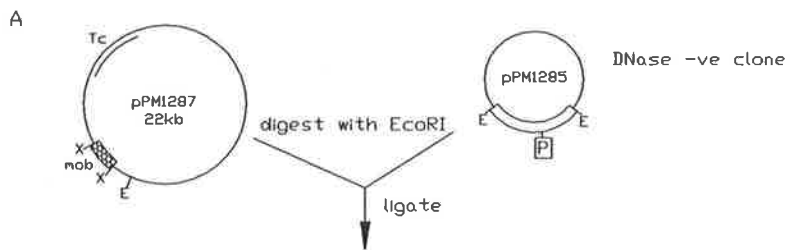
Resulting transconjugants were *rif*, Km^R, Ap^R and Tc^R.

(C) Plasmid pPH1JI (Gm^R) belongs to the IncP group and was transferred by conjugation into this *V.cholerae* strain. Gm^R, *rif*, Ap^R colonies were selected and screened for both Tc and Km sensitivity.

(D) *V.cholerae* cells in which the antibiotic resistance free mutation (Km^S) had recombined into the chromosome were isolated, and designated strain V556.

The restriction endonuclease sites are as follows:

E:*Eco*RI; P:*Pst*I, X:*Xho*II.



made in DNase-2. This mutation was constructed similar to that of DNase-1, where a frameshift in the structural gene by the introduction of linkers resulted in a DNase negative phenotype.

Plasmid pPM1290, (see Figure 5.6) was used to construct this mutant. *Bam*HI linkers were added to the *Hind*III site of DNase-2 after end-filling to create pPM1293. This plasmid had a DNase negative phenotype, and was introduced into V556 (*rif*,*Km*^S,*Ap*^R). After selecting for the loss of the plasmid pPM1294 by the introduction of pPH1JI (*Gm*^R), colonies were screened for *rif*,*Km*^S,*Ap*^S,*Tc*^S,*Gm*^R (Figure 5.14). Again, only 3 of 2,100 colonies screened had this phenotype. These colonies were purified and tested in the same way for bacteriophage sensitivity and *V.cholerae* specific antiserum agglutination. An example of this strain was designated V751.

5.2.6.4 Construction of a plasmid free, antibiotic free, DNase negative *V.cholerae* strain.

V.cholerae strains that were DNase negative and had lost their chromosomally located antibiotic resistances, however, still retained *Gm*^R,*Spec*^R,*Cm*^R encoded on the plasmid pPH1JI. This plasmid was very stable in *V.cholerae*, and was retained even after attempts to cure the strain by repeated generations of non-selection or elevated temperature shifts.

The plasmid pPH1JI was cured from the DNase negative strain V751, by introducing the plasmid pME305 (Rella *et al.*, 1985), which also belongs to the IncP incompatibility group. Plasmid pME305 is *Tc*^R gene, and selection for *Tc*^R *V.cholerae* resulted in the loss of plasmid pPH1JI. Plasmid pME305 was then easily lost from this *V.cholerae* strain, as it was temperature sensitive for replication, and incubation at 42°C overnight resulted in the immediate loss of this plasmid (Figure 5.15). The resulting *V.cholerae* strain V752 was free of all plasmids and all introduced antibiotic resistances, and had a *rif*, DNase negative phenotype.

Genomic DNA was isolated from this strain and probed with plasmids pPM1202 and pBG50 by Southern hybridization (Figure 5.16). When strain V752 was

Figure 5.14 Introduction of an antibiotic resistance free mutation of the DNase-2, into the Ap^R DNase negative *V.cholerae* strain V556.

(A) To replace the Ap^R cartridge present in the structural gene of DNase-2, an antibiotic resistance free mutation of this DNase present in pPM1290, was constructed to give pPM1293. The open box indicates the added *Bam*HI linker to the *Hind*III site present in the DNase structural gene.

(B) Plasmid pPM1293 was mobilized from *E.coli* strain S17-1 into *V.cholerae* strain V556.

Resulting transconjugants were Rf^R, Ap^R and Tc^R.

(C) Plasmid pPH1JI (Gm^R) belongs to the IncP group and was transferred by conjugation into this *V.cholerae* strain. Gm^R, Rif^R colonies were selected and screened for both Tc and Ap and sensitivity.

(D) *V.cholerae* cells in which the antibiotic resistance free deletion mutation (Ap^S) had recombined into the chromosome were isolated, and designated V751.

The restriction endonuclease sites are as follows:

B:*Bam*HI; **E:***Eco*RI; **H:***Hind*III; **P:***Pst*I; **X:***Xho*II.

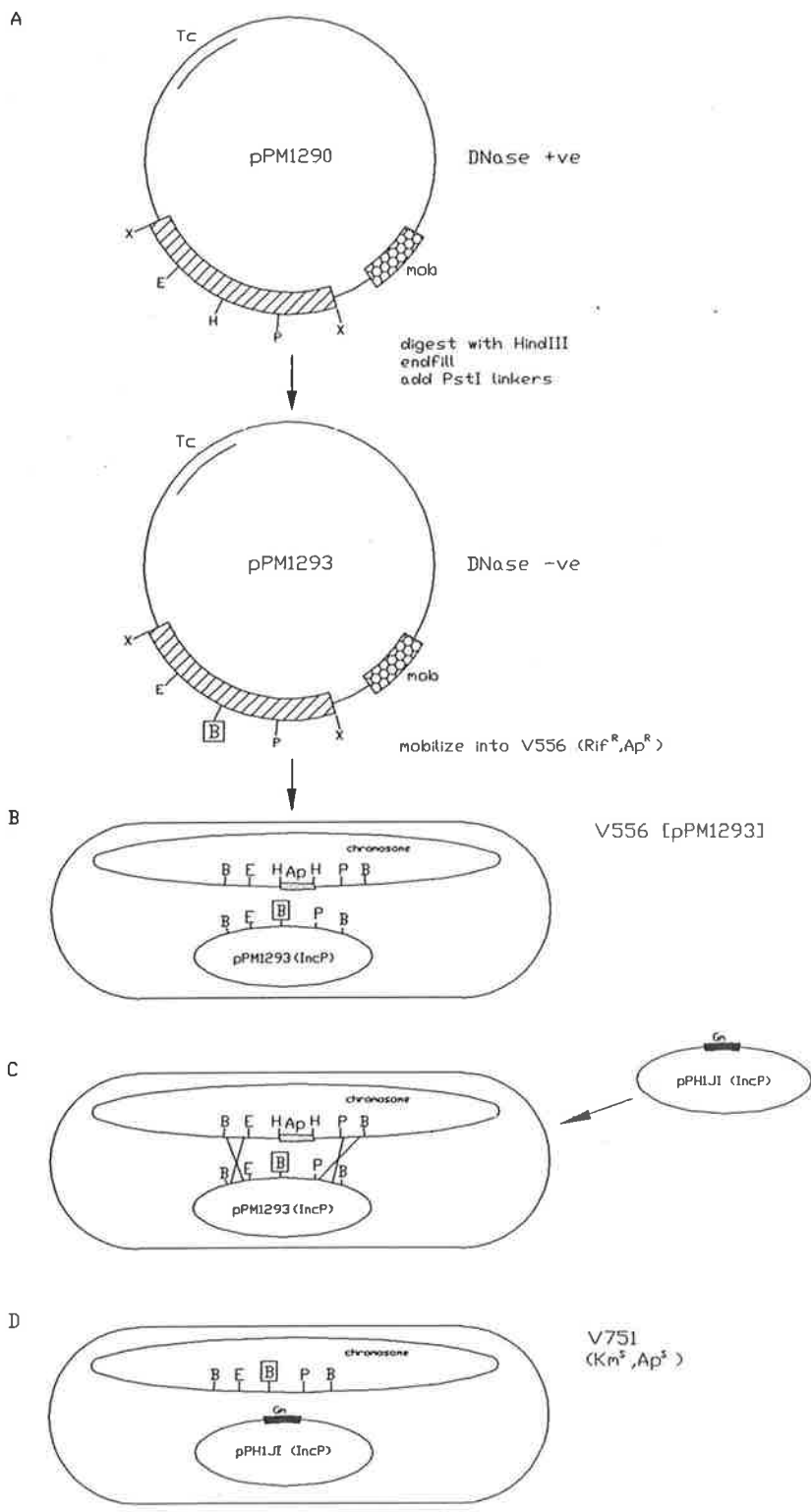
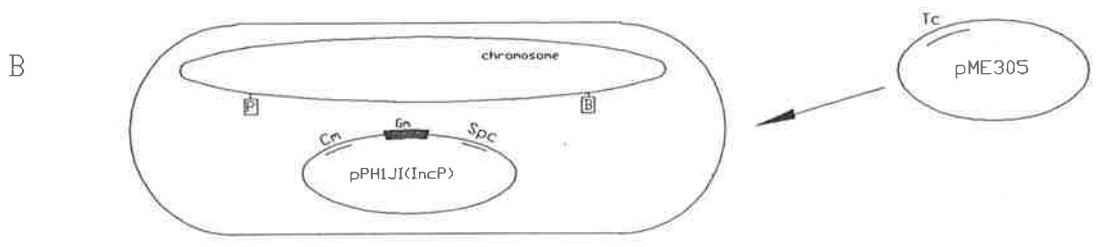
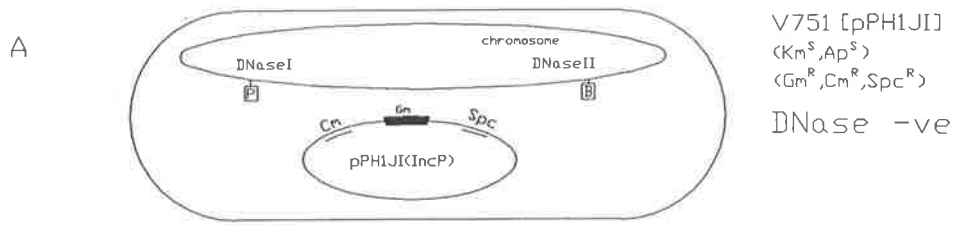


Figure 5.15 Construction of a plasmid-free, DNase negative *V.cholerae* strain.

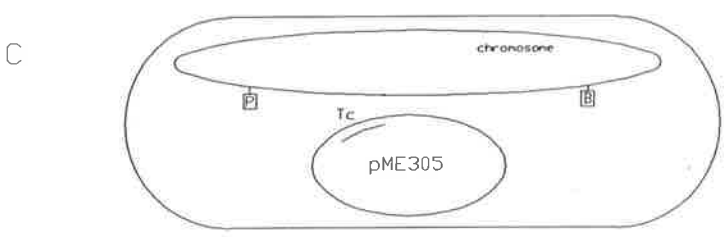
(A) and (B) The plasmid pPH1JI, which was used to cure *V.cholerae* strains of the pRK290 based plasmids containing the DNase mutations constructed *in vitro*, also encoded resistance to the antibiotics Sp and Cm as well as Gm. This plasmid is very stable in *V.cholerae*. The open boxes represent the added *Pst*I and *Bam*HI linkers in the DNase-1 and DNase-2 structural genes respectively.

(C) Introduction of the plasmid pME305 also belonging to the IncP incompatibility group, resulted in the loss of pPH1JI.

(D) A temperature shift from 30° to 42°C resulted in the loss of the plasmid pME305, which is temperature sensitive for replication. Colonies which had lost this plasmid were screened for Tc sensitivity, with the resultant strain being designated V752.



incubate at 30°C



incubate at 42°C

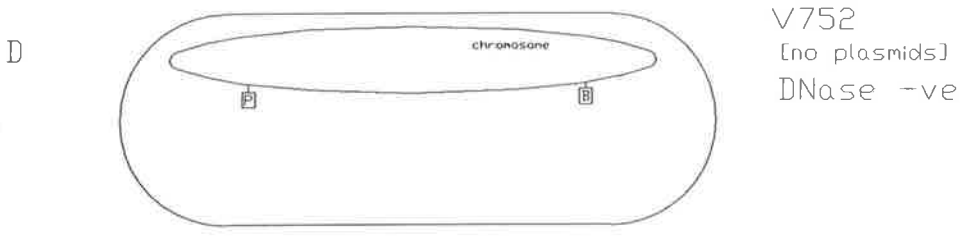
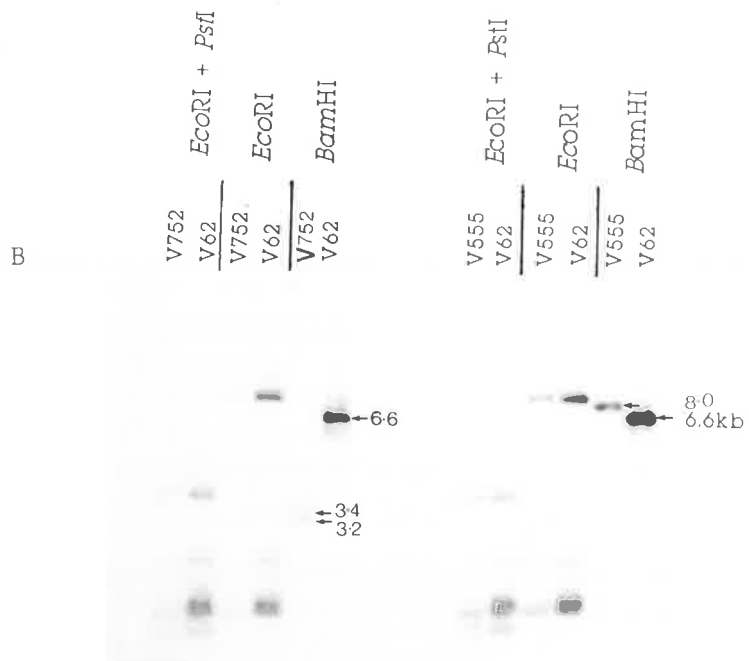
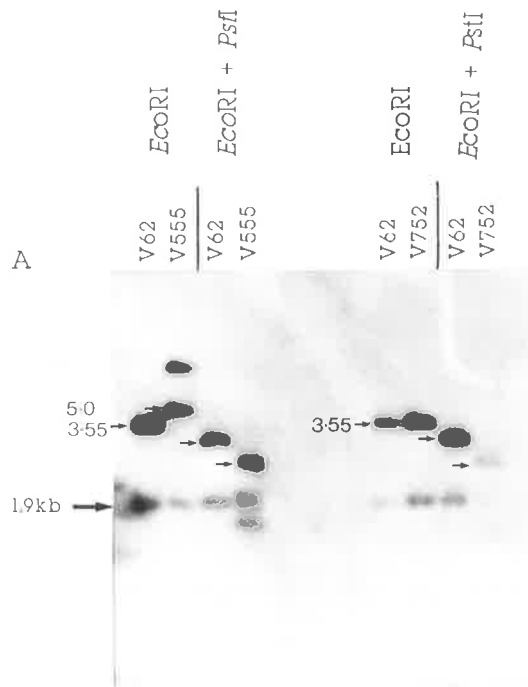


Figure 5.16 Southern hybridization analysis of *V.cholerae* strains V62, V555 and V752.

Whole genomic DNA was digested with the indicated restriction endonuclease and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filters were probed with α -[³²P]-dCTP labelled pPM1202 (Panel A) or pBG50 (Panel B). The *V.cholerae* strain V62 represents the wild type organism, while V555 has a mutation in both the DNases with the antibiotic resistance cartridges, and V752 has had the antibiotic resistances deleted from the chromosome.

(A) The extra band at 1.9kb present in all the tracks, is detected due to an additional 300bp of *V.cholerae* DNA present beyond the *EcoRI* site of the clone. The arrow present at 3.55kb is the size of the *EcoRI* fragment in the wild type *V.cholerae* strain. Strain V555 shows the resultant increase in size to 5.0kb when digested with *EcoRI*, while V752 has a similar size band to V62 (3.55kb) as the extra 8bp due to the presence of *PstI* linkers in the structural gene of DNase-1 cannot be detected. The mutation in V752 can only be detected if chromosomal DNA is digested with *PstI* and *EcoRI*.

(B) The 6.6kb *BamHI* fragment of DNase-2 present in V62, is seen as a 8.0kb band in V555 due to the insertion of the Ap^R cartridge, as indicated by the arrows. Digestion with *BamHI* in V752 results in two DNA bands present at 3.4kb and 3.2kb due to the added *BamHI* linker in the DNase-2 structural gene. This mutation however, cannot be detected when whole genomic DNA is digested with *EcoRI* or *EcoRI* and *PstI*.



probed with pPM1202, the *EcoRI* fragment is identical to the wild-type as an 8bp increase in length by insertion of *PstI* linker cannot be detected. When the chromosomal DNA was digested with *EcoRI* and *PstI*, the wild-type strain retains the same, single band, while the mutant strain V752 now has two smaller fragments by virtue of the inserted *PstI* site in the gene.

Similarly, when the *V.cholerae* strain with a mutation in DNase-2 was digested with *EcoRI* and *PstI*, an identical band to the wild type can be seen. However, a *BamHI* digest reveals two smaller bands due to the insertion of the *BamHI* linker, while the wild type retains a single band of 6.6kb. The Southern hybridization analyses of V752 confirm the presence of these defined mutations in the two DNase genes.

5.2.7.1 Effect of the DNases of *V.cholerae* on transformation.

5.2.7.2 Transformation of *E.coli* strains carrying the DNase gene.

The effect of extracellular DNases on the transformation efficiency of a bacterium, has been clearly demonstrated in *S.marcescens*, where the inactivation of nucleases produced by this organism, leads to the relatively efficient and reproducible ability to take up DNA (Reid *et al.*, 1982). Evidence for the idea that the extracellular DNase of *V.cholerae* is responsible for the inability of this organism to transform DNA, is provided by a series of transformations into *E.coli* strains harbouring plasmids producing the *V.cholerae* DNase-1. (Table 5.1).

The transformation efficiency of *E.coli* cells was significantly reduced in strains expressing DNase-1. This also correlates with the amount of DNase produced. For example, the plasmid pPM1207 which contains the DNase-1 gene in pUC18, results in several-fold more DNase activity than pPM1203 which has the DNase-1 cloned into pACYC184. The plasmid vector pUC18 has a higher copy number than pACYC184 (Chang and Cohen, 1978) and pPM1207 is also in the correct orientation to utilize the strong transcriptional signals in front of *lacZ*, while pPM1203 contains the DNase gene in the opposite orientation to the CAT promoter of pACYC184. Strains harbouring pPM1203 show a 60% decrease in

Table 5.1: Transformation of *Escherichia coli* K-12 harbouring various plasmids.

^aThe *E.coli* K-12 hosts are strain DH1 and its *tolA,B* mutant (periplasmic leaky) E852 (see Chapter 3; Section 3.2.10).

^bPlasmid pPM1204 corresponds to the *EcoRI* fragment of pPM1202 containing the gene for the DNase cloned into the *EcoRI* site of pACYC184. Plasmid pPM1207 is the *EcoRI/HindIII* fragment containing the DNase cloned into pUC18.

^cThe relative transformation frequencies were determined for both background strains. Approx. 1 μg of DNA was used to transform DH1[pACYC184] and E852 resulting in 1.5×10^5 and 1.69×10^5 transformants per μg respectively. These were given a relative frequency of 1.

Recipient ^a	Transforming ^b plasmid	Relative ^c transformation frequency
DH1[pACYC184]	pPM1207	1
DH1[pPM1204]	pPM1207	0.31
DH1[pPM1207]	pPM1204	0.008
E852[pACYC184]	pPM1207	1
E852[pPM1204]	pPM1207	0.062
E852	pPM1204	0.009

transformation frequency, with those harbouring pPM1207 resulting in a 99% reduction. It is clear that increasing the amount of DNase produced by the strain leads to a decrease in the yield of transformants. This is most dramatic in the *tolA,B* background which, because of its leaky phenotype, effectively mimics the DNase excretion seen with *V.cholerae*.

5.2.7.3 Transformation of *V.cholerae* strains with mutations in the DNase genes.

In order to evaluate the role of the DNases in the transformation of DNA into *V.cholerae*, strains with mutations in either or both of the DNases were compared with *E.coli* DH1 for their transformation efficiency using pBR322 DNA. The pBR322 DNA used in this study, was isolated from *E.coli* K-12 and purified on a CsCl gradient. Transformants were selected as either Ap^R or Tc^R. Examples of presumed transformants were confirmed to contain the 4.4kb pBR322 by plasmid extraction and agarose gel electrophoresis.

The transformation efficiency of *V.cholerae* was compared directly to that of *E.coli* DH1. *V.cholerae* strain V62 (*V.cholerae* 1621 *rif*) did not yield any transformants when selecting for either Tc or Ap. This is consistent with observations made in this laboratory over the past few years where no *V.cholerae* strain has been able to be transformed with plasmid DNA. Mutations in one or both DNases of *V.cholerae*, however, does result in transformants (Table 5.2).

The efficiency of transformation of V752 which is defective for both DNases is about 100-fold less than that of *E.coli*, but even so, sufficient numbers of transformants are obtained for this strain to be useful as a general transforming strain. Strain V553 (DNase-1⁻) can be transformed at a similar efficiency to V752, however, V557 (DNase-2⁻) is not as competent. There does not appear to be a significant additive effect.

Interestingly, transformants selected on Ap plates all gave rise to colonies after overnight incubation at 37°C. However, when Tc was used as selection the *V.cholerae* transformants required 48 hours to produce similar sized colonies. This was limited to the *V.cholerae* strains only. The reason for this is unknown,

Table 5.2: Transformation of *V.cholerae* DNase mutant strains.

V.cholerae DNase mutant strains were transformed with 1 μ g pBR322 DNA/10⁶ cells, and their transformation efficiency was compared with that of a wild type *V.cholerae* strain (V62) and the *E.coli* strain DH1. The strain V553 has a mutation in DNase-1, V557 a mutation in DNase-2 and V752 a mutation in both DNases.

^aThe *V.cholerae* strains with a mutation in either DNase-1 or DNase-2, retain a chromosomal antibiotic resistance due to the presence of the antibiotic^R cartridge in the inactivated DNase genes. The strain V752 with a mutation in both DNases has had these gene cartridges eliminated from the chromosome.

^bAll transformants, with the exception of V557, were selected for Ap^R. Since V557 was Ap^R due to the nature of the DNase-2 mutation, the plasmid pACYC184 was used to select for Cm^R.

^cThe relative transformation frequency was determined for DH1, resulting in 1.87 X 10⁵ transformants per μ g of pBR322 DNA, and this was given a frequency of 1.

Recipient ^a	Transforming plasmid	Relative ^c transformation frequency
DH1	pBR322	1
V62	pBR322	0
V553[Km ^R]	pBR322	0.035
^c V557[Ap ^R]	pACYC184	0.009
V752	pBR322	0.056

but it should be noted that *V.cholerae* strains are particularly sensitive to Tc and similar incubation times are required in conjugation experiments.

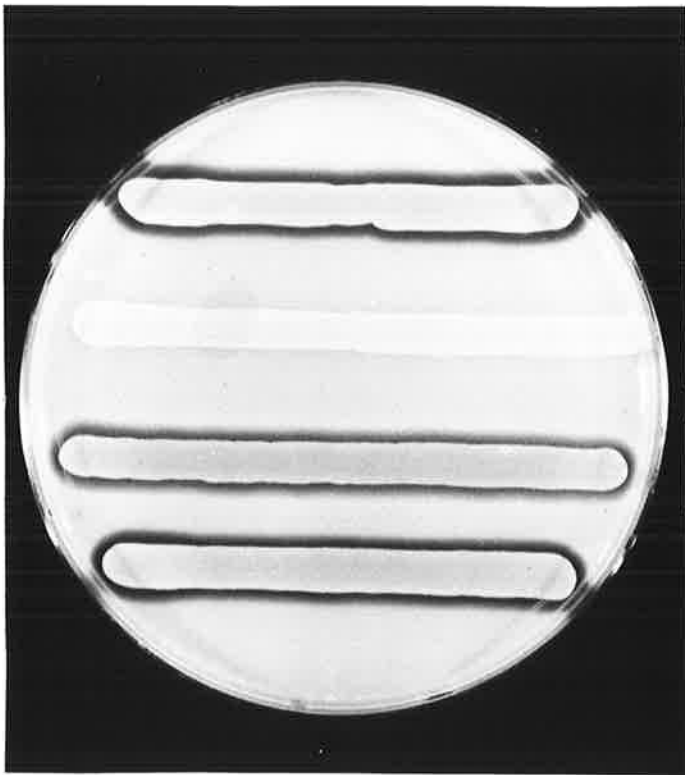
Finally, it was reasoned, that the lower transformation efficiency seen with *V.cholerae* strains when compared to *E.coli*, may have been due to restriction of the incoming pBR322 DNA by *V.cholerae*. Therefore pBR322 DNA was isolated from *V.cholerae* and purified on CsCl. No conclusions, however, could be drawn from the transformation experiments using this *V.cholerae* "modified" pBR322. No transformants were produced in any of the *V.cholerae* strains, selecting for either Ap or Tc, and the number of transformants in *E.coli* DH1 (a restriction minus host) were severely reduced ($< 1 \times 10^2$ cells/ μ g DNA). The reason for this is unknown, as the DNA appears identical to pBR322 isolated from *E.coli* when electrophoresed on an agarose gel. The very low level of transformants of *E.coli* DH1 seen with this plasmid DNA, suggests that the DNA may be modified in some way that is not compatible with transformation.

5.2.7.4 Transformation of a *V.cholerae* DNase negative strain with pPM1207 and pBG50.

To see if the DNase positive phenotype of the *V.cholerae* DNase negative strain V752 carrying mutations in both genes, could be restored, plasmids pPM1207 or pBG50 were transformed into this strain (Figure 5.17). At this stage, it was no longer necessary to mobilize or conjugate plasmids into *V.cholerae*, as this strain could now be simply transformed, selecting for either Ap^R (pPM1207) or Cm^R (pBG50). Upon acquiring either of these plasmids, the *V.cholerae* strain regained its DNase positive phenotype, thereby confirming that these cloned DNases were responsible for the DNase activity normally detected in *V.cholerae*.

Figure 5.17 DNA agar plate of V62, V752, V752[pBG50] and V752[pPM1207].

Strains were grown on DNA-agar and the plate flooded with 1N HCl. DNase activity was seen as zones of clearing. The DNase activity seen with the wild type strain can be restored by introducing the cloned DNase genes back into the DNase negative mutant V752.



V62

V752

V752 (pBG50)

V752 (pPM1207)

5.2.8.1 Virulence of the *V.cholerae* DNase negative strain V752 in the infant mouse cholera model.

V62 and its DNase negative variants were fed to infant mice at a range of concentrations up to 1×10^7 organisms/mouse (Attridge and Rowley, 1983a), and the mice incubated at 25°C for 48 hours. No difference was detected in the survival of the mice. In fact, no mice died in any of the groups, suggesting that V62 was totally avirulent.

V62 is a spontaneous *rif* mutant of *V.cholerae* El Tor 1621. The resulting change in outer membrane structure of these *rif* mutants apparently causes *V.cholerae* to lose its virulence, as these mutants do not survive as well in the infant mice (S. Attridge - personal communication). In order to see the effect of mutations in the DNase genes, these site-specific mutations had to be reconstructed in another *V.cholerae* strain which still retained its virulence.

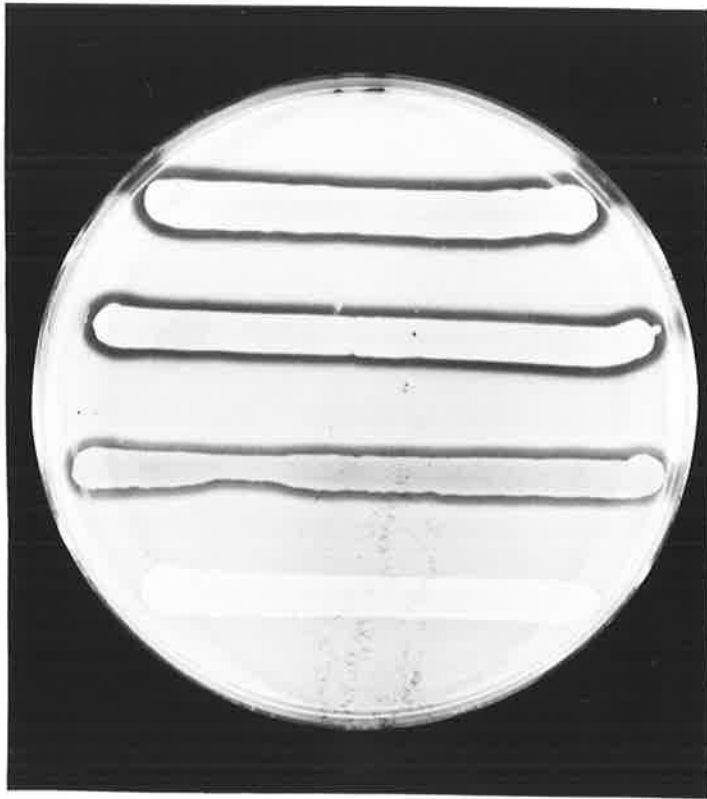
5.2.8.2 Construction of a DNase negative mutant in *V.cholerae* 017.

The *V.cholerae* strain 017 (El Tor, Ogawa) was chosen to introduce these mutations into the DNase genes, as it still retained its virulence, and was Sm^{R} which enabled selection of *V.cholerae* exconjugants. Mutants in each DNase gene, and both DNase genes were constructed essentially as described in Section 5.2.2.1 - 5.2.3.5, and free of the plasmid pPH1JI and pME305.

V.cholerae 017 mutants constructed were V753 (a mutation in the DNase-1), V755 (a mutation in the DNase-2) and V757 (a mutation in both DNases). The antibiotic resistance markers Km^{R} and Ap^{R} , were retained in these strains to ensure that the mutations were maintained. Mutations in a single DNase retained the DNase positive phenotype, while mutations in both DNases, resulted in a DNase negative phenotype (Figure 5.18). These strains were also checked by Southern hybridization analysis to ensure the mutation had recombined into the chromosome (Figure 5.19).

Figure 5.18 DNA-agar plate of *V.cholerae* 017 and the introduced DNase mutations.

V.cholerae strains were grown on DNA-agar and the plate flooded with 1N HCl. DNase activity was seen as zones of clearing. *V.cholerae* 017 represents the wild type strain, V753 has a mutation in DNase-1, V755 has a mutation in DNase-1, while V757 has a mutation in both DNases. A totally DNase negative phenotype is only seen when both DNases are inactivated.



V.cholerae 017

V753

V755

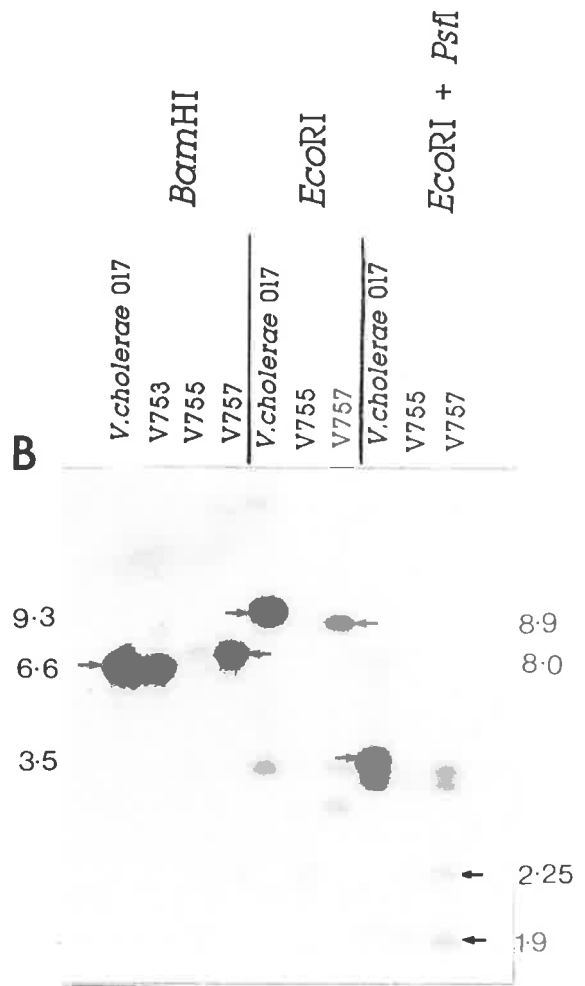
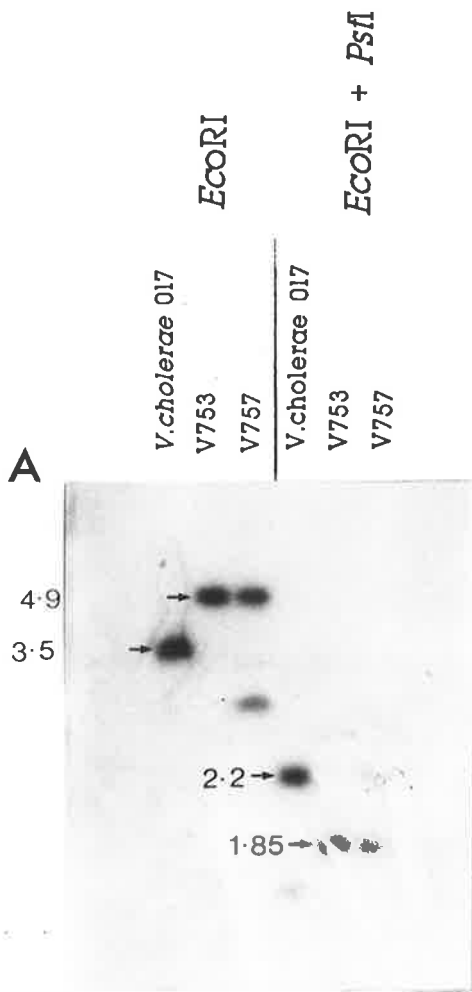
V757

Figure 5.19 Southern hybridization analysis of *V.cholerae* 017 recombinants with mutations in one or both DNase genes.

Whole genomic DNA was digested with the indicated restriction endonuclease, and electrophoresed on a 0.8% agarose gel. After transfer to nitrocellulose, the filter was probed with α -[³²P]-dCTP nick translated pPM1254 or pBG50.

(A) Panel A has been probed with pPM1254. *V.cholerae* 017 represents the wild-type strain, V753 has a mutation in DNase-1, while V757 has a mutation in both DNases. The arrows at 3.5kb and 2.2kb indicate the DNA fragment size in the wild-type strain 017, and the alteration in band sizes at 1.85kb and 4.9kb are seen when the constructed mutations are introduced into the chromosome.

(B) Panel B has been probed with pBG50. The recombinants are designated V755 (mutation in DNase-2) and V757 (mutation in both DNases). The arrows at 6.6kb, 9.3kb and 3.5kb indicate the size of the DNA fragment encoding DNase-2 in the wild-type strain 017, and the resulting change in fragment size of 8.0kb, 9.3kb and 2.25kb plus 1.9kb respectively in recombinants, when digested with *Bam*HI, *Eco*RI and *Eco*RI and *Pst*I. V753 has a mutation in the DNase-1 only, and no change in any bands are seen when probed with pBG50.



5.2.8.3 Virulence of *V.cholerae* 017 DNase negative mutants in the infant mouse cholera model.

V.cholerae 017 and its DNase negative variants were grown in Luria broth overnight at 37°C. Groups of 8 infant mice were orally administered with bacterial suspensions at varying concentrations (Table 5.3) and after 48 hours, the number of mice surviving within each group was noted. Concentrations were checked by both cell counts as well as viable counts. No loss in virulence was seen in any of the groups of mice with DNase mutants, and therefore appears that the elimination of the DNase(s) is not sufficient to affect the virulence of *V.cholerae*.

5.3 Conclusions.

Attempts to construct a DNase negative mutant of *V.cholerae* by chemical mutagenesis or by transposon insertion were unsuccessful. Site-specific mutagenesis, in which a defined mutation in the cloned DNase-1 was recombined into the chromosome of *V.cholerae* was used to determine the phenotypic effect of such a mutation. The mutation in the DNase gene was constructed with an internal antibiotic resistance marker, thus enabling selection for the recombination event. Recombinants with this mutated gene, were confirmed by Southern hybridization analysis, and the resulting phenotype of the *V.cholerae* strains were still DNase positive.

Another DNase (DNase-2) of *V.cholerae* has been cloned by Newland *et al.*, (1985). This clone was made available by Professor R.K. Holmes, and it has been compared to the DNase (DNase-1) described here. The restriction endonuclease cleavage data differ for both genes, as do the sizes for the proteins they encode. Southern hybridization analysis shows no homology between the two genes, and the DNase-1 appears to be more active, as *E.coli* cells expressing this DNase show zones of activity after overnight incubation while 48 hours is required before DNase-2 can be detected. The *xds* gene for DNase-2 cloned by Newland *et al.*, (1985), has been mapped between *pro* and *ile* on the *V.cholerae* El Tor chromosome and is immediately adjacent to the biotype locus. The chromosomal

Table 5.3 Comparison of virulence between *V.cholerae* 017 and its DNase mutant derivatives.

Bacterial concentration (organisms/mouse)	017	V753 survival ^a	V755	V757
5 X 10 ⁶	6	6	5	4
1 X 10 ⁷	2	3	2	1
5 X 10 ⁷	0	0	0	0

^aBaby mice (groups of 8) surviving 48 hours after oral administration of bacteria.

Assays were repeated two times with each concentration.

N.B. The *V.cholerae* strain V62 (*rif*) and its DNase mutant derivatives did not kill baby mice after 48 hours, even at 5 X 10⁸ organisms/mouse.

position of DNase-1, however, has not been mapped.

The introduction of a mutation in the *xds* locus by site-specific recombination, using an antibiotic marker to select for the event, still retains a DNase positive phenotype. However, the double mutant is DNase negative. It is clear that two genetically unrelated DNases are elaborated by *V.cholerae*, although their cross-reactivity at the protein level has not been tested.

The construction of a DNase negative *V.cholerae* mutant, allowed the evaluation of the significance of DNase(s) to transformation. These mutants were first constructed using antibiotic resistance markers to inactivate the genes, and these markers were subsequently eliminated.

V.cholerae DNase negative mutants are capable of transforming DNA, while the wild type *V.cholerae* are not. When compared to *E.coli*, *V.cholerae* does not yield as many transformants, but it must be remembered that the transformation system used has been optimized for *E.coli* (Hanahan, 1983). A mutation in the DNase-1, gives rise to a higher transformation frequency when compared to mutants in *xds*. The *V.cholerae* double mutant V752 is most efficient, and this strain is the most likely choice as a transforming strain. Introduction of the plasmids pPM1207 or pBG50 by transformation into this strain, restores a DNase positive phenotype. Clearly, the DNase(s) are involved in transformation in *V.cholerae*, and their inactivation permits transformation of exogenous DNA.

The loss of virulence in V62 due to the *rif* mutation, necessitated the introduction of both the DNase mutations into another strain. The DNase mutations were introduced into *V.cholerae* 017, a virulent El Tor strain, and tested for virulence in the infant mouse cholera model. No loss in virulence was detected in any of the DNase mutant strains. However, although these proteins by themselves do not seem to be essential virulence factors, they may still contribute in some way to the overall pathogenic process. Since such strains still retain virulence, these could be used as transforming strains to facilitate construction of mutations in other known virulence factors.

CHAPTER 6

Gene and Protein fusions of the DNase with TEM β -lactamase

6.1 Introduction

The movement of molecules from the site of synthesis to a new location is a fundamental property of biological systems. Gram-negative bacteria, for example, export polypeptides to the periplasmic and outer membrane subcompartments of their cell envelopes and secrete proteins into the extracellular milieu. Many bacterial species, but particularly those belonging to the Vibrionaceae and Pseudomonaceae, secrete numerous proteins, that are usually either toxins or degradatory proteins such as proteases and nucleases (Hirst *et al.*, 1984b; Howard and Buckley, 1985; Lory *et al.*, 1983). Most of these extracellular proteins are initially synthesized with amino-terminal signal sequences and are thought to initiate their secretion in a manner analogous to the export of periplasmic and outer membrane proteins (Randall and Hardy, 1984). However, the pathway taken by secreted proteins as they traverse the cell envelope in Gram-negative bacteria and the role of additional extragenic factors in the secretion of specific proteins remains to be identified.

E.coli on the other hand, does not usually excrete proteins except for a few specific exoproteins encoded on extrachromosomal elements and bacteriophages. For some proteins, such as α -haemolysin, bacteriocins and toxins (Mackman and Holland, 1984; Jakes and Model, 1979; Pugsley and Schwartz, 1985), the release of the exoprotein usually requires the presence of other gene products. This implies that *E.coli* is not normally endowed with a general extracellular secretory pathway.

A variety of exoprotein genes have been cloned and expressed in *E.coli*. The exoproteins appear to fall into two general classes with respect to their excretion properties. Firstly, most of the exoproteins, including those of *V.cholerae* (Guidolin and Manning, 1985), *Aeromonas hydrophila* (Howard and Buckley, 1986; Gobius and Pemberton, 1988) and various *Pseudomonas aeruginosa* proteins (Lory *et al.*, 1983; Lory and Tai, 1983, Coleman *et al.*, 1983), are found in the periplasm when expressed in *E.coli*. Some normally excreted proteins, however, are capable of being excreted from *E.coli*, and include the nuclease and serine protease of *Serratia marcescens* (Clegg and Allan, 1985; Ball *et al.*, 1987; Yanagida *et al.*, 1986), as well as the IgA protease of *Neisseria gonorrhoeae* (Pohlner *et al.*, 1987). These proteins appear to fall into the second class, i.e. self-secreting proteins.

The process of extracellular secretion (excretion) has been studied in two ways. By isolating mutants that are defective only in the excretion of extracellular proteins (Howard and Buckley, 1983; Wretlind and Pavlovskis, 1984; Hines *et al.*, 1988), it has been possible to identify a number of genes essential for the production of exoproteins. Gene fusions coding for hybrid proteins have also been used to determine whether a protein destined for export in one organism can cross the membrane barrier in another (Palva *et al.*, 1982; Ulmanen *et al.*, 1985). This chapter deals with the construction of DNase- β -lactamase protein fusions in an attempt to identify any additional intragenic information, contained in the DNase, which is necessary for the export of extracellular proteins. Since the DNase does not belong to the class of self-secreting exoproteins, these hybrid proteins were analyzed in *V.cholerae*, where an interaction with the "secretion machinery" not present in *E.coli*, could occur.

6.2 Results

6.2.1 Construction of translational fusions of the DNase to TEM β -lactamase.

The plasmid vector, pJBS633 (Broome-Smith and Spratt, 1986), was used to

construct translational fusions of the DNase to the TEM β -lactamase. This vector contains the coding region for the mature form of TEM β -lactamase which has been engineered to introduce a unique blunt end cloning site (*PvuII*) at the start of the coding region. In pJBS633 the β -lactamase is not expressed since there is no promoter, ribosome-binding site or signal sequence in front of the coding region.

A series of fusions were constructed to the carboxy-terminal end of the DNase fused to the amino-terminus of the mature form of β -lactamase (Figure 6.1). Plasmid pPM1254 was linearized by digestion with *EcoRV*, which cleaves 70 bp downstream of the DNase structural gene. A time course of 1 to 15 minutes of *Bal31* digestion was performed with the linearized DNA. The *Bal31* generated fragments were then digested with *HindIII* which cleaves 315 bp upstream of the start site of the DNase gene, and includes the proposed ribosome binding site and promoter region of this gene. These varying sized fragments were then cloned into pJBS633 digested with *PvuII* and *HindIII*. The *HindIII/PvuII* digestion removes all of the Tc^R gene present in pJBS633, and the various DNase carboxy-terminal deletions can then be fused with the β -lactamase at the *PvuII* site (Figure 6.1). Km^R, Tc^S transformants all contained *Bal31* generated fragments cloned into the vector pJBS633. The in-frame protein fusions were identified by patching transformants onto Luria agar containing 200 μ g/ml Ap. In-frame fusions resulted in the β -lactamase being translated in the correct frame, with hybrid proteins containing the active β -lactamase enzyme. Transformants with out of frame fusions did not grow on Ap plates, as the β -lactamase was not translated. In effect, when the β -lactamase was in the correct reading frame, it was under the control of the transcriptional and translational signals provided by the DNase gene. Analysis of the recombinant plasmids revealed that a series of DNase- β -lactamase fusions had been obtained, containing various amounts of the DNase upstream of the β -lactamase (Figure 6.2).

6.2.2 Nucleotide sequence of the fusion junctions in the DNase- β -lactamase hybrid proteins.

In order to determine the exact point of fusion of the DNase to the mature β -lactamase, recombinant plasmids containing the proposed DNase- β -lactamase hybrid genes were sequenced across the *PvuII* junction site. The plasmid

Figure 6.1 Construction of DNase- β -lactamase gene fusions

The plasmid pPM1254 (containing the cloned DNase gene) was digested and linearized with the restriction endonuclease *EcoRV*. This cleavage site was present 70bp after the DNase ORF. Incubation with *Ba*/31 exonuclease at time intervals of 1 to 15 minutes, resulted in a series of deletions of this gene at the carboxy-terminal end of the protein. These deletions were then digested with *HindIII*, and the DNA fragments containing all or part of the DNase gene were cloned into the *HindIII* and *PvuII* site of pJBS633. The plasmid pJBS633 contains the inactive *bla* gene, due to the deletion of its promoter, ribosome binding site and signal sequence. In-frame fusions of the DNase with the *bla* gene produced an active β -lactamase and were selected by screening for Ap^R.

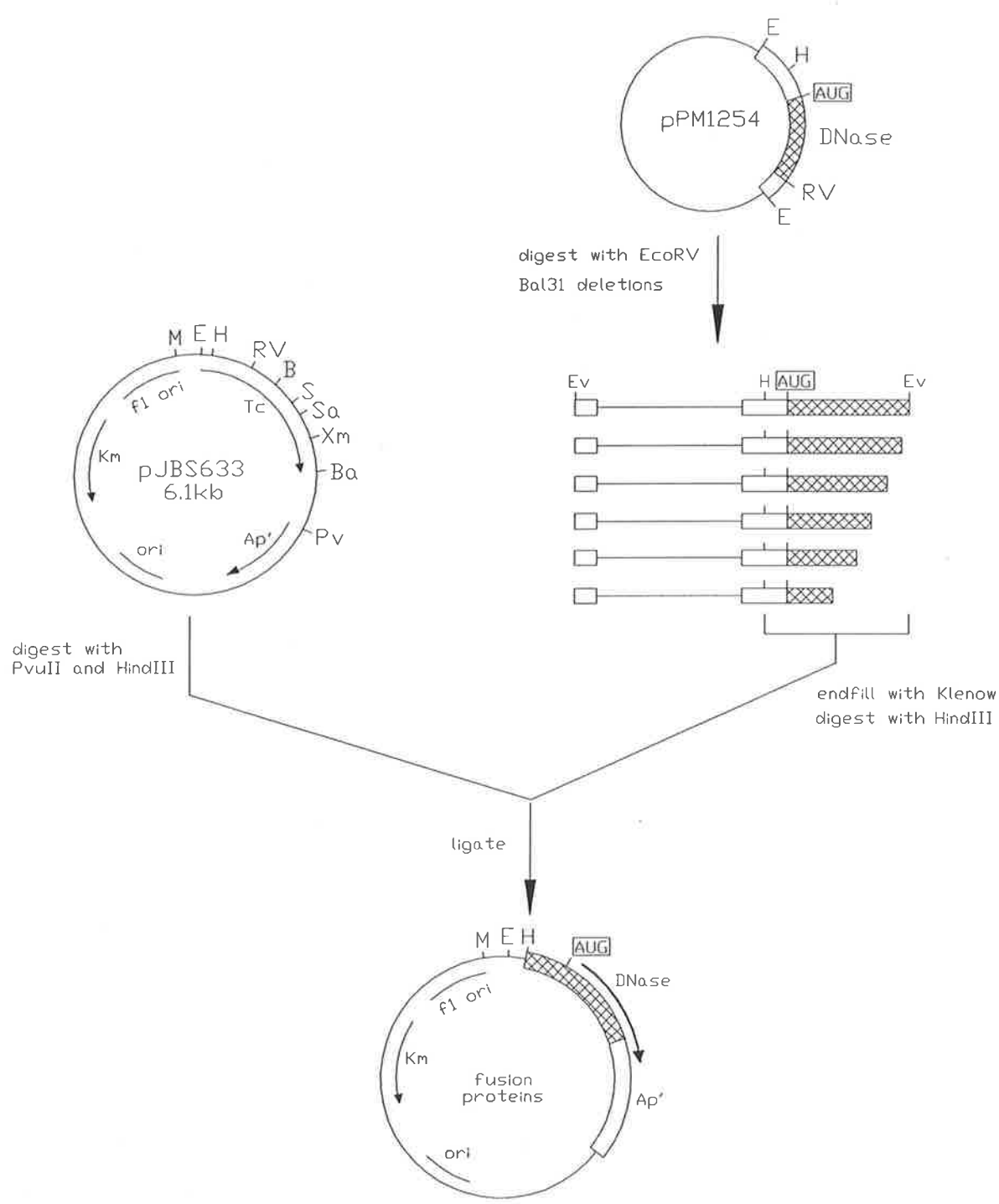
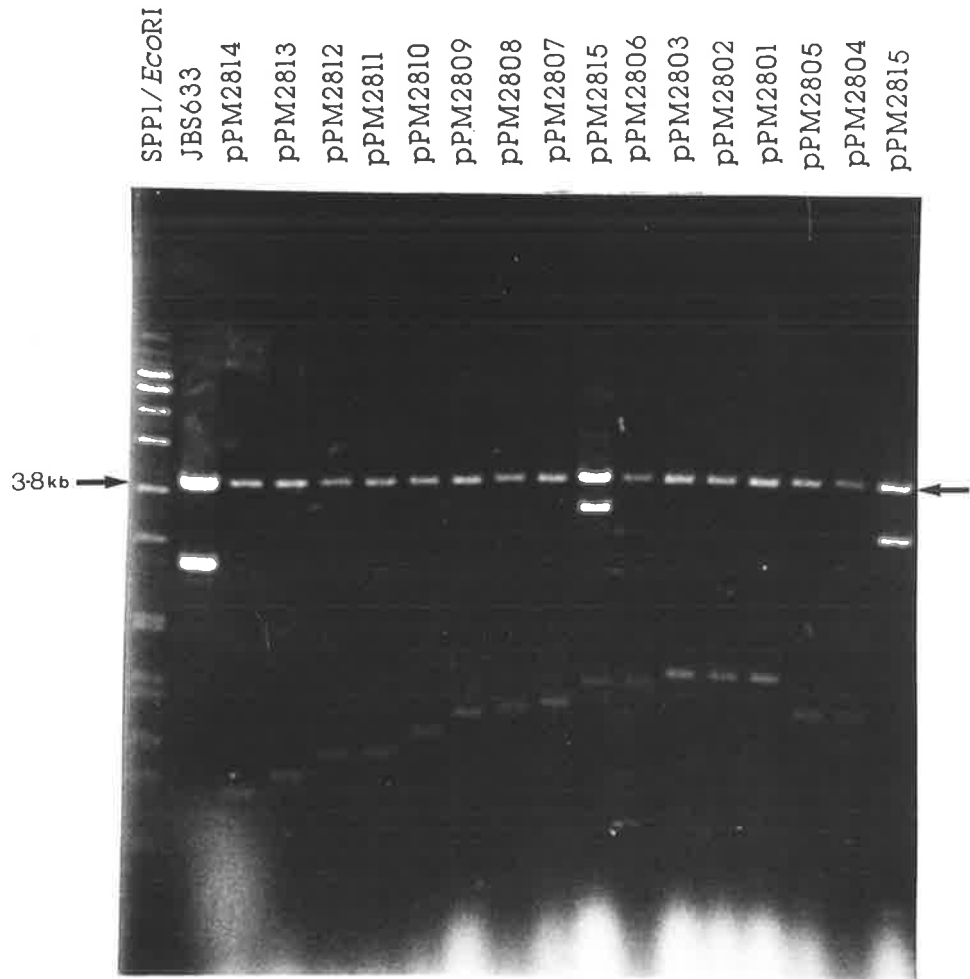


Figure 6.2 Gene fusions of the DNase with the β -lactamase of pJBS633.

A 0.8% agarose gel showing DNA fragments of pPM1254 cloned into pJBS633. The band at 3.8 kb is the vector pJBS633, while the smaller bands of varying size represent the *Ba/31* deletions of the DNase. The two larger bands present in pPM2815 and pPM2816 have had the vector portion of pPM1254 cloned into pJBS633 and are Ap^R due to the β -lactamase present on this vector (pUC18).

*Pst*I and *Hind*III



pJBS633 contains the pBR322 replication origin and it also has the phage f1 replication origin, thus enabling the production of single-stranded template DNA for dideoxy sequencing. A primer to the mature β -lactamase which hybridised 40 nucleotides prior to the fusion junction was used, thus enabling the sequence across the junction to be read (Figure 6.3).

Sequence data from these fusions verified that the Ap^R recombinant plasmids contained in-frame fusions of the β -lactamase gene to the upstream DNase gene (Figure 6.4).

6.2.3 Production of a β -lactamase specific antiserum.

The hybrid DNase- β -lactamase proteins in *E.coli* DH1 could not be visualized by Coomassie blue staining of cellular fractions. This was not surprising, since the genes for these hybrid proteins were under the control of the DNase expression signals. The DNase itself was poorly expressed and could not be detected with normal Coomassie blue staining (Chapter 3; Section 3.2). An attempt to overproduce and purify the DNase in order to raise an antiserum proved unsuccessful (Chapter 4, Section 4.0.0), so the highly expressed, periplasmically located β -lactamase was isolated and used to generate an antiserum.

E.coli cells harbouring pUC18 were grown to mid-exponential phase, sphaeroplasted and the shock fluids (periplasmic fractions) of these cells were run in SDS on a 15% polyacrylamide gel (Figure 6.5). A portion of this gel was stained with Coomassie blue, while the remainder was washed in distilled water to remove the SDS. The stained portion of the gel was aligned with the unstained gel, and the region corresponding to the β -lactamase was cut out and homogenized in 5 parts of paraffin oil/1 part Span85. A rabbit was then injected subcutaneously, by three repeated injections at fortnightly intervals. The antiserum was absorbed overnight with *E.coli* K-12, to remove cross-reacting *E.coli* antibodies before use.

Figure 6.3 Sequencing strategy of the DNase- β -lactamase gene fusions.

The phasmid pJBS633 contains both a ColE1 and f1 origin of replication. Single-stranded DNA of this vector was obtained by packaging into the f1 phage variant IR1. The point of fusion of the DNase to the β -lactamase was determined by using a primer 40 nucleotides prior to the start of the *bla* gene, and sequencing back through the fusion point and into the DNase gene.

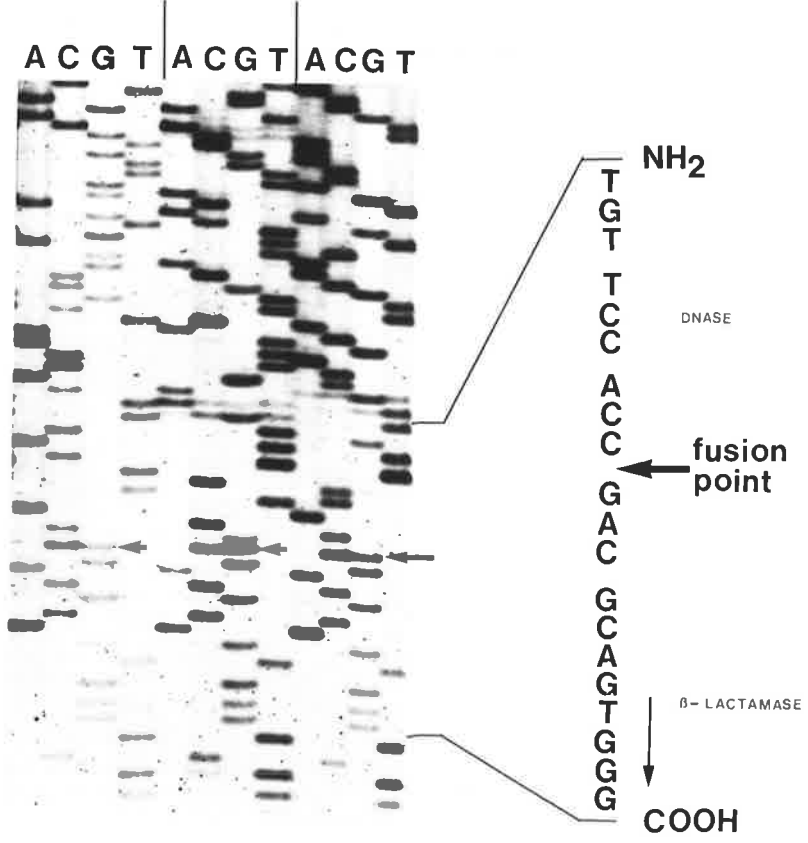
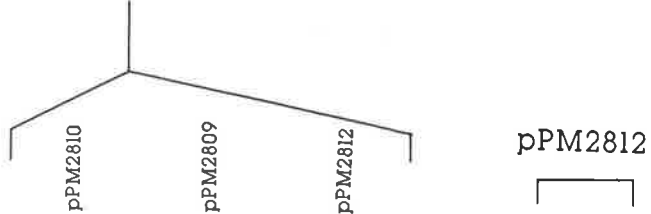
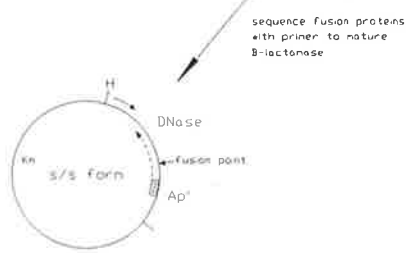
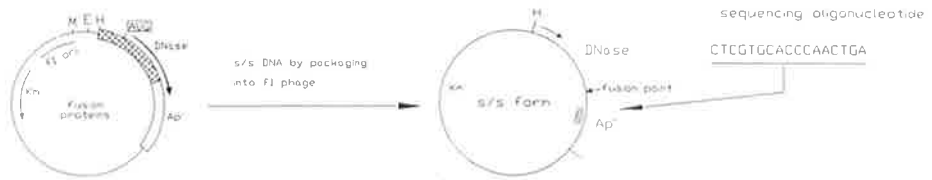
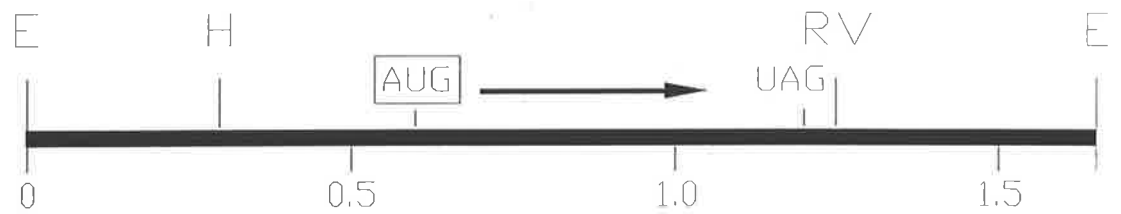


Figure 6.4 Carboxy terminal deletion of the DNase fused to β -lactamase.

Sequencing of the gene fusions identified the point of insertion of the DNase gene to β -lactamase. The plasmid pPM2801 has the complete DNase of 230aa fused to the β -lactamase, while the remaining plasmids are progressively deleted at the carboxy-terminus. The numbers in the right hand column represent the remaining aa's of the DNase protein, while the aa immediately adjacent to the gene is the point of the fusion.



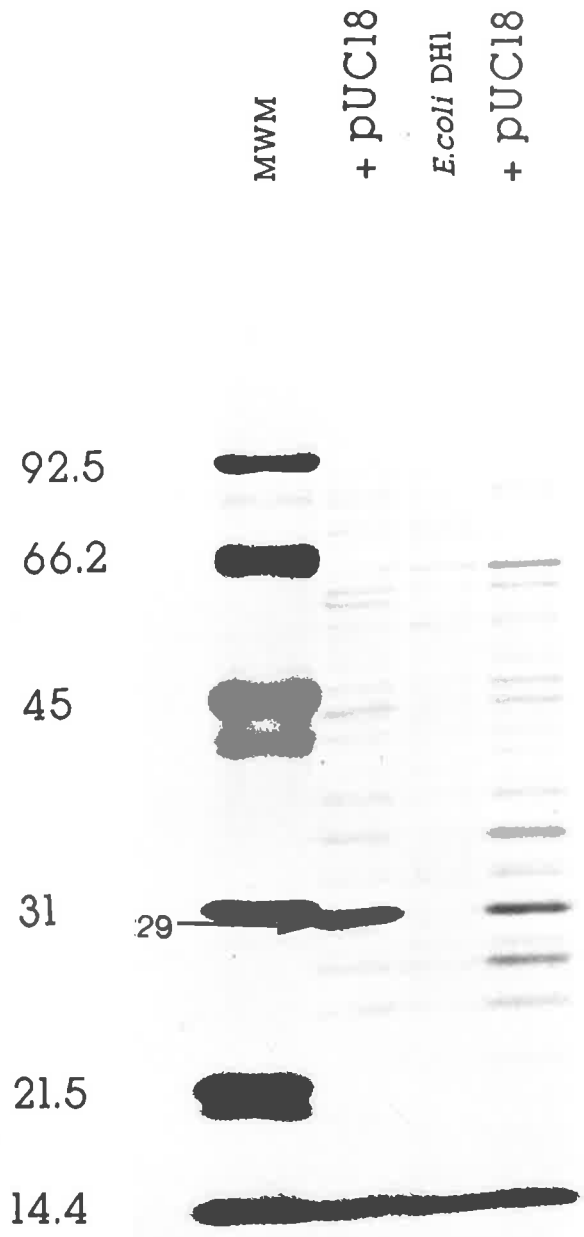
DNase protein

230aa

pPM2801		Asn	230aa
pPM2802		Leu	229aa
pPM2803		Cys	228aa
pPM2804		Val	224aa
pPM2805		Phe	223aa
pPM2806		Val	216aa
pPM2807		Ala	171aa
pPM2808		Ala	169aa
pPM2809		Arg	159aa
pPM2810		Gly	129aa
pPM2811		Arg	100aa
pPM2812		Gly	99aa
pPM2813		Arg	68aa
pPM2814		Gly	46aa

Figure 6.5 Polyacrylamide gel electrophoresis of periplasmic fractions of *E.coli* cells harbouring pUC18.

Periplasmic fractions of *E.coli* cells expressing β -lactamase (harbouring pUC18) were prepared and run on a 15% polyacrylamide gel in SDS. The band present at 29kDa represents the β -lactamase protein of pUC18, while this protein is not present in the *E.coli* DH1 cells, alone. A portion of the gel was stained with Coomassie Brilliant Blue to visualize the protein bands, while the remainder of the gel was washed in distilled water to remove the SDS and then aligned to the stained gel. The 29kDa region was cut out of the unstained gel, homogenized in 5 parts to 1 of Span85 and Paraffin oil and injected subcutaneously into a rabbit to raise anti β -lactamase antiserum. The numbers on the left hand side represent the protein sizes in kDa.



6.2.4 Western immunoblotting analysis of hybrid proteins in *E.coli*.

E.coli expressing the gene fusions were screened for hybrid proteins using the β -lactamase specific antiserum which reacts with the carboxy-terminal portion (β -lactamase) of such proteins. Periplasmic fractions were run on a 15% polyacrylamide gel and after transfer to nitrocellulose, were blotted with the β -lactamase antiserum. It was reasoned that since the DNase had a functional signal sequence, the hybrid proteins would be exported into the periplasm. Furthermore, both wild type DNase and β -lactamase are normally found in the periplasmic space in *E.coli*.

Western blot analysis of the periplasmic fractions of *E.coli* strains harbouring these gene fusions, reveals a series of hybrid proteins of different sizes (Figure 6.6). The difference in size of each of the proteins corresponds to the amount of the carboxy-terminus deleted from the DNase gene before fusion to the β -lactamase.

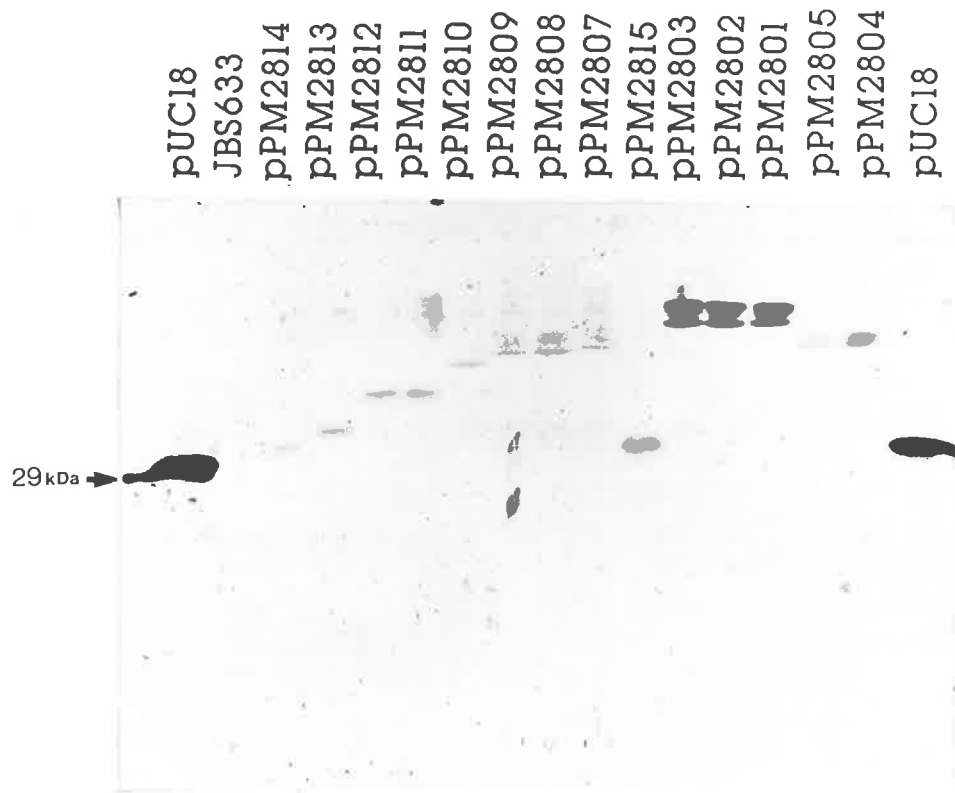
When staining of the hybrid proteins is compared with the pUC18 encoded β -lactamase under the control of its own promoter (Figure 6.6), the expression of the 29kDa wild type β -lactamase is more marked. This presumably reflects the increased expression of β -lactamase under its own expression signals, while the levels of the hybrid proteins are indicative of the low level of expression which is normally seen with the DNase. As seen in Figure 6.4, the band corresponding to the β -lactamase is easily seen after Coomassie blue staining, while the DNase is difficult to detect even after silver staining (Chapter 3; Figure 3.2.9).

The signal sequence of the DNase is still functional in the hybrid proteins, as they are all exported from the cytoplasm to the periplasm. Therefore, while the signal sequence of the DNase may not be sufficient for the eventual extracellular location of this protein in *V.cholerae*, it is sufficient to transport both the wild type DNase and the hybrid proteins from the cytoplasm across the inner membrane to the periplasm. Perhaps this is also the case in *V. cholerae*, where the signal sequence of the DNase is just required for export to the periplasm. There the mature protein is then free to interact with a specific "secretion apparatus" which determines its final extracellular destination. This concept will be discussed in greater detail later in the chapter. Many of the hybrid proteins also appear to have

Figure 6.6 Western blot analysis following SDS-PAGE of periplasmic fractions of *E.coli* cells harbouring the DNase- β -lactamase gene fusions.

The blot was developed with absorbed (10^9 *E.coli* cells/ml) rabbit antiserum to TEM β -lactamase, followed by goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase. The band at 29kDa represents the β -lactamase present on pUC18. The bands of increasing size seen in the other tracks represent the DNase- β -lactamase gene fusions (hybrid proteins) with serial carboxy-terminal deletions of the DNase. The plasmid pPM2815 has a 29kDa protein band present, as this is not a gene fusion, but has the Ap^R gene of pUC18 cloned into pJBS633. The doublets present with pPM2801, pPM2802 and pPM2803 correspond to the precursor and processed forms of these hybrid proteins. All tracks contained β -lactamase in the sample buffer.

REDUCING PAGE



an extra band which is about 2 kDa larger than their normal hybrid size. This is suggestive of a precursor (pro-DNase- β -lactamase) and a mature, processed form (DNase- β -lactamase). The fact that these proteins are hybrids could result in the inefficient or improper processing of the proteins.

6.2.5 Detection of DNase activity in DNase- β -lactamase hybrid proteins.

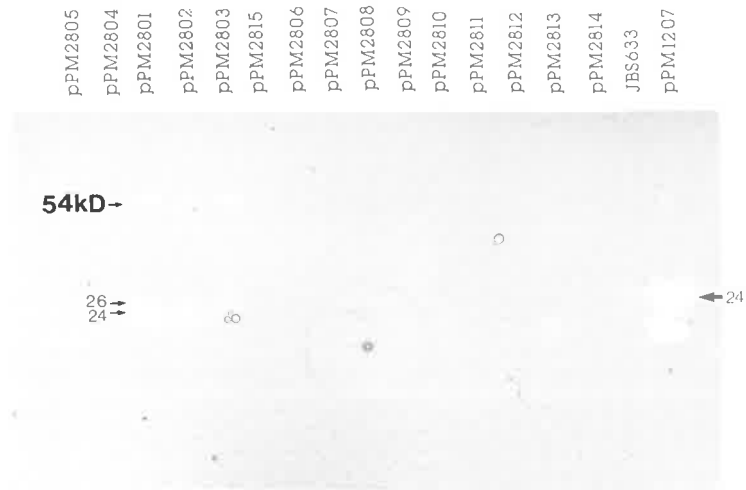
The recombinant plasmids pPM2801, pPM2802 and pPM2803, coding for the largest hybrid proteins have both DNase and β -lactamase enzyme activity. Periplasmic fractions of *E.coli* cells with the hybrid proteins, were examined on duplicate non-denaturing polyacrylamide gels. The previous Western blot (Figure 6.6) was run under reducing/denaturing conditions (β -mercaptoethanol added to the sample buffer), but in order to detect DNase activity after polyacrylamide gel electrophoresis no β -mercaptoethanol can be present (Chapter 3; Section 3.2.8). One gel was transferred to nitrocellulose and immunoblotted, while the other was layered onto DNA-agar (Figure 6.7). The plasmids pPM2801, pPM2802 and pPM2803, all expressed DNase activity which was present as a band of about 54kDa. Surprisingly, a further two bands were detected at about 26kDa and 24kDa. These two bands probably represent the DNase protein after cleavage from the β -lactamase; the 26kDa form represents the precursor and the 24kDa form is the mature protein. Again, the inefficiency of processing can be attributed to the hybrid proteins being altered in their folding which affects the rate of cleavage or accessibility to the signal peptidase. However, it is important that at least, in the case of the larger hybrid proteins, both enzymatic properties can be displayed by the one protein. Presumably distinct domains exist, and a specific or non-specific proteolytic cleavage results in the separation of the two proteins because of their distinct individual folding. However, Western blot analysis (Figure 6.7) fails to detect the 29kDa form of the cleaved β -lactamase in the periplasmic fraction. One possibility is that the 54kDa protein seen in the Western blot is bifunctional and present in this form in the periplasm. The 26kDa and 24kDa active DNase proteins could result from the cleavage of the bifunctional hybrid proteins prior to their release into the periplasm. Plasmids pPM2801, pPM2802

Figure 6.7 DNase activity and Western blot analysis following SDS-PAGE of periplasmic fractions of E.coli cells harbouring the DNase- β -lactamase gene fusions.

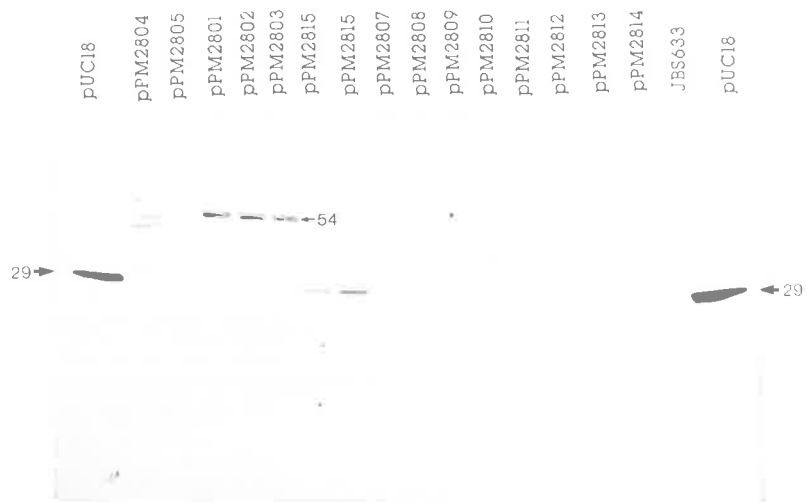
(A) After SDS-PAGE, the gel was washed in 40mM Tris pH7.5, 2mM MgCl₂ for 3 hrs. to remove the SDS and layered onto a DNA-agar plate. After overnight incubation at 37°C, the plate was flooded with 1N HCl and zones of activity were visualized as regions of clearing. The wild type DNase in pPM1207 has a protein size of 24kDa, while the gene fusions of pPM2801, pPM2802 and pPM2803 also retain DNase activity. These plasmids have bands of activity at 54kDa, 26kDa and 24kDa. The 54kDa band represents the DNase- β -lactamase, while the 26kDa band corresponds to the precursor form of the wild type DNase and the 24kDa the processed mature form of the DNase. No β -mercaptoethanol was present in the sample buffer.

(B) A Western blot of a duplicate gel following SDS-PAGE. The blot was developed with rabbit antiserum to TEM β -lactamase, followed by goat antirabbit immunoglobulin G coupled with horseradish peroxidase. The band at 29kDa represents the wild type β -lactamase of pUC18, while only the 54kDa hybrid form of pPM2801, pPM2802 and pPM2803 can be detected. No β -mercaptoethanol was present in the sample buffer.

A NON-REDUCING PAGE/
DNA-AGAR OVERLAY



B NON-REDUCING PAGE



and pPM2803 which express both DNase and β -lactamase activity have carboxy terminal deletions that still retain the last residue of the DNase at aa 228 (Figure 6.2). This cysteine is probably involved in disulphide bonding elsewhere in the DNase (Section 3.2.8), and helps to mark the end of the DNase as distinct from the β -lactamase. If the fusion proteins have a similar mode of post-translational translocation as is normally displayed by the wild type β -lactamase (Koshland and Botstein, 1982), proteolytic cleavage of the proteins at the fusion point could occur, with the DNase segment containing the signal sequence being exported to the periplasm, and the resulting β -lactamase remaining in the cytoplasm or cytoplasmic membrane. Interestingly, expression from plasmids pPM2804 and pPM2805 in which only four and five more codons than pPM2803 have been deleted at the carboxy-terminus of the DNase (see Figure 6.4), results in a DNase negative phenotype. These codons again include Cys₂₂₈, and so it is not surprising that their loss leads to the loss of activity.

6.2.6 Export of the DNase- β -lactamase hybrid proteins in *V.cholerae*.

Since *V.cholerae* normally excretes the DNase into the external environment, the hybrid proteins have been examined in their natural host, to see how they interact with components of the "secretion apparatus". It was hoped that some insight could be gained into whether the hybrid proteins could also be exported out of the cell, and if specific sequences (information) present in the DNase itself were required. The construction of the DNase negative *V.cholerae* mutant V752 (Chapter 5;Section 5.2.6.4), made this strain ideal for such a use. Firstly, the background activity of the chromosomally encoded DNases was eliminated, and secondly the ability of this strain to be transformed, simplified the introduction of the various plasmids into *V.cholerae*.

V.cholerae strain V752 transformed with the plasmids encoding the hybrid proteins and the derivatives, were analyzed for their ability to excrete these proteins. Western blot analysis did not reveal the presence of the hybrid proteins in the extracellular fractions. The wild type 29kDa β -lactamase protein encoded on pUC18 could not be detected in the extracellular medium.

When the periplasmic fractions of the *V.cholerae* strains were examined, (Figure 6.8A) a significant difference was detected in the expression of the hybrid proteins compared to *E.coli*. The 29kDa band corresponding to the mature β -lactamase could be detected in each case. This suggests that the DNase- β -lactamase hybrids are probably cleaved after they have crossed the cytoplasmic membrane, and the entire hybrid protein has entered the periplasmic space. This cleavage may or may not be specific, but it is interesting to note that it seems to be precise and quite efficient, as the 29kDa β -lactamase is quite prominent, with only the larger hybrid proteins showing the higher molecular weight bands as well. This may be indicative of a specific interaction of the DNase with a component not present in *E.coli*.

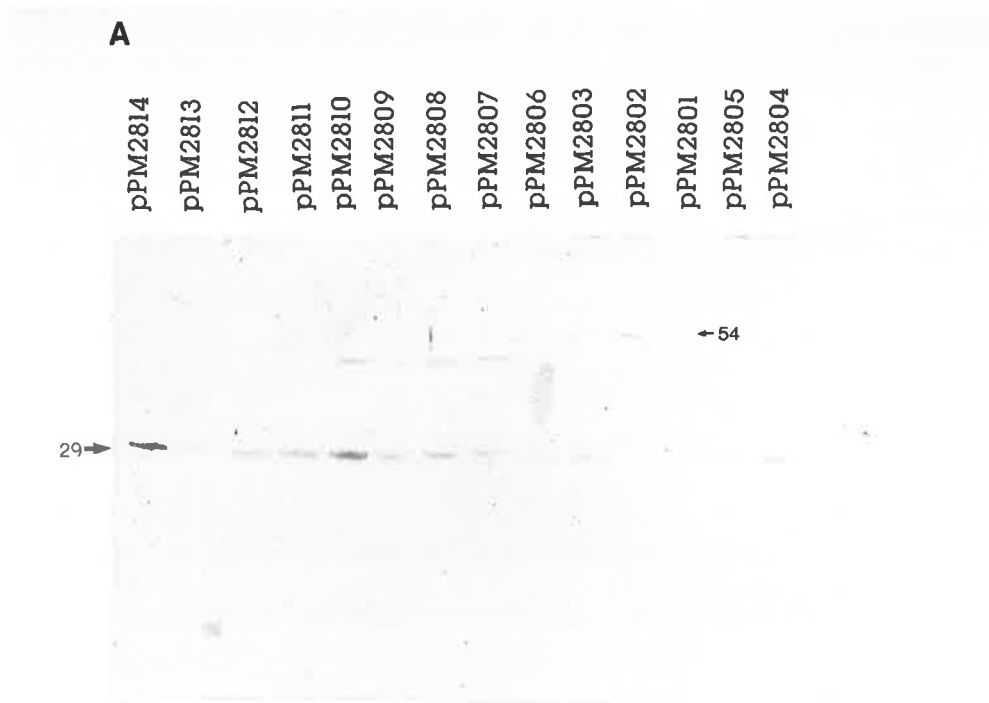
When the extracellular fractions of *V.cholerae* harbouring pPM2801, pPM2802 and pPM2803 were examined, the DNase activity could be detected (Figure 6.8B). This represents true excretion as extracellular fractions of a strain containing just the more highly expressed wild type β -lactamase, did not reveal the presence of this periplasmic protein, and hence no generalized leakage of periplasmic proteins is involved. The DNase activity is associated with a 24kDa protein whereas the 54kDa hybrid protein remains in the periplasm. The fate of the DNase portion of the other fusion proteins is unknown because they are DNase negative and the antiserum only reacts with the β -lactamase. There is clearly a need to produce an antiserum to the DNase in order to determine the location of the cleaved carboxy-terminal deleted DNase segments of the hybrid proteins. Lack of excretion by any of these deletions would presumably identify any regions present in the mature DNase protein capable of interacting with the "secretion apparatus".

The periplasmic location of the hybrid proteins may also be an intermediate step in the excretion of the DNase from the cell in normal circumstances. Previous experiments (Chapter 3: Section 3.2.8) failed to detect a periplasmic intermediate of the DNase in *V.cholerae*, but its increased expression when introduced on a high copy number plasmid results in a detectable level of DNase activity being present in the periplasm. This is presumably due to the build up of the periplasmic pool of DNase, which under normal circumstances is transient and quickly exported outside the cell as is the case with cholera toxin (Hirst and Holmgren, 1987). This would suggest that the DNase is exported to the periplasm

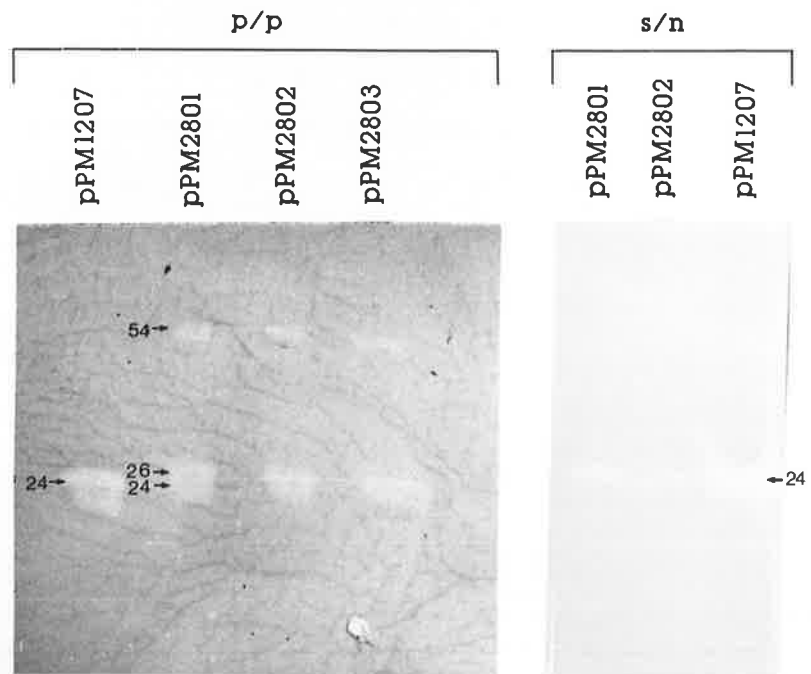
Figure 6.8 DNase- β -lactamase protein fusions in *V.cholerae*

(A) Western blot analysis following SDS-PAGE of periplasmic fractions of *V.cholerae* cells harbouring the DNase- β -lactamase gene fusions. The blot was developed with absorbed rabbit antiserum to TEM β -lactamase, followed by goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase. Although the hybrid proteins of varying sizes can be seen as with *E.coli*, the 29kDa β -lactamase protein can also be detected. The 29kDa protein is not detected in *E.coli* and is presumably due to a protease present in the periplasm of *V.cholerae* but not in *E.coli*.

(B) After SDS-PAGE, the gel was washed in 40mM Tris pH7.5, 2mM MgCl₂ for 3 hrs. to remove the SDS and layered onto a DNA-agar plate. The plate was incubated overnight at 37°C, and then flooded with 1N HCl. Zones of activity were detected as distinct bands of clearing. The wild type DNase can be detected in the periplasm of *V.cholerae* at 24kDa and this presumably represents an intermediate in the export process. The plasmids pPM2801, pPM2802 and pPM2803 which still retain DNase activity, are all found in the periplasm (p/p) of *V.cholerae* in 3 forms; the 54kDa hybrid protein, the 26kDa cleaved DNase precursor form and the 24kDa wild type DNase. However, only the 24kDa form of the hybrid proteins is found exported to the outside of the cell (s/n). Sample buffer in both the Western blot and the DNase activity assay, did not contain β -mercaptoethanol



B SDS-PAGE/DNA-AGAR OVERLAY



in *V.cholerae* by means of the signal peptide, as it also does when cloned into *E.coli*, and subsequent excretion from the *V.cholerae* cell occurs due to the interaction with specific "secretion machinery" which is not present in *E.coli* and therefore explains its periplasmic location in *E.coli*.

6.2.7 Levels of Ap^R conferred by β -lactamase fusion proteins

To determine the level of Ap^R conferred by each fusion protein, an overnight broth of each strain producing a fusion protein was diluted 10⁵ fold and 4 μ l spotted onto NA plates containing increasing concentrations of Ap. The levels of Ap^R conferred by the fusion proteins were compared with the wild type β -lactamase present on pUC18. Plasmid pUC18 yielded minimal inhibitory concentrations (MIC's) of 1600 μ g/ml of Ap, whereas the fusion proteins all gave MIC's between 500-600 μ g/ml in either *E.coli* or *V.cholerae*. Since these fusion proteins are under the control of the DNase expression signals, the MIC is an indicator of the strength of the promoter and ribosome binding site of the DNase gene. The overall level of expression of β -lactamase activity is much lower than the wild type, and the expression signals of the DNase appear to be utilized with the same efficiency in *E.coli* and *V.cholerae* since they both give similar MIC's.

6.3 Conclusions.

A series of carboxy-terminal deletions of the DNase protein fused to the mature form TEM β -lactamase has allowed the analysis of the export of the DNase in both *E.coli* and *V.cholerae*. In-frame fusions were identified by the ability of cells expressing these hybrid proteins to grow in the presence of high levels of Ap.

The proposed signal peptide of the DNase, discussed in Chapter 4, appears to be functional in the hybrid proteins as it allows their translocation across the cytoplasmic membrane. Both the DNase and β -lactamase are normally found in the periplasm when present in *E.coli*, and this appears to be the case with the hybrid proteins. The largest protein fusions, encoded on plasmids pPM2801,

pPM2802 and pPM2803 have both DNase and β -lactamase activities. These protein fusions can be identified as bands at 54kDa in both Western blots and after overlay onto DNA agar plates. The DNA agar plates, also show two bands of DNase activity at 26kDa and 24kDa. These are presumably the precursors and mature forms of the DNase after cleavage of the hybrid proteins. These two bands are not detected in a Western blot, as would be expected because the antiserum is directed only against the β -lactamase portion of the fusion proteins. On the other hand, the 29kDa β -lactamase band should be visualized in the Western blot of *E.coli* periplasmic fractions if cleavage of the hybrid protein was occurring in the periplasm. It may be that the cleavage that does occur, is at the cytoplasmic membrane or in the cytoplasm and prevents the 29kDa mature β -lactamase entering the periplasm as it does not possess its own signal peptide. The secretion of β -lactamase is post-translational and has been shown to involve three steps (Fitts *et al.*, 1987). The first step involves recognition and translocation through the membrane. This is followed by cleavage of the signal peptide, and finally the β -lactamase is released into the periplasm. A mutation affecting any of these steps results in a membrane bound protease-sensitive form of the protein (Fitts *et al.*, 1987). Therefore, the cleavage that does occur in *E.coli* to give the active 26kDa and 24kDa DNase proteins could be at the cytoplasmic membrane, resulting in a membrane bound β -lactamase.

The DNase- β -lactamase fusion proteins expressed in *V.cholerae*, also appear to be exported to the periplasm. One notable difference is the presence of both the larger fusion protein and the 29kDa cleavage product. Cleavage between these two proteins appears to occur in the periplasmic space, as both forms of the protein are seen in a Western blot. When the largest hybrid proteins containing the active DNase are examined on DNA-agar plates, the 54kDa, 26kDa and 24kDa forms are seen in the periplasm. The cleavage seen with these fusion proteins is possibly non-specific, but it may also be the result of the interaction of these proteins with a component present in the *V.cholerae* periplasm but not in *E.coli*.

The extracellular secretion of the DNase may be similar to that of cholera toxin. Cholera toxin is transiently present in the periplasm before being secreted to the outside of the cell (Hirst and Holmgren, 1987) and this is also true for HlyA (Mercurio and Manning, 1985; Alm, R. and Manning, P.A., manuscript in

preparation). This periplasmic intermediate can also be detected in *V.cholerae* when the DNase is present on a multicopy plasmid. Both cholera toxin and presumably the DNase are then free to interact with the "secretion machinery" present in the periplasm or the outer membrane, and be exported to the outside of the cell.

The B subunit of cholera toxin appears to have the structural domains present for recognition with the secretion apparatus, and the A subunit is secreted by virtue of its assembly to the B subunit prior to export (Hirst *et al.*, 1984b). While the DNase⁺ fusion proteins are present in several forms in the periplasm, the 54kDa hybrid protein, the 26kDa and 24kDa DNase and the 29kDa β -lactamase, only the 24kDa protein is exported out of the cell. Both the β -lactamase and hybrid protein remain in the periplasm. The β -lactamase therefore is not secreted with the DNase although it is attached, as is the case with the A subunit of cholera toxin. The carboxy-terminal deletions of the DNase, however, may help to determine any structural domains or specific sequences necessary for the interaction with the "secretion apparatus". This remains to be determined as antiserum specific for the DNase is required to establish the fate of these truncated proteins. If the recognition process by the "secretory apparatus" requires a folded or structural domain this may not be possible with the truncated proteins. In that case, internal deletions or size specific mutagenesis may be required before this process can be analyzed further. The need for a DNase specific antiserum is apparent for such experiments.

CHAPTER 7

DISCUSSION

7.1 Introduction

V.cholerae secretes a variety of extracellular proteins. These include proteases (Schneider *et al.*, 1981), haemagglutinins (Hanne and Finkelstein, 1982), haemolysins (Manning *et al.*, 1984; Richardson *et al.*, 1986), neuraminidase (Ada *et al.*, 1961; Vimr *et al.*, 1988), cholera toxin (Finkelstein, 1969) and DNases (Tsan, 1978). Many of these proteins are thought to be involved in the pathogenesis of this organism, with the most notable virulence factor being the cholera toxin (Finkelstein, 1969).

This study has centred on the cloning and characterization of one of these extracellular proteins, the DNase. The role of the extracellular DNase in both the pathogenesis of the organism and restricting transformation in *V.cholerae* has been examined, and the question of why this protein belongs to the class of extracellular proteins has also been addressed.

7.2 Cloning and characterization of the gene encoding the DNase.

In this study an *E.coli* K-12 DH1 clone expressing the DNase of *V.cholerae* was obtained from a cosmid library based on *V.cholerae* 017 (El Tor, Ogawa). One positive clone was identified after screening on DNA-agar plates for colonies surrounded by zones of hydrolysis. The cosmid clone containing this gene was designated pPM1201.

By deletion analysis, a 1.25 kb *EcoRI/HindIII* DNA fragment still exhibiting DNase activity was generated shown to be present in both the cosmid clone pPM1201 and the *V.cholerae* 017 chromosome.

By transposon insertion mutagenesis of the cloned DNA, the maximum coding region for the gene encoding the DNase was determined to be 1.07kb. These insertions were all clustered within a 0.6kb region resulting in a DNase negative phenotype, although no DNase positive insertions in this region were detected. By use of linker insertion mutagenesis, an *EcoRV* site at 1.07kb distal to the *HindIII* end was identified that did not affect DNase production. The maximum size protein that could be encoded on this DNA was limited to 35kDa, with a minimum estimate of 22kDa being established by the transposon mutants.

7.3 Identification of protein products.

Plasmids expressing the active DNase and transposon insertion mutations were introduced into the minicell strain DS410. Subsequent visualization of plasmid encoded products showed a 24kDa protein in the DNase producing plasmid pPM1202, and this was lost in the transposon insertion derivatives with a DNase negative phenotype. No truncated protein products were seen with the transposon insertion plasmids, but this may simply have been due to degradation of the smaller polypeptides. A similar size band of 24kDa is also seen when protein products of the plasmids containing the DNase gene are examined with an *in vitro* coupled transcription/ translation system. The DNase gene product does not appear to be expressed well, as the intensity of this band is greatly reduced when compared with the 30 kDa β -lactamase (Achtman *et al.*, 1979) encoded by the plasmid vector, or the 25 kDa chloramphenicol transacetylase (Close and Rodriguez, 1982) encoded by the transposon Tn1725 (Ubben and Schmitt, 1986) even though it contains 5 methionine residues.

A method was developed to visualize the DNase protein and determine its size by means of its enzymic activity, i.e. DNA hydrolysis. This method was a modification of the procedure of Rosenthal and Lacks (1977), who used DNA-containing polyacrylamide gels to determine the molecular weight of nucleases.

Polyacrylamide gels after electrophoresis were stained in ethidium bromide, and areas which did not take up the ethidium bromide were assigned as regions of nuclease activity. Such a technique, however, proved inconsistent and difficult to interpret and therefore the polyacrylamide gels were run without the addition of DNA. When these these gels were overlaid onto large DNA agar plates, and incubated overnight at 37°C, distinct bands of DNA hydrolysis were detected on the DNA agar plates due to diffusion of the enzyme from the gel. Again, a protein of 24kDa consistent with the radiolabelled protein seen in minicells was visualized. Interestingly, DNase activity was only seen when the reducing agent β -mercaptoethanol, was not present in the sample buffer. This suggested that disulphide bonding was involved in the formation of the active enzyme complex.

7.4 Nucleotide sequence determination.

The nucleotide sequence of the 1.25kb *EcoRI/HindIII* DNA fragment encoding the DNase has been discussed extensively in Chapter 4, and the salient features of this nucleotide sequence can be summarized here.

Firstly, the *EcoRI/HindIII* DNA fragment contains an ORF of 690bp which would code for a protein of 26kDa. This ORF is within the limits set by transposon and linker mutagenesis, and is preceded by a consensus-like promoter region (Rosenberg and Court, 1979; Hawley and McClure, 1983). Although the promoter region is suggestive of a good transcriptional initiator (Gentz and Bujard, 1985), the ribosome binding site may not be an efficient translational initiator due to the poor homology exhibited with the consensus Shine-Dalgarno sequence (Shine and Dalgarno, 1974). Protein fusions described in Chapter 6, where the β -lactamase has been fused downstream to the DNase have been used to measure the efficiency of these expression signals, based on the minimal inhibitory concentrations (MIC's) of Ap required to kill growing cells. While the β -lactamase under its natural promoter has an MIC of 1600 μ g/ml, protein fusions using the DNase expression signals have MIC's of under 600 μ g/ml. Therefore, while the DNase appears to have its own promoter, the combination of transcription and translation expression signals lead to this protein being

expressed at lower levels than the wild type β -lactamase. This observation is consistent with the low levels of DNase protein which appear to be expressed.

Primer extension analysis identifies the mRNA start point just prior to this promoter region, which confirms that this area of consensus promoter homology is indeed the DNase promoter. In order to increase the expression of this gene, a gene fusion was constructed in which both the strong *tac* promoter and ribosome binding site were introduced prior to the start of the ORF. The anticipated overexpression of the DNase protein did not result, although expression of the gene was now under the control of the inducible *tac* promoter. The reasons for the lack of high levels of protein product even under such strong expression signals are not known, but it may be that the gene fusion construct itself was not stable, possibly at the mRNA level.

7.5 Signal sequence of the DNase.

Although the ORF codes for a protein size of 26kDa, and a 24kDa DNase has been detected, the extra 2kDa can easily be accounted for by the presence of a signal sequence which is cleaved to give the mature protein. An N-terminal signal sequence is usually found associated with exported proteins like the DNase. Signal sequences generally exhibit little nucleotide or amino acid homology, but the presence of positively charged amino acid residues at the extreme N-terminal end and a long hydrophobic core are necessary components of a signal peptide. The properties of the signal peptide of the DNase have also been discussed in detail in Chapter 4, and it is suffice to mention here that the DNase signal sequence conforms well with the rules established for signal peptides (Inouye and Halegoua, 1980; von Heijne, 1983). This signal peptide also appears to be functional *in vivo* as both the DNase and fusion proteins with the DNase signal sequence are exported to the periplasm of *E.coli* (Chapter 6).

As the signal peptides from proteins inserted into, or translocated through, the membranes of Gram-negative bacteria have little sequence homology, they have not been considered to contain information related to the different final localization of the proteins. However, the comparison of 43 signal peptide amino acid

sequences of proteins with different final localizations in *E.coli*, by a multivariate data analysis (where each amino acid was characterized by 20 physio-chemical properties), yielded a different property profile for each class of signal peptide (Sjostrom *et al.*, 1987). Signal peptides from proteins localized to the periplasmic space, the outer membrane, or the extracellular milieu (excreted proteins), were separated into distinct groups. As a general rule, the hydrophobicity of the N-terminal segment and the size of the hydrophobic core of the signal peptides increased with an increased distance from the cytoplasm of the final localization for the corresponding proteins.

Experiments with the outer membrane protein LamB have indicated that the signal sequence alone does not suffice for its correct localization and that additional information, present in the mature protein, is required (Benson and Silhavy, 1983; Hall *et al.*, 1982). Excretion of extracellular proteins has also been shown to involve sequences present in the mature protein, with these regions being identified at the carboxy-terminus in both the IgA protease of *N.gonorrhoeae* (Pohlner *et al.*, 1987) and the α -haemolysin of *E.coli* (Gray *et al.*, 1987). The long signal peptide of the *Bacillus subtilis* amylase which has been cloned and is excreted in *E.coli* (Yang *et al.*, 1983) is grouped with the signal peptides of *E.coli* excreted proteins (Sjostrom *et al.*, 1987). This *Bacillus* signal peptide can also mediate secretion of the periplasmic protein β -lactamase out of the cell in *E.coli* (Nakazawa *et al.*, 1986). As the proposed signal sequence of the DNase does not export the wild type DNase or the DNase- β -lactamase fusions beyond the periplasm in *E.coli*, it does not belong to this class of signal peptides for secreted proteins. The DNase- β -lactamase fusion proteins containing the proposed DNase signal peptide are also not excreted in *V.cholerae* and remain localized in the periplasm. This suggests that the signal peptide of the DNase may not be involved in the final excretion of the protein, and supports the idea that the excretion process involves two steps; the initial signal peptide dependent translocation across the inner membrane, and the signal peptide independent interaction with the "secretion machinery" present in *V.cholerae*. This evidence is however, indirect, and the construction of site specific mutations in the signal sequence of the DNase which resulted in the accumulation of precursors in either the cytoplasm or inner membrane (with none being found in the periplasm) would

confirm such an hypothesis.

7.6 Comparison of the DNase with DNA-binding proteins

Structural studies on proteins which interact with DNA, such as the cI repressor of bacteriophage lambda (Ohlendorf *et al.*, 1983) and the catabolite activator protein (CAP) (McKay and Steitz, 1981), reveal highly conserved regions of the proteins which form DNA binding domains with a common helix-turn-helix structure (Sauer *et al.*, 1982; Pabo and Sauer, 1984).

A protein helical structure similar to that adopted by gene repressors and activators is also evident in some nucleases (Argos, 1985; Saporito and Cunningham, 1988). The type I restriction endonucleases have secondary structure predictions which display two helical regions in conserved domains, which may act in a fashion similar to that proposed for repressor and activator molecules; namely, interaction with double-stranded DNA through helices and in two successive major grooves on the same DNA side. One helical motif could provide the specific recognition site and the other, the restriction site (Argos, 1985). The *nfo* gene of *E.coli* K-12 which encodes endonuclease IV also displays this helix-turn-helix structure, although it does not share any similarity with the functionally related endonuclease III of *E.coli* (Dempfle *et al.*, 1986; Saporito and Cunningham, 1988).

The predicted secondary structure of the DNase according to Chou and Fasman (1974a, 1974b, 1975) does not reveal any helix-turn-helix domains (Figure 4.8), normally seen with DNA-binding proteins, but this may simply reflect the fact that the DNase does not have a specific interaction with the DNA. There are also 10 cysteine residues within the protein which are presumably crosslinked in the active enzyme, thus accounting for the sensitivity to β -mercaptoethanol (Chapter 3, Section 3.2.8). However, without further data on the possible conformation of this protein, it is not possible to predict the interactions in the tertiary structure and how the cysteine residues are involved in the maintenance of the stable active enzyme.

Nucleotide and protein sequence comparisons with the type I restriction

endonucleases and endonuclease III and IV of *E.coli*, did not reveal any significant homology. A computer search of both nucleotide and protein sequences using the GenBank and EMBL Gene/Protein Sequence Database, also did not reveal any sequences with extensive similarity to the DNase.

7.7 Construction of a DNase negative *V.cholerae* mutant.

The construction of a DNase negative *V.cholerae* mutant using conventional methods such as chemical and transposon insertion mutagenesis were unsuccessful. Site-directed mutagenesis was attempted, in which a mutation constructed *in vitro* in the cloned DNase was re-introduced into *V.cholerae* and recombined into the chromosome, displacing the wild type copy. In order to select for this recombination event, a Km^R gene cartridge was introduced into the DNase structural gene, such that incorporation of this constructed mutation would give rise to *V.cholerae* with the cartridge integrated into the chromosome. *V.cholerae* strains isolated with this mutation still exhibited a DNase positive phenotype, indicating that a second DNase was probably being expressed. This fact also accounted for the failure to obtain a DNase negative mutant by chemical or transposon insertion mutagenesis, as potential mutants were screened for the phenotypic loss of DNase activity.

An extracellular DNase of *V.cholerae* has also been cloned by Newland and co-workers (1985). This DNase (DNase-2) appears to be unrelated to the one cloned here (DNase-1). The reported protein size of DNase-2 is 100kDa compared with the 24kDa protein of DNase-1. Analysis of *V.cholerae* fractions failed to detect any other proteins with DNase activity apart from the 24kDa protein. As predicted, Southern hybridization analysis does not demonstrate any homology between the two DNase genes, since they are present on different sized fragments in the *V.cholerae* chromosome.

A mutation in DNase-2, introduced into the *V.cholerae* chromosome by site directed mutagenesis in a manner similar to that for DNase-1 also resulted in a DNase positive phenotype. However, a *V.cholerae* strain containing a mutation in both DNases gave a completely DNase negative phenotype. The re-introduction

of plasmids containing either DNase gene into this *V.cholerae* double mutant restored the DNase positive phenotype.

Clearly, the two DNases which are expressed in *V.cholerae*, are distinct from each other at the DNA level. Whether this is also true at the protein level remains to be determined, as antiserum directed against either DNase is at present unavailable. DNase-2, encoded by the *xds* locus, has been mapped between *pro* and *ile*, and this region of the chromosome is immediately adjacent to the biotype locus of *V.cholerae* (Newland *et al.*, 1985). The position of DNase-1 on the *V.cholerae* chromosome has not been mapped, but the insertion of a Km^R cartridge into the structural gene for DNase-1, makes the screening for exconjugants receiving this region of the chromosome a simple matter, as the Km^R can be selected for in the recipient strain. The construction of the DNase negative strain V555 with one antibiotic resistance (Km^R) inserted in the DNase-1 gene and another (Ap^R) in the DNase-2 gene also allows the mapping of these two genes relative to each other, as the antibiotic resistance markers can be used to screen and select each locus. Although the maximum sized DNA fragments encoding these DNases (1.25kb for DNase-1 and 3.5kb for DNase-2) are identical in both El Tor and Classical strains, the larger DNA fragment (3.55kb for DNase-1 and 6.6kb for DNase-2) these genes were isolated from, vary between biotype strains. Whether this is due to them being in an area of biotype variation, or the result of restriction fragment length polymorphism, remains to be seen. The two DNases also share the same distribution within the Vibrionaceae, with homologous DNA being present in *V.mimicus* and *V.cholerae* non-01 strains, while the DNase-2 also shows some homology with *V.parahaemolyticus* and *V.fluvialis*. Presumably these homologous bands represent DNases, although this has not been determined.

7.8 The role of the DNase in *V.cholerae*.

The construction of mutations in the DNases of *V.cholerae* permitted the evaluation of their roles in the virulence of the organism. The use of the infant mouse cholera model to test either single DNase mutants or double DNase

mutants, did not reveal any loss of virulence in these organisms. The infant mouse model is a sensitive assay for virulence. The DNase mutations first constructed in a *rif V.cholerae* strain could not be tested properly due to the reduction of virulence resulting from the nature of the rifampicin resistance mutation. Although *V.cholerae* strain 017, lacking either or both DNases shows no loss of virulence, they may still be contributing factors in the pathogenesis of the disease. The process of disease in cholerae requires several virulence attributes, ranging from cholera toxin (Finkelstein, 1969) to motility (Guentzel and Berry, 1975), chemotaxis (Freter *et al.*, 1981) and adherence (Taylor *et al.*, 1987a). While cholera toxin (Mekalanos *et al.*, 1983) and the TcpA pilus (Taylor *et al.*, 1987a) contribute greatly to this process, the role of the DNase may be minor. Thus, taken individually it does not greatly affect the virulence of the organism, but taken collectively with other minor virulence determinants a more dramatic effect could be seen.

The DNase negative *V.cholerae* mutants were also tested for their ability to be transformed with plasmid DNA. *E.coli* strains harbouring plasmids containing the DNase showed a reduction in transformation efficiency, with the most marked reduction demonstrated in *toIA,B* strains leaky for the periplasmic DNase, thereby effectively mimicking the extracellular secretion of this protein seen in *V.cholerae*.

DNase-1 has the most marked effect on transformation with plasmid DNA. Thus, strains lacking DNase-1 or both DNases can be reproducibly transformed but not as efficiently as *E.coli*. It must be remembered that the transformation procedure used has been optimized for *E.coli* (Hanahan, 1983) and the plasmid DNA was of *E.coli* origin. Attempts to use isolated plasmid DNA from *V.cholerae* resulted in a severe reduction in transformants with both *V.cholerae* and *E.coli*. The reason for this is unknown.

The ability to transform DNA into an organism is very important, especially in the case of *V.cholerae*, to facilitate genetic manipulations in an effort to produce a suitable vaccine candidate. The relatively recent method of electroporation, has been used to introduce DNA into a variety of eukaryotic cell types including both mammalian cells and plant cell protoplasts. Attempts to use this method to permeabilize small cells such as bacteria have met with only limited success. The modified procedure of Calvin and Hannawalt (1988) has allowed this technique to

be used with bacteria, with the successful uptake of DNA by *V.cholerae* being demonstrated in one reported case (Stoebner and Payne, 1988). The efficiency of this method of introducing DNA into *V.cholerae*, however, has not been documented, and a major shortcoming of such a technique is the requirement for the necessary apparatus which may not be readily available. The recent development of a more efficient and time saving method of transformation by Chung *et al.*, (1989) makes this a more desirable alternative.

7.9 Export of the DNase in *V.cholerae*.

An understanding of the genetic information that enables proteins to be exported, and is responsible for their accurate targeting to non-cytoplasmic compartments, is of basic biological interest. Much of our understanding of this process has derived from work based on protein fusions. Early work involved mainly the use of N-terminal portions of exported proteins fused to the cytoplasmic enzyme β -galactosidase (Benson *et al.*, 1985; Silhavy and Beckwith, 1985). However, the realization that β -galactosidase cannot be translocated across the cytoplasmic membrane imposes severe limitations on the use of β -galactosidase fusions for many studies of protein export (Tommassen *et al.*, 1985).

The use of β -lactamase circumvents the problems encountered with β -galactosidase, as β -lactamase is normally a periplasmic protein. Protein fusions with β -lactamase have resulted in its insertion into the outer membrane when fused with the outer membrane protein, PhoE (Tommassen *et al.*, 1983), while fusions with the periplasmic enzyme alkaline phosphatase result in the hybrid protein remaining in the periplasm (Manoil and Beckwith, 1985). The outer membrane protein LamB has sorting signals present in the mature protein required for its correct localization to the outer membrane (Benson *et al.*, 1984) while the B subunit of cholera toxin contains the export signals necessary for the excretion of the cholera toxin AB subunit complex (Hirst *et al.*, 1984b). The nature of these signals could vary, from perhaps structural requirements necessary for incorporation into the membrane in the case of LamB, to the recognition signals required for interaction with the "secretion machinery" for extracellular export in

the case of CtxB.

DNase- β -lactamase fusions were used to try to identify any intragenic sequences of the DNase which may be responsible for directing DNase export from the cell. The export of the lipase (*lip*) gene of *Staphylococcus hyicus* has been studied in a similar manner, (Liebl and Gotz, 1986) and carried out in *Staphylococcus carnosus* which simulates the conditions of the natural host but avoids the background activity of a chromosomally encoded lipase. The amount of hybrid proteins secreted was influenced by the length of the N-terminal lipase portion. A significant concentration of secreted lipase- β -lactamase hybrid proteins, comparable to that of the native lipase, was only found when the lipase portion of these hybrids comprised more than 101 aa's, with the proposed lipase signal peptide being 36 aa's long. Hybrid proteins containing fewer than the 101 aa's of the N-terminus of the lipase preprotein were detected in the cellular fraction, indicating that the lipase possesses a domain adjacent to the signal peptide that is essential for secretion. Similar studies with the N-terminal end of Braun's lipoprotein fused to β -lactamase, have shown that at least 9 aa's as well as the signal peptide of the lipoprotein are required for the localization of the β -lactamase to the outer membrane of *E.coli* (Ghrayeb and Inouye, 1984). DNase- β -lactamase protein fusions in *E.coli* were all found in the periplasm, as is the case normally with either wild type protein. The fusions are not secreted to the outside of the cell in *V.cholerae* but remain in the periplasmic space. However, unlike in *E.coli* these fusion proteins are cleaved in the *V.cholerae* periplasmic space, and although this may represent non-specific proteolytic cleavage between the structural domains of the fused proteins, this protease may be the soluble-haemagglutinin protease responsible for the "nicking" and activation of cholera toxin in *V.cholerae* (Gill, 1976). When the genes for cholera toxin are cloned into *E.coli*, the A subunit is found in an unnicked form (Pearson and Mekalanos, 1982), while the LT of *E.coli* which is usually unnicked is found to be "nicked" when introduced into *V.cholerae* (Neill *et al.*, 1983). Although the hybrid proteins or the mature β -lactamase are not excreted in *V.cholerae*, the fate of the DNase portion of the cleaved fusion proteins is unknown as the antiserum is specific for the β -lactamase. The largest fusion proteins containing both an active DNase and β -lactamase enzyme, show the 24kDa DNase to be excreted from the cell after

cleavage. This can be detected as a 24kDa band of DNA hydrolysis in the extracellular medium of such strains. The production of antisera directed against the DNase, would facilitate the localization of the progressively carboxy-terminal deleted DNase fragments. This, however, is based on the assumption that only primary sequences are involved in the interaction of exported proteins with the "secretion apparatus".

LT of *E.coli* which is closely related in mode of action, subunit structure and immunochemistry to CT of *V.cholerae* (Mekalanos, 1983), can be efficiently excreted when introduced into *V.cholerae* (Neill *et al.*, 1983). However, there are differences in the primary aa sequence (Moseley and Falkow, 1980) and it seems unlikely that these sequences are solely responsible for their cellular localization in *V.cholerae*. Some structural requirements may also be necessary. CT and LT must also utilize the same pathway for excretion in *V.cholerae*, as mutants defective in CT excretion are also defective in LT excretion (Neill *et al.*, 1983).

In the case of the DNase- β -lactamase hybrid proteins, the only proteins to be excreted are the cleaved DNase molecules containing the last Cys (Cys₂₂₈) residue. These molecules probably retain a similar folding to the wild type DNase as they are still enzymatically active. If there is also a structural requirement for excretion, the introduction of mutations that affect the folding of this protein should be examined. Site directed mutagenesis of the cysteine residues present in the DNase would seem to be the most likely first choice.

The introduction of a plasmid encoding the wild type DNase in *V.cholerae*, results in the accumulation of DNase in the periplasm not normally seen in an otherwise wild type *V.cholerae* strain. This is probably a consequence of an increase in the number of DNase molecules due to gene dosage caused by the high copy number plasmid. This periplasmic intermediate suggests that the DNase may follow a two-step secretion step as is seen with cholera toxin (Hirst and Holmgren, 1987) and haemolysin (Mercurio and Manning, 1985; Alm and Manning, man. in prep.) Therefore, the periplasmic localization of the DNase seen in *E.coli* may represent the first and intermediate step also occurring in *V.cholerae*, and the lack of further export in *E.coli* being due solely to the lack of any further "secretory apparatus" to enable the DNase to be exported in the second and final step of the process. Extracellular proteins such as the DNase,

however, should be distinguished from the class of self-secreting proteins of *S.marcescens* (Hines *et al.*, 1988) and IgA protease of *N.gonorrhoeae* (Pohlner *et al.*, 1987) which may be independent of such a system.

7.10 Concluding Remarks.

The unique property of some proteins to be exported out of the bacterial cell is an interesting phenomenon. This has important implications in areas as widely separated as the role of some excreted proteins in virulence (eg. haemolysin, toxin) and the purely economic considerations of using bacteria to manufacture a desired product that can be readily purified from a culture supernatant. In the case of the extracellular DNase of *V.cholerae*, this property is limited to the *V.cholerae* host, implicating the existence of a specific excretion system not found in *E.coli* K-12. The DNase itself must also possess some signal enabling it to be exported from the cell. While this system or "secretion apparatus" present in *V.cholerae* has not yet been identified, recognition signals have been found in some other exported proteins (Hirst and Holmgren, 1987; Tommassen *et al.*, 1983).

DNase- β -lactamase hybrid proteins are not excreted in *V.cholerae*. Further research is required to establish whether this is solely due to the DNase needing to maintain a correct conformation and therefore be able to interact with the "secretion machinery". It may simply be that specific sequence signals are involved. A DNase specific antiserum may provide the answer by determining the ultimate location of the cleaved DNase protein fusions which have been deleted at the carboxy-terminus.

The DNase negative *V.cholerae* mutants created by site specific mutagenesis can be readily transformed, and many exciting possibilities now exist for further genetic manipulation of other postulated virulence factors. This method is not limited to the strains described here but in theory may be applied to any *Vibrio* isolate. For the first time these organisms may be manipulated in a similar manner to other genetically well defined species such as *E.coli* and *Salmonella* spp. This

is particularly important in the context of research into live cholera vaccines. DNase negative *V.cholerae* strains could be used as the basis for vaccine strains because of the ease of introducing DNA required for the necessary genetic engineering. This, together with their apparently unchanged virulence properties in the animal model described here make them prime candidates for this type of research.

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